

Helicobacter pylori in the 21st Century

Edited by Philip Sutton and Hazel M. Mitchell



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ADVANCES IN MOLECULAR AND CELLULAR MICROBIOLOGY

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***Helicobacter pylori* in the 21st Century**

Edited by

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Advances in Molecular and Cellular Microbiology

Through the application of molecular and cellular microbiology we now recognize the diversity and dominance of microbial life forms that exist in all environments on our planet. These microbes have many important planetary roles, but for humans a major problem is their ability to colonize our tissues and cause disease. The same techniques of molecular and cellular microbiology have been applied to the problems of human and animal infection since the 1990s and have proved to be immensely powerful tools in elucidating how microorganisms cause human pathology. This series has the aim of providing information on the advances that have been made in the application of molecular and cellular microbiology to specific organisms and the diseases they cause. The series is edited by researchers active in the application of molecular and cellular microbiology to human disease states. Each volume focuses on a particular aspect of infectious disease and will enable graduate students and researchers to keep up with the rapidly diversifying literature in current microbiological research.

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A catalogue record for this book is available from the British Library, London, UK.

Library of Congress Cataloging-in-Publication Data

Helicobacter pylori in the 21st century / edited by Philip Sutton, Hazel Mitchell.
p. ; cm. -- (Advances in molecular and cellular microbiology ; 17)

Includes bibliographical references and index.

ISBN 978-1-84593-594-8 (alk. paper)

1. *Helicobacter pylori* infections. I. Sutton, Philip, 1965- II. Mitchell, Hazel, 1949- III. Title: *Helicobacter pylori* in the twenty-first century. IV. Series: Advances in molecular and cellular microbiology ; 17.

[DNLM: 1. *Helicobacter* Infections. 2. *Helicobacter pylori*. WC 200 H47533 2010]

QR201.H44H462 2010
616.3'3014--dc22

2009049449

ISBN-13: 978 1 84593 594 8

Commissioning editor: Nigel Farrar
Production editor: Tracy Head

Typeset by Columns Design Ltd, Reading, UK.
Printed and bound in the UK by CPI Antony Rowe, Chippenham.

Contents

Contributors	vii
Foreword <i>B. Marshall</i>	ix
PART I: PATHOLOGIES AND TREATMENT	
1 Epidemiology of <i>Helicobacter pylori</i> Infection <i>H.M. Malaty</i>	1
2 <i>Helicobacter pylori</i> Infection in Asia <i>K.M. Fock</i>	13
3 Gastric Adenocarcinoma <i>P. Correa and M.B. Piazuelo</i>	24
4 Antimicrobial Resistance and Approaches to Treatment <i>F. Mégraud</i>	45
5 Extragastric Manifestations of <i>Helicobacter pylori</i> Infection <i>H.M. Mitchell, N.O. Kaakoush and P. Sutton</i>	69
PART II: HOST RESPONSE	
6 <i>Helicobacter pylori</i>-induced Acquired Immunity and Immunoregulation <i>K. Robinson and J.C. Atherton</i>	94
7 Host Genetic Factors in Susceptibility and Resistance to <i>Helicobacter pylori</i> Pathogenesis <i>P. Sutton, A.L. Every and S.N. Harbour</i>	116
8 Innate Immune Initiators and Effectors in <i>Helicobacter pylori</i> Infection <i>M. Kaparakis, C.C. Allison, M.L. Hutton and R.L. Ferrero</i>	142

9	<i>Helicobacter pylori</i> Vaccines <i>T.G. Blanchard and J.G. Nedrud</i>	167
PART III: BACTERIAL MECHANISMS OF PATHOGENESIS		
10	Lipopolysaccharides of <i>Helicobacter pylori</i>: Importance in Gastric Adaptation and Pathogenesis <i>A.P. Moran</i>	190
11	Virulence Factors of <i>Helicobacter pylori</i> <i>S. Backert, H. Mimuro, D.A. Israel and R.M. Peek Jr</i>	212
12	<i>Helicobacter pylori</i> Adhesion to the Gastric Surface <i>S.K. Lindén, A. Arnqvist, S. Teneberg and A.P. Moran</i>	248
13	Helicobacteromics – Genomics of a Highly Variable, Well-adapted and Persistent Pathogen <i>H. de Reuse, M.M. Heimesaat and S. Bereswill</i>	269
	Index	293

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Foreword

Unlike books that have been written before about *Helicobacter pylori*, this volume, 30 years after Warren's original description of a case at Royal Perth Hospital, presents a mature understanding of how the bacterium colonizes its host and causes disease. As such, it will stay on my laboratory shelf for years to come, perhaps with a few minor details added as new signalling pathways are discovered.

Thirty years after Robin Warren first saw spiral bacteria on human gastric biopsies, Sutton and Mitchell's new book is a timely reminder that many clinical and basic aspects of *H. pylori* science are still controversial and worthy of further research. While much of our knowledge of the bacterium is a logical extrapolation of findings in other infectious diseases, the unique ability of *Helicobacter* to colonize the human gastric mucosa for many decades provides important insights into host-pathogen interactions, mucosal immunity and the whole immune system.

The editors themselves have been entrenched in *Helicobacter* microbiology for many years and are very well respected in their fields. Associate Professor Phil Sutton, an immunologist, has spent the past several years developing new animal models of *Helicobacter* infection whereby the fine details of the mucosal immune response can be isolated and teased out with the goal of designing vaccines against the organism. Similarly, Professor Hazel Mitchell is now Professor of Microbiology at the University of New South Wales, with many publications on all aspects of *Helicobacter* since she was one of the first in the world to complete a PhD on the biology and immunology of this intriguing organism.

With such eminent editors, it is no surprise that their co-authors include the leading lights in specific aspects of *Helicobacter* science. These specialists have each spent many years supervising teams of scientists and no doubt any one of them could easily produce an authoritative book on their special area of interest if they had the time. However, thanks to years of experience, every one of them has produced a concise chapter that not only emphasizes the proven facts but also mentions aspects of the disease that are unusual, controversial or defy explanation.

The chapter on epidemiology is written by Hoda Malaty, who trained in David Graham's faculty in Houston and who managed many of the seminal epidemiological studies using endoscopy, ELISA serology tests and ¹³C-urea breath tests. There is no doubt that *Helicobacter* is less common now as the standard of living increases around the world, but questions of transmission, acquisition and spontaneous loss of the infection are not all that well understood. Clinicians treating refractory patients need to consider these puzzling aspects of the disease. Twin studies, too, have been described here, to give a taste of the ongoing controversy, which is: 'Are host factors or bacterial factors the most important in determining disease outcome?'

Many of the unsolved dilemmas are then addressed further by Professor Kwong Fock from Singapore, a well-known academic and clinician with first-hand experience of the great disparities between *Helicobacter* incidence and *Helicobacter*-related diseases in the Asia Pacific region. Of particular interest is the question: 'If *Helicobacter* causes gastric cancer, why is it that some ethnic and geographic groups have one without the other?' Once again the host/cultural factors versus bacterial factors, especially various types of toxigenic *Helicobacter* strains, are carefully considered. Recent molecular/epidemiological studies of the mosaicism and phosphorylation sites of the gene encoding the cytotoxin-associated gene A product (CagA) could explain some of this variation. Presently, however, one must admit that a unifying hypothesis does not exist to explain the great disparities in disease phenotypes in Asia. Fock's chapter points out where modest efforts into carefully selected new studies are likely to pay off.

In a related chapter, the whole subject of gastric carcinogenesis, its histology, aetiology and relationship with *Helicobacter*, is placed into perspective by Pelayo Correa, a pathologist and pioneer of gastric cancer histological studies, and M. Blanca Piazuelo. With direct experience in the USA and South America, particularly the high cancer areas in Colombia, Correa and Piazuelo establish the background knowledge relating cancer risks to diets and lifestyle and then bring us up to date with new advances in the basic science of gastric carcinogenesis, particularly the exciting areas of *Helicobacter* toxin types, nitric oxide generation and oxidative stress. This well-referenced chapter is essential reading for anyone starting out in the field of gastric carcinogenesis.

Rounding off the clinical section is the chapter on antimicrobial resistance by Francis Mégraud of Bordeaux, who expertly addresses this emerging problem that is leading to global reductions in cure rate. Mégraud has authored many clinical studies of *Helicobacter* treatment and therefore has studied the resistance patterns of persisting isolates. His chapter is up to date with authoritative explanations of the pharmacokinetics of antimicrobials as they apply to the human stomach. In addition, he explains new technologies being introduced to rapidly evaluate antimicrobial resistance using molecular methods which thus avoid the several days' delay of 'old-fashioned' bacterial culture.

Bridging the clinical and basic sections, the chapter on taxonomy and extragastric *Helicobacter* infections by Hazel Mitchell, Nadeem Kaakoush and Phil Sutton will still be of great interest to clinicians. Mitchell has orchestrated a careful and impartial review of the non-gastric diseases that might be associated with *H. pylori* infection. Actually, it is very likely that some of these so-called associations are coincidental, related to socio-economic status for example. But in response to this, Mitchell has put the most robust linkages at the front of the chapter ('idiopathic thrombocytopenic purpura' and iron deficiency), then those with highly variable associations and likely reporting bias, most notably atherosclerosis, are discussed later. The review of the epidemiologic and basic research for each of these associations is an excellent resource for hypothesis-driven research plans. Similarly, attempts to unravel the highly variable association between *H. pylori* and atherosclerotic diseases could justify a career in clinical epidemiology. The chapter then flows easily into an expertly crafted discussion of the 'hygiene' link to allergic and 'autoimmune' disorders diseases, e.g. asthma and colitis, again using a clinical review followed by an in-depth description of hypothesis-driven basic immunological studies. Surprisingly, these have already revealed potential causative pathways that could lead to novel therapies for atopic diseases. With half the world still infected with *H. pylori*, modest increases in 'non-gastric' disease risk could actually drive numerically large amounts of morbidity, so it is very easy to justify further investigation into this emerging area of *Helicobacter* discovery.

In their chapter on *H. pylori*-induced acquired immunity and immunoregulation, Karen Robinson and John Atherton start off by listing the many paradoxes associated with *Helicobacter* immunology – for example, that B-cell-deficient animals can still be immunized and that some immunizations can actually increase *Helicobacter* colonization. To whet our appetite further,

they discuss tantalizing data whereby some human diseases characterized by hyperactive immune systems might be ameliorated or prevented by chronic gut infections such as *Helicobacter*.

Phil Sutton, Alison Every and Stacey Harbour's chapter on host genetic factors and susceptibility to *H. pylori* pathogenesis systematically describes the known workings of the mucosal immune system and how its components are influenced by various *Helicobacter* infections in several animal models. Since *Helicobacter* cannot usually be eradicated by even a robust serological immune response, it is important to understand the workings of the cell-mediated immune response too, as this is the component that correlates with protection from *Helicobacter* in animal models. Interaction between *Helicobacter* immunity and other pathogens, such as helminths, is discussed as part of the 'clean' versus 'dirty' human bacterial ecology hypothesis as it could affect the human propensity to develop hyperactive immune responses in autoimmune diseases, particularly atopic conditions such as asthma.

Maria Kaparakis, Cody Allison and Melanie Hutton from Richard Ferrero's group at Monash University in Melbourne start their review of innate immune initiators and effectors in *H. pylori* infection by pointing out the apparent paradox: namely that gastric epithelial cells are not usually considered to be a component of the immune system, yet colonization of this mucosa generates mucosal inflammation and a strong antibody response. However, these gastric epithelial cells are all very competent innate immune responders and this immediate part of the immune response might be used by *H. pylori* to improve its nutrition in the gastric mucus layer. The chapter then catalogues evidence for and against a major role for each of the relevant cytokines, assigning importance to the Toll-like receptors (TLR) 4 and TLR2 (from epithelia) and the neutrophil-activating protein HP-NAP (from *H. pylori*), but not lipopolysaccharide or flagellin (to which TLR5 is unreactive). Ferrero's specialty is the nucleotide-binding oligomerization (NOD) receptors, an interesting group that carries out an inflammatory role but might also trigger release of the cellular antibiotics such as 'defensins'. The authors make the point that much work is needed to further clarify this area.

Thomas Blanchard and John Nedrud have prepared a careful review of *H. pylori* vaccines, reflecting their long experience in this area. So that immunologists new to the field do not try to 'reinvent the wheel', the authors systematically explain the evolution of vaccines for *H. pylori* with tales of success and failure in various animal models, finishing with comprehensive lists of antigens, administration routes and results. They critically review the outcome of many studies, explaining results according to whether the vaccine causes protection, sterilizing immunity, or some lesser degree of effect. In each case they characterize the underlying immune response, T-helper 1 or T-helper 2 type, and consider why the results might not have been appropriate or sufficient for human use.

Going into some detail, Blanchard and Nedrud explain, similarly to the other immunological chapters, how the *H. pylori* immunity is unrelated to the serological immune response and that, in some models, antibodies actually enhance the colonization with *H. pylori* compared with a knockout mouse unable to produce antibody (μ MT antibody-deficient mice). These data are reminiscent of the human situation, where *H. pylori* has never been a major pathogen in AIDS or immune-deficient patients, supporting the hypothesis that robust immunity might be necessary for conversion from the asymptomatic to the symptomatic (ulcer) phenotype. In their chapter, these authors try to make sense of a plethora of experimental data but it seems that there is much work to be done. With so many adjuvants, mouse strains, vaccination routes and even knockout animals to test these days, it is difficult to say which will mimic the human situation and be an appropriate model. Of interest is a description of some animal models whereby gastritis worsens or activates when an *H. pylori* challenge is given subsequent to a vaccine. Thoughtful consideration of this effect, which seems to resolve after *H. pylori* eradication, is worthwhile in case there are autoimmune implications to empirical attempts to eradicate *H. pylori* in humans. However, as far as we know, *H. pylori* disease is not the cause of idiopathic autoimmune gastritis.

In great detail Blanchard and Nedrud catalogue even more obscure factors that interact with gastric immunity, most notably the adipokine leptin. The fact that leptin influences immunization against *H. pylori* is an example to us that there may be other as yet undiscovered factors that are playing a key role in the mucosal defence, or lack thereof, against *H. pylori*. After discussing poor results from studies in monkeys and humans using live *Salmonella* vectors and various other strategies, the authors summarize by saying that there are many strategies that should work, but have yet to be properly trialled, so *H. pylori* vaccinology will remain an exciting and busy area of research for years to come.

In a personal chapter, based on a lifelong interest in the lipopolysaccharides of *H. pylori* and their importance in gastric adaptation and pathogenesis, Professor Tony Moran provides a very detailed review of the *H. pylori* lipopolysaccharide, cell wall structures, interactions with the immune systems and the intricacies of the Lewis antigen mimicry. Clearly, fundamental understanding of the bacterial cell wall structures, particularly its lipid, is essential if we are to develop new means of eradicating or immunizing against *Helicobacter*. Not just a list of references and summaries of each, Moran's well-known personal expertise in this area allows him to synthesize the data and critically review the field so that new investigators can see what has been done, what strengths, weaknesses and inconsistencies there are in the existing body of work, and what is needed to make progress in this exciting but tough scientific niche.

In a very comprehensive chapter about virulence factors of *H. pylori*, accompanied by beautiful and detailed illustrations, Steffen Backert, Hitomi Mimuro, Dawn Israel and Richard Peek from Nashville summarize current knowledge in this area. Their long experience in the field is reflected first in a relevant critique of the latest methodology, especially cell biology and new transgenic animal models. They successfully tease out the interactions between the many adhesins, cytokines, toxins and signalling proteins, with carefully linked text and illustrations. Their synthesis incorporates new information from the post-genomic/proteomic era to explain the fundamental workings of the vacuolating cytotoxin VacA and CagA. By carefully reading the text and following the numbered steps in the illustrations readers will see why, for 60,000 years, *H. pylori* has been able to successfully colonize – but not kill – its unlucky human host. This is a very detailed, heavy-duty chapter about the workings of VacA and CagA, a really great reference especially valued because of its thoughtful and expertly done illustrations.

In a related chapter on *H. pylori* adhesion to the gastric surface, authors Sara Lindén, Anna Arnqvist, Susanne Teneberg and Tony Moran represent three of the world's premier groups studying the interaction between *H. pylori* and the various carbohydrate moieties present on the gastric epithelial cells. Put simply, these structures help make *H. pylori* stick. In a complicated area, these experts have created a structured chapter starting with consideration of the oral cavity, salivary, then gastric and epithelial adhesive structures. Some of these are well studied, such as the Bab adhesins, but others remain a mystery. Clearly, however, *H. pylori* has an efficient means of reaching and then tethering itself to the gastric mucosa, and a comprehensive explanation of the current state of the art is presented here.

Hilde de Reuse, Markus Heimesaat and Stefan Bereswill provide a fast-moving and readable overview of the *H. pylori* genome, 'Helicobacteromics', emphasizing the variability and plasticity as vast bacterial numbers balance their existence against the human immune system on the quite variable environments of the human gastric mucosa. *H. pylori* does this by genome shuffling and randomly disabling or enabling its outer membrane proteins to vary their interactions with the host, aided by its rather poor DNA repair repertoire, so that small mutations continually occur whereby new variations of all its genes are constantly being tested. Understanding how the *H. pylori* genome works also explains how the bacterium imports blocks of genes such as the *cagA* pathogenicity island, a secretion system that has a distinctly different G+C content from the rest of the organism's DNA, showing that it was imported 'en bloc' from a different organism, though which one we have no idea! After finding a home in the human stomach, *H. pylori* then discarded many genes because it now lived exclusively in a rather hostile but constant environmental niche, with few competitors, and its excursions into

the outside world were probably rare and rather short-lived, as its new host was almost always in the same human family. The authors explain how tracing human migrations using polymorphisms in essential housekeeping genes (via multilocus sequence typing, MLST) has become an area of research with wide interest and general implications for the human family. These authors enhance their chapter with their own deep insights into the genomic strategies whereby *H. pylori* survives in its acidic niche.

To sum it up then, this is an industrial-strength book containing up-to-date reviews on *Helicobacter* science, each presented by a world expert in that particular area. The first third of the book contains reviews of the issues of interest to clinicians, i.e. epidemiology, microbiology, pathophysiology and therapeutics. The rest of the book delves minutely into a diverse range of fundamental issues arising in the bacterium itself (carbohydrates, lipids and toxins) and their effects on the host's immune systems, innate and adaptive. This book is action-packed, fact-filled and thoughtfully assembled. I look forward to having it on my laboratory shelf for years to come.

Barry Marshall
December 2009

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1 Epidemiology of *Helicobacter pylori* Infection

H.M. MALATY

1.1 Introduction

Helicobacter pylori infection is now recognized as a worldwide problem. *H. pylori* infection is the most common cause of chronic gastritis, and has been strongly linked to peptic ulcer disease and gastric cancer. *H. pylori* is estimated to infect one-half of the world's population. The epidemiology of infection reveals that given the right circumstances it is readily transmissible. Infection is generally acquired in childhood, but disease manifestations typically do not appear until adulthood and often only after long periods of latency. The infection has a high morbidity rate, but a low mortality rate, and is curable with antibiotic therapy.

1.2 Relationship between *H. pylori* Infection and Associated Diseases

H. pylori infection is causally related to chronic gastritis and peptic ulcer disease, and indirectly related to gastric adenocarcinoma and primary gastric B-cell lymphoma (Forman *et al.*, 1990, 1991; Tytgat *et al.*, 1993; Peura and Graham, 1994). Infection with *H. pylori* leads to the development of gastric damage, and of those infected approximately 25% may ultimately develop low gastric acid production (achlorhydria), which is associated with an increased susceptibility to the development

of gastric cancer (see Correa and Piazuelo, Chapter 3, this volume; Graham *et al.*, 1988). Approximately 17% of infected patients develop peptic (gastric and duodenal) ulcers, and one-quarter of such patients experience an ulcer complication (Tytgat *et al.*, 1993). Numerous trials have shown that ulcer relapse is prevented after cure of the infection (Graham *et al.*, 1992). Histological and serological studies have also shown that infection precedes the ulcer and thus *H. pylori* infection is now accepted as one of the two major causes of peptic ulcers, the other being use of non-steroidal anti-inflammatory drugs (NSAIDs). In an evaluation of 100 consecutive duodenal ulcer and 154 gastric ulcer patients in a Veteran Affairs population in Houston, Texas, 99% of those with duodenal ulcers were found to be positive for *H. pylori*, 30% of whom were also found to be using NSAIDs (Al-Assi *et al.*, 1996), and 92% of patients with gastric ulcers were infected with the bacterium, 58% of whom were also taking NSAIDs. These findings were confirmed in a recent study by Gisbert and Calvet (2009) that reviewed the real prevalence of *H. pylori*-negative duodenal ulcer and its possible causes. This study reported that, in truly *H. pylori*-negative patients, the single most common cause of duodenal ulcer is, by far, the use of NSAIDs.

More sophisticated approaches looking for evidence of cyclooxygenase-1 inhibition

have shown that NSAID use is much more common than expected in peptic ulcer patients (Al-Assi *et al.*, 1996) and probably accounts for a sizeable proportion of cases. Additional possibilities include other infections such as herpes simplex virus and other drugs such as alendronate (Gisbert and Calvet, 2009), as well as misdiagnosis by the endoscopist. Peptic ulcer disease is a chronic relapsing disorder, with typical morphological and endoscopic appearances. One important reason for the observed differences in the ulcer prevalence and ulcer recurrence rates in clinical trials is the interpretation of the findings by endoscopists, who are possibly the most likely source of variability in such studies.

The exact prevalence of *H. pylori* infection in gastric cancer patients remains difficult to estimate, because infection can be lost from individuals with cancer or its precursor conditions (see Correa and Piazuelo, Chapter 3, this volume). In a study by Yamaji *et al.* (2002), sera from 10,234 consecutive Japanese individuals who participated in a health examination programme were tested for the presence of antibodies against *H. pylori* by immunoglobulin G (IgG) ELISA. While this study confirmed the relationship between serum positivity and gastric cancer, these authors pointed out that, particularly in the elderly, a weak *H. pylori* antibody response carried a high risk for gastric cancer. A Swedish population-based case-control study, using an immunoblot for the cytotoxin-associated gene A protein (CagA) (rather than an IgG ELISA) to detect infection, showed the CagA immunoblot to be a more sensitive assay for detecting past infection. The study reported that the estimated proportion of non-cardia adenocarcinoma attributable to *H. pylori* was 71% for both histological subtypes (odds ratio (OR) = 21, 95% confidence interval 8.3, 53.4), ranking with the association reported between smoking and lung cancer (Ekstrom *et al.*, 2001).

Atrophic gastritis and intestinal metaplasia are well-established precursor lesions of the intestinal type of gastric cancer (see Correa and Piazuelo, Chapter 3, this volume). A study from Japan followed up 1526 patients with duodenal ulcers, gastric ulcers, gastric hyperplasia or non-ulcer dyspepsia, of whom

1246 had *H. pylori* infection and 280 did not, for a mean period of 7.8 years (Uemura *et al.*, 2001). Gastric cancer was shown to develop in 36 (2.9%) of the infected subjects compared with none of the uninfected patients. Among the patients with *H. pylori* infection, those with severe gastric atrophy, corpus-predominant gastritis and intestinal metaplasia were at significantly higher risk for gastric cancer. The authors detected gastric cancers in 21 (4.7%) of the 445 patients with non-ulcer dyspepsia, ten (3.4%) of the 297 with gastric ulcers, five (2.2%) of the 229 with gastric hyperplastic polyps, and none of the 275 with duodenal ulcers. The study concluded that gastric cancer develops in persons infected with *H. pylori* but not in uninfected persons. Another study from Japan reported the results of a multicentre study to evaluate the relationship between *H. pylori* infection, atrophic gastritis and intestinal metaplasia (Asaka *et al.*, 2001). The study reported that the prevalence of atrophic gastritis increased from 9.4% in individuals less than 20 years of age to >70% in those aged 60 years or older, and was strongly associated with *H. pylori* infection. The overall prevalence of atrophic gastritis in *H. pylori* infection was 82.9%, compared with 9.8% among those uninfected (OR = 44.8). Intestinal metaplasia was present in 43.1% of *H. pylori*-positive persons compared with 6.2% among the uninfected (OR = 11.5). The authors concluded that atrophic gastritis and intestinal metaplasia were strongly associated with *H. pylori* and not with ageing. These data suggest that the risk of development of early gastric cancer will continue to remain high in Japan.

1.3 Geographical Distribution of the Prevalence of *H. pylori* Infection

The prevalence of *H. pylori* infection varies from country to country, with the largest differences being observed between developed and developing countries (Megraud *et al.*, 1989; Graham *et al.*, 1991a; Taylor and Blaser, 1991; Bardhan *et al.*, 1998; Redlinger *et al.*, 1999) (Fig. 1.1). The epidemiology of *H. pylori* infection in developing countries such

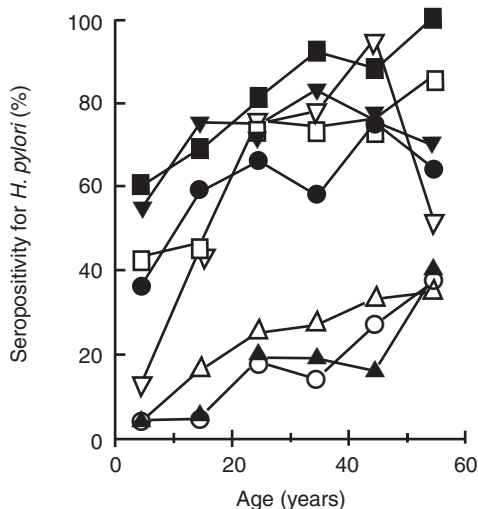


Fig. 1.1. Comparison of the prevalence of *Helicobacter pylori* infection in industrialized and non-industrialized countries (■, India; □, Saudi Arabia; ▼, Ivory Coast; ●, New Guinea; ▽, Vietnam; ▲, UK; ○, Australia; △, France), as indicated by the presence of serum immunoglobulin G antibodies to *H. pylori* antigens. In both regions acquisition takes place primarily in childhood, and the higher rate of acquisition among children in developing countries accounts for the large difference in the prevalence between industrialized and non-industrialized countries. (Adapted from Graham *et al.*, 1991a.)

as India, Saudi Arabia and Vietnam is characterized by a rapid rate of acquisition of infection such that approximately 80% of the population is infected with the bacterium by the age of 20 (Megraud *et al.*, 1989; Al-Moagel *et al.*, 1990; Graham *et al.*, 1991a). Because the infection is most often acquired in childhood (Mitchell *et al.*, 1992; Malaty *et al.*, 1996a, 1999, 2001, 2002), unless the infection is treated, it persists during adulthood. The prevalence of infection varies between and within countries and within subpopulations within the same country (Pateraki *et al.*, 1990; Perez-Perez *et al.*, 1990; Graham *et al.*, 1991b; EUROGAST Study Group, 1993; Breuer *et al.*, 1996). For example, a study conducted in Houston, Texas, of 485 asymptomatic individuals (50% black and 50% white) aged 15–80 years of age showed that the prevalence of *H. pylori* infection increased with age (Graham *et al.*, 1991b). These results are similar to patterns observed

in other developed countries such as the UK, Australia and France where an increasing prevalence of infection with age has been observed. The increase in prevalence of infection with age can be attributable either to new acquisition of infection among the adult population or to the presence of different birth cohorts, each with a different rate of acquisition in childhood, within the population. Current data suggest that the increase with age is actually related to different birth cohorts, and reflects that each successively younger cohort has had a lower rate of acquisition of infection than those born earlier (Parsonnet *et al.*, 1992; Banatvala *et al.*, 1993). *H. pylori* infection has been shown to follow the routes of human migration by their geographical origin, and several studies have examined the effect of immigration on the prevalence of the infection. One recent study examined *H. pylori* strains among three major ethnic groups in Malaysia (namely the Malay, Chinese and Indian populations), reporting that while the majority of the Malay and Indian *H. pylori* isolates share the same origin, the origin of the Malaysian Chinese *H. pylori* is distinctive (Tay *et al.*, 2009). The study concluded that the Malay population was likely to have been initially *H. pylori*-free and gained the pathogen recently from cross-infection from other populations.

It has been also established that the prevalence of *H. pylori* is inversely related to socio-economic status (Graham *et al.*, 1991b; Malaty *et al.*, 1996a,b, 2001) with the major variable being the status during childhood, the period of highest risk of acquisition. Attempts to understand the different rates of infection in defined groups have focused on differences in socio-economic status as defined by occupation, family income level and living conditions. Each of these variables measures a different component of the socio-economic complex. Within the USA, studies in a cohort of blacks and white Hispanics examined the relationship between current and childhood socio-economic status and the prevalence of *H. pylori* infection (Malaty *et al.*, 1992). The study showed that there was an inverse correlation between low socio-economic status during childhood and the prevalence of *H. pylori* infection, irrespective

of the present social class. These results were later confirmed in a study conducted in Korea (Malaty *et al.*, 1996a). For populations in which the social class is more or less homogeneous, such as China and Russia, density of living has been shown to be the most significant risk factor (Sitas *et al.*, 1991; Mitchell *et al.*, 1992; Malaty *et al.*, 1996b). Similar findings have been reported in a number of other countries (Monno *et al.*, 2008; Cheng *et al.*, 2009). A study of the effect of childhood conditions carried out in monozygotic and dizygotic twins, reared together or apart and differing in socio-economic status, revealed that the strongest effects on the acquisition of *H. pylori* infection were density of living and low household income during childhood (Malaty *et al.*, 1998). Variation in acquisition of infection among ethnic and racial groups appears to be primarily related to differential exposure (e.g. cultural background; social, dietary and environmental factors) (Graham *et al.*, 1991b; Mitchell *et al.*, 1992; Malaty *et al.*, 1998) and not to possible differences in genetic predisposition (Malaty *et al.*, 1992).

The finding regarding socio-economic status during childhood holds true for all subgroups in the USA. For instance, in a paediatric study conducted in Arkansas, children (aged 11–20 years) from families with an income of <US\$5000/year had prevalence rates of up to 60%, whereas those with a family income of >US\$25,000/year had only a 15% rate of infection (Fiedorek *et al.*, 1991). This phenomenon has also been observed in other countries. In recently developed countries such as Korea, although approximately 80% of individuals more than 20 years of age are infected (reflecting the fact that Korea has become a developed country only within the past 20 years) the prevalence of infection in young children is inversely related to the socio-economic class of their family. Those aged 10–19 years who were from a high socio-economic class had a 20% frequency of *H. pylori* infection, while those of the same age who were from a low socio-economic class had a 60% frequency (Malaty *et al.*, 1996a). A cross-sectional study conducted in children residing in industrial and rural areas of Italy reported that the seroprevalence of *H. pylori* infection was significantly higher in children

residing in rural areas compared with those residing in industrial areas (Dore *et al.*, 2002). The study also found that, in rural areas, children having dogs (most commonly shepherd dogs) were at greatest risk for *H. pylori* acquisition, but this effect was not observed among children from the industrial areas.

1.4 Changing Prevalence of *H. pylori* Infection

While the rate of *H. pylori* infection in the USA is low among white and economically advantaged Americans, minority populations have a high frequency of infection. Data from the USA indicate that black children aged 5–9 years have an overall infection frequency of 30%, which is three times higher than that in white children (Malaty *et al.*, 2002). Around the world, the frequency of infection in children aged 3–19 years ranges from approximately 35% in Russia, to 20% in China and Poland, 12% in Korea and the USA, and <10% in France, Belgium and Finland (Ashorn *et al.*, 1995; Malaty *et al.*, 1996a,b; Matysiak-Budnik *et al.*, 1996; Mitchell, 1997). Despite high rates of infection in certain pockets of the globe, the frequency of *H. pylori* infection is declining worldwide. For instance, in one study conducted in Matsumoto, Japan, the rate of infection declined by up to 20% between 1986 and 1994 among individuals aged 9–70 years (Kumagai *et al.*, 1998).

In two cross-sectional studies conducted in Russia, the effect of recent improvements in the standard of living on the prevalence of *H. pylori* infection was examined in Russian children resident in St Petersburg. In both the first study conducted in 1995 and the second conducted a decade later, *H. pylori* status was evaluated using the same ELISA method to measure anti-*H. pylori* IgG (Malaty *et al.*, 1996b; Tkachenko *et al.*, 2007). These studies showed that the overall prevalence of *H. pylori* infection in 1995 was 44%, which decreased to 13% 10 years later. In both studies, the prevalence of infection increased with age. Interestingly, while in 1995 the prevalence of infection among children younger than 5 years of age was 30%, a decade later the prevalence in the

same age group was only 2%. The study concluded that improvements in the standard of living in Russia had resulted in a marked reduction in *H. pylori* transmission. Different rates of acquisition of *H. pylori* form the basis for the differences in the prevalence of infection between and among populations. The changes in Russia are a dramatic example of how sensitive *H. pylori* acquisition appears to be to improvement in standards of living and/or the availability of *H. pylori* treatment (Malaty *et al.*, 1996b; Tkachenko *et al.*, 2007).

1.5 Routes of Transmission

To date, there is still an ongoing debate about the exact mode of transmission of *H. pylori* infection. Person-to-person transmission through faecal–oral or oral–oral transmission has been supported by numerous research studies. The majority of data support the notion that transmission is within families (Mitchell *et al.*, 1993; Weyermann *et al.*, 2009). In one study, *H. pylori* status was determined in 41 families, with ‘family’ being defined as a husband and wife living together with biological children (Malaty *et al.*, 1991). Prior to disclosure of the results of *H. pylori* testing,

one of the parents was randomly selected as the index case. The results revealed that, if the index case (either the mother or father) was positive, the children and spouse in that family were also likely to test positive for *H. pylori* (Fig. 1.2). If the index case was negative, then the children and spouse were likely to be negative as well. Hence in that study performed in the USA, there was no difference whether the index case was the father or the mother (Malaty *et al.*, 1991). *H. pylori* strains isolated from two Hispanic families living in Houston were characterized on the basis of their *cagA*, *vacA* (vacuolating cytotoxin) and *iceA* (induced by contact with the epithelium) genotypes. While *H. pylori* isolates from children and their mothers had the same genotype, they were different from those associated with the children’s father (Yamaoka *et al.*, 2000). This finding emphasizes the importance of contact between mother and child for the transmission of the bacterium. In countries where the father has little contact with the children (e.g. in Japan), infection appears to be almost always linked to the mother (Malaty *et al.*, 2000a). Thus, close contact and level of household sanitation appear to be the important variables (Nouraie *et al.*, 2009; Weyermann *et*

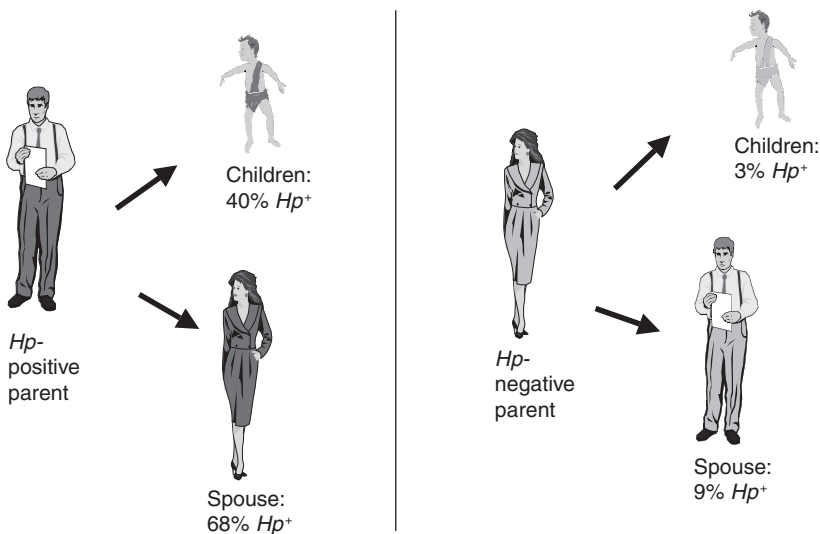


Fig. 1.2. The dynamics of transmission of *Helicobacter pylori* (*Hp*) infection in family members and *Hp* status of the index parent. (Adapted from Malaty *et al.*, 1991.)

al., 2009). Another study examined the entire genome of *H. pylori* by PCR-based, random amplified polymorphic DNA (RAPD) fingerprinting, among 32 members of 11 families, for the presence of genetic homogeneity (Roma-Giannikou *et al.*, 2003). In this study RAPD fingerprinting confirmed that closely related *H. pylori* strains were involved in intra-familial dispersion and strongly supported the hypothesis of transmission of *H. pylori* from person to person or from a common source. Transmission between siblings by their birth order has also been reported. A study conducted on 684 rural Colombian children aged 2–9 years found that the odds of infection increased with the number of 2–9-year-old siblings in the household (Goodman and Correa, 2000). That study concluded that *H. pylori* infection seems to be transmitted most readily among siblings who are close in age, and most frequently from older siblings to younger ones. These findings support the concept that the likely sources of transmission are person-to-person transmission and/or exposure to a common source of infection.

The rationale for the oral–oral route of transmission is based on the premise that *H. pylori* is present in the gastric juice and thus could reach the oral cavity through reflux or vomitus. An early study reported that *H. pylori* can be cultivated uniformly from vomitus and concluded that *H. pylori* is potentially transmissible during episodes of gastrointestinal tract illness, particularly with vomiting (Parsonnet *et al.*, 1999). The mechanism of spread of the organism, whether by the faecal–oral or oral–oral route, raises the possibility of transmission of this organism from infected patients to hospital staff, particularly those involved in endoscopy. Evidence for an increased risk of *H. pylori* infection in endoscopists is contradictory, varying from none to a fivefold increase (Nelson and Muscarella, 2006).

The transmission of *H. pylori* through exposure to contaminated food or water is still very controversial. There is mounting evidence which suggests that the prevalence of *H. pylori* infection has a strong correlation with access to clean water, suggesting a transmission route to the host. Studies from

Peru and Kazakhstan found contaminated water to be a risk factor for *H. pylori* transmission (Klein *et al.*, 1991; Nurgalieva *et al.*, 2003). A study among unrelated asymptomatic individuals between 10 and 60 years of age examined various aspects of the local household environment and access to water (Nurgalieva *et al.*, 2003). The authors reported that the transmission of *H. pylori* could be waterborne or water-washed or both (related to poor sanitary practices) and concluded that reducing the rate of *H. pylori* transmission will require an improvement in overall sanitation, including waste disposal, clean water and safe food, as well as in household hygiene practices. In a study from India that examined 500 adults of varying ages ranging from 30 to 79 years, three biopsy samples were collected from each subject to assess *H. pylori* infection (Ahmed *et al.*, 2007). Based on detection by PCR amplification of the gene encoding 16S rRNA from *H. pylori*, the prevalence of infection among people who drank water from wells was 92%, compared with 75% in those who drank tap water ($P < 0.001$). *H. pylori* infection prevalence was found to be higher in people with a low clean water index (88%) than in those with a higher clean water index (33%) ($P < 0.001$). The results of the study suggested that the risk of acquisition and transmission of *H. pylori* can be prevented to a large extent by regular boiling of water used for drinking purposes. However, the lack to date of established culture methods for the detection of viable *H. pylori* in the environment, in particular drinking water supplies, has prevented the development of true epidemiological and risk assessments.

A study from Japan investigated the presence of *H. pylori* in cow's milk as this is one of the foods that most Japanese children consume (Fujimura *et al.*, 2002). Detection of *H. pylori* was demonstrated by semi-nested PCR, a culture method and by electron microscopy. *H. pylori* was cultured from one raw milk sample, whereas it was not cultured in pasteurized milk samples. The study concluded that there is a possibility that cow's milk is a transmission vehicle in childhood *H. pylori* infection, although the study failed to confirm the survival of *H. pylori* in pasteurized milk.

1.6 Changes in the Environment and Human Behaviour that Influence the Prevalence of *H. pylori* Infection

It is now apparent that the decrease in the age-adjusted prevalence of *H. pylori* infection in industrialized countries is related to both improved living conditions during childhood and loss of infection. Although *H. pylori* infection is chronic, and possibly lifelong, spontaneous elimination of the infection was reported as early as 1992 using serological tests and was confirmed using breath tests (Klein *et al.*, 1994) and histology (Guelrud *et al.*, 1994) in both developed and underdeveloped countries. These observations have subsequently been confirmed in a number of populations (Matysiak-Budnik *et al.*, 1996; Cranstrom *et al.*, 1997; Roosendaal *et al.*, 1997). A longitudinal study, conducted over a 12-year period in 212 black and white children living in the same community and attending the same schools, has provided additional insights into the changing pattern of infection (Malaty *et al.*, 1999). The study showed differences to exist between the two races. At ages 7–9 years, 19% of children had *H. pylori* infection (40% of

blacks versus 11% of whites; $P = 0.001$). After a 12-year period of observation, more black children remained infected (or were more likely to become re-infected) compared with white children, in whom the infection was lost in 50% during the observation period. This suggested that the higher rate of acquisition and the lower rate of loss of infection among black children might be due to differences in access to healthcare facilities or more intense exposure, for whatever reason. The high rate of loss of *H. pylori* infection among white children was not related to *H. pylori* 'eradication therapy' as the infection had not yet been diagnosed.

A cohort study conducted in Japanese children and adults from a typical mountain village evaluated the seroepidemiology of *H. pylori* infection over a 9-year period, and also compared the results with the age-specific seroprevalence from two independent cross-sectional surveys conducted in the same population, over the same period (Kumagai *et al.*, 1998). The study also found the rate of disappearance of *H. pylori* infection to be greater than the rate of acquisition, and these results were confirmed in the two cross-

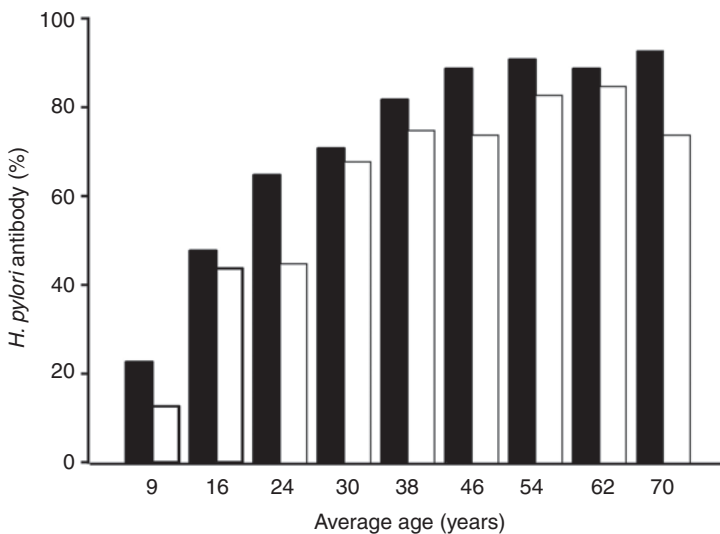


Fig. 1.3. The seroprevalence of *Helicobacter pylori* infection in two cross-sectional studies conducted in 1986 (■) and 1994 (□). Each pair of bars represents the prevalence of *H. pylori* infection in persons of a specific age range (birth cohort) in 1986 and the same cohort 8 years later (e.g. the 24-year-old age group shows the seroprevalence for the 20–27-year-old age group in 1986 and the 28–35-year-old age group in 1994). The prevalence of *H. pylori* infection declined overall and in each birth cohort.

sectional studies. Of particular interest was the observation that there was a decrease in prevalence in every age group (Fig. 1.3). The fall in prevalence that occurred during the observation period did not reflect changes in the rate of acquisition of *H. pylori* in childhood but rather the higher rate of loss of infection. The continuing change in the epidemiology of *H. pylori* infection complicates our understanding of the actual prevalence of *H. pylori*. The higher rate of loss of infection as compared with acquisition may be related to changes in standards of living in successive generations, or to changes in medical practices leading to increased use of antimicrobials for other common infections.

It is impossible to entirely separate environmental factors from genetic influences. An early twin study in the Swedish population suggested a genetic component for acquiring *H. pylori* infection (Malaty *et al.*, 1994) and a follow-up study in the same twin population has questioned if there are genetic influences for peptic ulcer disease in common with genetic influences for *H. pylori* infection (Malaty *et al.*, 2000b). A comparison of monozygotic and dizygotic cross-twin and cross-trait correlations in this Swedish population demonstrated that, despite the similarity in heritability for the two traits (peptic ulcer disease and *H. pylori* infection), the genetic influences for susceptibility to peptic ulcer disease were independent of the genetic effects for acquiring *H. pylori* infection. It is feasible that the relationship between *H. pylori* and peptic ulcer disease could be mediated by familial environmental factors, such as environmental experiences or situations that are shared by family members. Examples of familial environmental factors that may mediate the association between *H. pylori* and peptic ulcer disease are diet, smoking and drug consumption (e.g. alcohol, caffeine and/or NSAID consumption).

1.7 The Prevalence of Infection-induced Ulcers is Falling in Western Countries

Since the early 1980s, the occurrence of peptic ulcer disease has declined remarkably in the

USA, Europe, Australia and Japan (Vogt and Johnson, 1980; Wylie, 1981; Smith, 1997). The risk of peptic ulcers was highest among those born at the beginning of the 20th century and has decreased in all subsequent generations (Susser, 1982; Sonnenberg *et al.*, 1985). However, this is not exactly the case in Asia (see Fock, Chapter 2, this volume). Due to the rapidity of this change in the pattern of peptic ulcer disease in successive generations (birth cohort phenomenon), it is far more likely to be due to changes in environmental factors rather than to changes in the genes of the affected patients (Sonnenberg *et al.*, 1985). Another possibility is that a change has occurred in the prevalence of particularly virulent *H. pylori* strains (e.g. *cag* pathogenicity island-positive; see Backert *et al.*, Chapter 11, this volume). However, serological studies have shown that the prevalence of identified *H. pylori* putative virulence factors has not changed in any population over time, which can be interpreted in two ways. Either we have not examined the critical factors, or this is not the correct explanation. The latter appears most likely.

In the past there has been interest in identifying genetic factors that might play an important role in the aetiology of peptic ulcer disease (Jensen, 1972; McConnell, 1980). However, the pattern of inheritance is not simple Mendelian and it has been suggested that the genetic basis of peptic ulcer disease is multifactorial (see Sutton *et al.*, Chapter 7, this volume). Initially, the strongest evidence to support a genetic influence in peptic ulcer disease came from studies showing an increased risk of duodenal ulcer in individuals with hyperpepsinogenaemia (Neiderman *et al.*, 1964; Rotter *et al.*, 1979). This evidence was discounted, however, when it became evident that elevated serum pepsinogen I was also a feature of *H. pylori* infection (Asaka *et al.*, 1992; Fraser *et al.*, 1992). The fact that family members of patients with peptic ulcer disease are at increased risk can be explained by the fact that they are at increased risk of infection (clustering effect of infection in families), they are more likely to have a similar strain, or they share(d) a common environment. The marked geographical variation in the incidence rates of peptic ulcer disease in different parts of the world along with the rapid rate of change in

incidence provides strong evidence that environmental factors are the most important features that modulate the development of an ulcer.

1.8 Conclusions

Epidemiological studies of *H. pylori* show acquisition in early childhood. However, infection often remains asymptomatic in children and, except for peptic ulcer disease (which is rare in childhood), a relationship between abdominal pain and *H. pylori* infection is not demonstrated. It is inappropriate to prescribe anti-*H. pylori* therapy without a firm diagnosis. The use of multiple antibiotic-containing regimens in a paediatric patient with an ulcer but without *H. pylori* cannot provide any benefit to the patient or the community. Most studies are in favour of intra-familial transmission, and *H. pylori* prevalence in mothers is a crucial determinant for the child's risk of being infected. The prevalence of *H. pylori* infection in a community depends on the rate of acquisition of the infection (i.e. incidence of the infection). The rate of acquisition of *H. pylori* infection varies remarkably between populations and is higher in non-industrialized countries than in industrialized countries. Several gastrointestinal and non-gastrointestinal diseases (see Mitchell *et al.*, Chapter 5, this volume) have been reported to have a significant association with *H. pylori* infection. We therefore need to discover why ulcers or cancer occur in so few of those infected with *H. pylori* and how this subgroup can be identified and treated.

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2 *Helicobacter pylori* Infection in Asia

K.M. Fock

2.1 Introduction

Helicobacter pylori is a micro-aerophilic, spiral-shaped, Gram-negative bacterium that colonizes the human stomach. Globally, *H. pylori* infection affects 50% of the population (Correa and Piazzuelo, 2008). Although *H. pylori* was first described in 1892 by the Italian pathologist Giulio Bizzozzero, its role in the pathogenesis of gastritis and peptic ulcer disease was discovered in Perth, Australia, by Barry Marshall and Robin Warren. This discovery has led to a major change in the treatment of peptic ulcer disease. In 1994, the International Agency for Research on Cancer (IARC) listed *H. pylori* as an aetiological factor in the pathogenesis of gastric carcinoma (Anwar *et al.*, 1994). As two-thirds of cases of stomach cancer occur in Asia, *H. pylori* and gastric cancer has been the subject of intensive research in the Asia Pacific region. This chapter focuses on the epidemiology of *H. pylori* infection and gastric cancer in Asia, strategies for preventing gastric cancer targeted at *H. pylori* eradication and treatment regimens used in Asia.

2.2 Epidemiology of *H. pylori* Infection

A study of the epidemiology of *H. pylori* in the Asia Pacific region reveals that there is a wide

variation in the prevalence of *H. pylori* infection, both between countries and within countries, as well as between ethnic communities within countries. In general, the seroprevalence rates in developing or less-developed countries are higher than in developed countries (Table 2.1). In Iran, the reported *H. pylori* seroprevalence rate (from children to adults) was 71% (Alizadeh *et al.*, 2009). In India, the reported overall seroprevalence rate was 79% (Graham *et al.*, 1991). In Vietnam, the *H. pylori* seroprevalence rate was 75% (Hoang *et al.*, 2005). On the other hand, the seroprevalence rates in more developed countries in the Asia Pacific region are generally lower. For example in Australia, the overall seroprevalence rate has been reported to be 15% (Moujaber *et al.*, 2008). In Asian countries that became developed or industrialized in recent years, the seroprevalence rates are higher than in Australia, but still considerably lower than in less-developed countries. Among North-east Asian countries, the overall seroprevalence rate was 58% in China (Wang and Wang, 2003), 39% in Japan (Fujisawa *et al.*, 1999), 55% in Taiwan (Teh *et al.*, 1994) and 60% in South Korea (Yim *et al.*, 2007). Among South-east Asian countries, the reported seroprevalence rate was 36% in Malaysia (Goh and Parasakthi, 2001), 31% in Singapore (Fock, 1997) and 57% in Thailand (Deankanob *et al.*, 2006).

Table 2.1 shows that, in general, countries with a high seroprevalence of *H. pylori*

Table 2.1. Seroprevalence of *Helicobacter pylori* and gastric cancer incidence in Asia.

Country/region	<i>H. pylori</i> seroprevalence (%)	Age-standardized incidence rate of gastric cancer (per 100,000)		Reference(s)
		Males	Females	
<i>Low gastric cancer risk</i>				
Australia	15.1	9.8	4.1	Ferlay <i>et al.</i> (2004); Moujaber <i>et al.</i> (2008)
India	79	5.7	2.8	Graham <i>et al.</i> (1991); Ferlay <i>et al.</i> (2004)
Thailand	57	4.3	2.9	Ferlay <i>et al.</i> (2004); Deankanob <i>et al.</i> (2006)
<i>Intermediate gastric cancer risk</i>				
Hong Kong	58.4	19.3	9.6	Wong <i>et al.</i> (1999)
Malaysia				Goh and Parasakthi (2001); Fock <i>et al.</i> (2008)
Overall	35.9			
Chinese	26.7–57.8	11.9	8.7	
Malay	11.9–29.3	2.6	1.3	
Indian	49.4–52.3	12.9	7.9	
Singapore				Fock (1997); Fock <i>et al.</i> (2008)
Overall	31			
Chinese	48.3	21.4	10.8	
Malay	27.9	6.6	3.8	
Indian	48.1	7.8	6.1	
Taiwan	54.5	18.6	10.5	Teh <i>et al.</i> (1994); Fock <i>et al.</i> (2008)
Vietnam	74.6	21.8	10.0	Ferlay <i>et al.</i> (2004); Hoang <i>et al.</i> (2005)
<i>High gastric cancer risk</i>				
China	58.07	41.4	19.2	Wang and Wang (2003); Ferlay <i>et al.</i> (2004)
Japan	39.3	62.1	26.1	Fujisawa <i>et al.</i> (1999); Ferlay <i>et al.</i> (2004)
Korea	59.6	69.7	26.8	Ferlay <i>et al.</i> (2004); Yim <i>et al.</i> (2007)
Iran	71	51.2	15.4	Derakhshan <i>et al.</i> (2004); Alizadeh <i>et al.</i> (2009)

infection have a high or intermediate risk of gastric cancer, with the exceptions of Thailand, India and possibly Vietnam. Much research has been undertaken to understand this phenomenon, variously referred to as the 'Indian dilemma', the 'Thai dilemma', etc. This phenomenon could be due to differences in host genetic factors or bacterial virulence factors in *H. pylori* strains prevalent in these regions – a topic addressed in Section 2.4 on molecular epidemiology.

In each country, differences in seroprevalence rates between different geographic regions and also between different ethnic groups have been reported. In Australia, for example, the Anglo-Celtic population has been shown to have a lower seroprevalence rate compared with the Aboriginal population, the rates being 38% (Lin *et al.*, 1998) and 68% (Windsor *et al.*, 2005), respectively. In China, seroprevalence rates have been noted to be higher in regions with higher gastric

cancer incidence rates. For example, the seroprevalence of *H. pylori* in Changde, northern China, was reported to be 80% (Wong *et al.*, 1999) compared with 62.5% in Guangzhou, southern China (Chen *et al.*, 2007). In Malaysia, the seroprevalence rate has been reported to be lower in west Malaysia (26–31%) than in east Malaysia (43–55%) (Goh and Parasakthi, 2001). In Taiwan, the highest seroprevalence rate (63%) has been reported in rural areas where the aborigines live and in which gastric cancer rates are highest. This compares with a prevalence rate of 40.5% in urban areas, where gastric cancer rates were lowest (Teh *et al.*, 1994). In Vietnam, differences in *H. pylori* seroprevalence rates have been reported between an urban area (Hanoi; 79%) and a rural area (Hatay; 69%) (Hoang *et al.*, 2005).

While it is generally agreed that *H. pylori* infection has been declining in Asia, the data supporting this impression are limited. In a study from Guangzhou province in China, the overall *H. pylori* seroprevalence rate was reported to have decreased from 62.5 to 47% in the period between 1993 and 2003 (Chen *et al.*, 2007). Similarly, in Japan, the overall seroprevalence rate declined from 73% in 1974 to 55% in 1984 and declined even further to 39% in 1994 (Fujisawa *et al.*, 1999). In South Korea, the seroprevalence rate decreased from 67% in 1998 to 60% in 2005 (Yim *et al.*, 2007). In particular, lower seroprevalence rates have been observed in the younger population.

In Australia, the prevalence rate in those aged 1–4 years has been reported to be 4% but increased to 23% among those aged 50–59 years (Moujaber *et al.*, 2008). In Malaysia, among those aged less than 45 years, reported seroprevalence rates ranged from 25 to 41%, whereas among those aged more than 45 years, the rates ranged from 31 to 57% (Goh and Parasakthi, 2001). In Singapore, the seroprevalence rate in those aged less than 3 years has been reported to be 3%, but rose to 71% among those aged more than 65 years (Fock, 1997). In Taiwan, the seroprevalence rate among subjects less than 10 years of age was 27% compared with 72% in adults older than 40 years (Teh *et al.*, 1994). In a study of asymptomatic subjects from New Delhi, India, a stepwise increase in seroprevalence rate was evident with increasing age. Among subjects

less than 10 years old, the rate was 39%, which increased to 52% among those aged 10–19 years and 60% among those aged 20–29 years; by 30–39 years, the seroprevalence rate was 68% (Chen *et al.*, 2007). In Thailand, among those aged 5–9 years, the seroprevalence rate has been reported to be 17.5%, which was found to increase to 75% among those aged 30–49 years (Moodley *et al.*, 2009).

Current evidence would therefore indicate that in most countries in Asia the rate of *H. pylori* infection has been decreasing over the last 40–50 years, with an overall decline in *H. pylori* seroprevalence in Asia similar to that observed in Western developed countries. While the awareness and diagnosis of *H. pylori* infection have led to increased use of eradication therapies, the major decline in *H. pylori* seroprevalence is probably associated with socio-economic development in Asia. With development, there is an improvement in public health measures, personal hygiene and living conditions. Consequently, childhood infections have decreased, leading to a lower seroprevalence rate of *H. pylori* in the younger generations, thus lowering the overall seroprevalence rate in the population.

The decline in *H. pylori* infection in Asia has been matched by a decline in gastric cancer incidence and gastric cancer mortality, although there are uncertainties regarding the quantification of this association. In Japan, for example, with the fall in *H. pylori* prevalence between 1986 and 1996 the fall in gastric cancer mortality has been greater in younger age groups (20–39 years) in comparison with the overall gastric cancer mortality rate, which has long been declining (Kobayashi *et al.*, 2004).

A study in Singapore, where the population comprises immigrants from China and India and the Malays who were early settlers in the area, has demonstrated that Singaporean Chinese and Singaporean Indians have similar *H. pylori* seroprevalence rates and yet have significantly different gastric cancer incidence rates (Ang *et al.*, 2005). The fact that these ethnic groups live on a small island with an area of 704 km² makes it ideal for studying host genetic factors, bacterial virulence factors or dietary factors that could influence carcinogenesis.

2.3 Gastric Cancer Epidemiology in Asia

Gastric cancer arises as a consequence of a complex interaction between host factors, *H. pylori* infection and environmental factors. Correa's hypothesis outlines the steps towards the development of gastric cancer (see Correa and Piazuelo, Chapter 3, this volume). Gastric cancer is the fourth most common cancer in the world, with 934,000 new cases diagnosed annually. It is the second most common cancer in terms of mortality, with 700,000 deaths. Two-thirds of the cases are found in Asia and about 42% of patients are from China. The Asia Pacific can be subdivided into regions of high, intermediate and low gastric cancer risk. High-risk regions are mainly localized in East Asian countries such as China, Japan and Korea, where the age-standardized incidence rate (ASR) is greater than 20 per 100,000. The intermediate-risk areas, such as Singapore, Malaysia and Taiwan, have an ASR of 11–20/100,000. Low-risk areas (ASR < 10/100,000) include countries such as India, Australia and New Zealand. As stated above, gastric cancer risk correlates in general with *H. pylori* prevalence, with notable exceptions being the Indian subcontinent and the Indian immigrant populations of the South-east Asian countries of Singapore and Malaysia. The other exception is Thailand, where ethnic Chinese have a higher gastric cancer incidence than indigenous Thais, despite similar *H. pylori* infection rates. Parkin *et al.* (2002) estimated that about 63% of the gastric cancer cases worldwide are attributable to *H. pylori* infection. In a landmark study, Uemura *et al.* (2001) prospectively studied 1526 Japanese patients, of whom 1246 had *H. pylori* infection and 280 were uninfected. Subjects underwent endoscopy with biopsy at baseline and between 1 and 3 years after enrolment. Over a mean follow-up period of 7.8 years, gastric cancer developed in 2.9% of patients with *H. pylori* infection, but none of the uninfected patients developed gastric cancer, resulting in a relative risk of 34.5 (95% confidence interval (CI) 7.1, 166.7) for gastric cancer. This Asian study showed conclusively that *H. pylori* is an important aetiological agent in gastric carcinogenesis in Asia.

2.4 Molecular Epidemiology of *H. pylori*

The first complete genome sequence of *H. pylori*, reported in 1997 (Tomb *et al.*, 1997), showed that *H. pylori* strain 26695 has a circular genome of 1,667,867 base pairs and 1590 predicted coding sequences, and that it has well-developed systems for motility, for scavenging iron, and for DNA restriction and modification (see de Reuse *et al.*, Chapter 13, this volume). In addition, multiple adhesins, lipoproteins and other outer membrane proteins (OMPs) have been identified (see Moran, Chapter 10, this volume; Lindén *et al.*, Chapter 12, this volume). There is an immense diversity in *H. pylori* strains in terms of these characteristics. The clinical outcome of *H. pylori* infection is also diverse and includes chronic gastritis, with or without intestinal metaplasia, atrophic gastritis, peptic ulcer disease and gastric cancer. The reason for the differences observed in clinical outcome following *H. pylori* infection have been related to variability in bacterial virulence factors, host genetics (see Sutton *et al.*, Chapter 7, this volume), diet or a combination of these factors.

Multilocus sequence typing of the seven core housekeeping genes (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ure1*, *yphC*) of *H. pylori* isolates from different geographic regions has been conducted. On the basis of these analyses, six main geographic strains were identified, termed hpAfrica1, hpAfrica2, hpNEAfrica, hpEastAsia, hpAsia2 and hpEurope (Linz *et al.*, 2007). The hpEurope strain is common in Europe and countries colonized by Europeans, while hpEastAsia characterizes strains from East Asia. The hpEastAsia strain has been further classified into hspMaori (Polynesians), hspAmerind (native Americans) and hspEAsia (East Asia) subpopulations. The hpAsia2 strain was isolated from South and South-east Asia. It has been observed that populations with high gastric cancer rates correspond almost exactly to populations with hpEastAsia strains (Yamaoka *et al.*, 2008). In South Asian countries where *H. pylori* seroprevalence rates are high but gastric cancer prevalence rates are low, *H. pylori* strains have

been reported to be predominantly hpAsia2. Similarly, in Africa, most strains have been shown to be hpNEAfrica, hpAfrica1 or hpAfrica2, and the gastric cancer rates are also correspondingly lower than in East Asia (Linz *et al.*, 2007).

A number of *H. pylori* putative virulence genes have been proposed to play key roles in gastric carcinogenesis. These include the genes encoding cytotoxin-associated gene A protein (CagA), vacuolating cytotoxin (VacA) (see Backert *et al.*, Chapter 11, this volume) and OMPs (McNamara and El-Omar, 2008).

The presence of the *cagE* gene, which is located on the *cag* pathogenicity island (*cagPAI*) and has attracted considerable attention, has been associated with gastric cancer in some studies (Erzin *et al.*, 2006; Fock *et al.*, 2006a), but studies that failed to show this association have also been reported (Chomvarin *et al.*, 2008; Ghasemi *et al.*, 2008).

The *cagA* gene, also situated on the *cagPAI*, has also been associated with gastric cancer. A meta-analysis of 16 studies (comprising a total of 2284 cases and 2770 controls) showed that infection with CagA-positive strains of *H. pylori* increased the risk for gastric cancer (Huang *et al.*, 2003). Individually, *H. pylori* and CagA seropositivity significantly increased the risk for gastric cancer by 2.28- and 2.87-fold, respectively. However, among *H. pylori*-infected populations, infection with CagA-positive strains further increased the risk for gastric cancer by 1.64-fold (95% CI 1.21, 2.24) overall and by 2.01-fold (95% CI 1.21, 3.32) for non-cardia gastric cancer. In Asia, however, where the prevalence of CagA seropositivity is high, many reports have failed to demonstrate an association between *cagA* genotypes and gastric cancer.

More recent studies have shown that CagA is characterized by the presence of repeated five-amino-acid sequences (Glu-Pro-Ile-Tyr-Ala), designated EPIYA motifs, that are located at the C terminus of the protein. Four different EPIYA motifs (EPIYA-A, EPIYA-B, EPIYA-C and EPIYA-D) have been defined and, based on these, the CagA protein has been classified into Western and East Asian types (see Correa and Piazuelo, Chapter 3, and Backert *et al.*, Chapter 11, this volume). The Western type, prevalent in Europe,

America, Australia and Africa, contains EPIYA-A and EPIYA-B, followed by up to five repeated sequences of EPIYA-C, whereas the East Asian strain, which is dominant in Japan, Korea and China, possesses EPIYA-A, EPIYA-B and EPIYA-D (Nguyen *et al.*, 2008). The East Asian strain has been shown to be more virulent than the Western CagA strains with respect to clinical outcomes. Azuma *et al.* (2004) demonstrated that, in the gastric antrum and body, grades of inflammation, activity and mucosal atrophy are significantly higher in patients infected with East Asian CagA-positive strains than in those infected with Western CagA-positive strains. In addition, Satomi *et al.* (2006) have shown that in Okinawa, Japan, where both Western and East Asian CagA are present, the prevalence of East Asian CagA-positive strains is significantly higher in patients with gastric cancer (85%) compared with patients with duodenal ulcer (27%), with Western strains predominating in patients with duodenal ulcers. Similar results have been reported by Vilaichone *et al.* (2004), where both East Asian and South Asian types are found in infected groups in Thailand. The authors showed that 85% of *H. pylori* isolates from ethnic Chinese people living in Thailand harboured East Asian CagA, whereas only 18% of isolates from indigenous Thais carried East Asian CagA (Vilaichone *et al.*, 2004). Interestingly, gastric cancer is common among Thais of Chinese stock but rare among indigenous Thais. This study supports the theory that the differences in the EPIYA motifs of CagA may be associated with the risk of developing gastric cancer. In Malaysia and Singapore, which have multi-ethnic populations comprising Chinese, Malays and Indians, gastric cancer rates are significantly higher among the Chinese than the Malays and Indians. Interestingly, although the seroprevalence of *H. pylori* in Chinese and Indians is similar, the gastric cancer incidence in Chinese is three times that in Indians (Ang *et al.*, 2005). A recent collaborative study between Singapore, Malaysia and Australia showed that the East Asian strain was present in 90% of *H. pylori* strains isolated from Chinese subjects, compared with 38% from Malay subjects and only 7% from Indian subjects (Schmidt *et al.*, 2009). This observation could

explain the lower incidence of gastric cancer in Indian Singaporeans compared with Chinese Singaporeans.

The protein VacA, encoded by the *vacA* gene, is generally thought to be a virulence factor (see Backert *et al.*, Chapter 11, this volume). Genotypic variations in the *vacA* gene have been reported in *H. pylori* strains from different geographic regions and postulated to be associated with different disease outcomes (McNamara and El-Omar, 2008). Variations in the *vacA* gene structure can occur at both the signal region (*s1* and *s2*) and the middle region (*m1* and *m2*). The *s1/m1* genotype is more toxic for a wider range of epithelial cells than is *s1/m2* and gastric cancer patients usually are infected with the *s1/m1* type. The *vacA s2/m2* strains are virtually non-toxic to epithelial cells. All East Asian *H. pylori* strains are *vacA s1*. Within East Asian countries, the *m1* type is predominant in Japan and Korea, which have a higher incidence of gastric cancer. In contrast, the *m2* type is predominant in southern parts of East Asia (Vietnam, Hong Kong), where the incidence of gastric cancer is relatively lower. This suggests that the *s1/m1* type could be more virulent than the *s1/m2* type. The *vacA s1/m2* genotype is also predominant in South Asia, where the incidence of gastric cancer is lower (Yamaoka *et al.*, 2008). However, a comparative study from Singapore that compared the prevalence rate of *vacA s1/m1* genotypes in Chinese subjects with gastric cancer, and in patients with functional dyspepsia, failed to show any difference between the two groups (Fock *et al.*, 2006a). This was a cross-sectional study and it is possible that infected patients with functional dyspepsia could develop gastric cancer with increasing age.

Genomic differences in the structure of the *oipA* gene, encoding outer inflammatory protein A, have been noted between East Asian strains and Western strains of *H. pylori* and may account for the geographic differences in gastric cancer rates (Fock *et al.*, 2006b; Yamaoka *et al.*, 2008). Functional and intracellular signalling differences associated with AlpAB adhesin have also been observed between Western and East Asian strains (Lu *et al.*, 2007). Currently it is not established with certainty which of these genotypes of OMPs is crucial for gastric carcinogenesis.

2.5 Host Factors

Scientific evidence clearly supports the importance of host factors in gastric cancer pathogenesis (for details see Sutton *et al.*, Chapter 7, this volume). The risk of developing gastric cancer is increased up to threefold in individuals with an immediate relative suffering from gastric cancer, and 10% of cases of gastric cancer show familial clustering (Fox and Wang, 2007). Strong associations have been shown between host genetic polymorphisms in the interleukin (IL)-1 β gene cluster (El-Omar *et al.*, 2000; Machado *et al.*, 2001; Furuta *et al.*, 2004; Palli *et al.*, 2005), tumour necrosis factor- α , IL-10 (El-Omar *et al.*, 2003) and in the IL-8 promoter (Lee, *et al.*, 2005; Taguchi *et al.*, 2005), and the risk of gastric cancer in Caucasian populations. In contrast, results from studies conducted in Asia are conflicting, with a meta-analysis examining the role of polymorphisms in the genes encoding IL-1 β and IL-1 receptor antagonists in gastric cancer risk showing an association in Caucasians but not in Asians (Camargo *et al.*, 2006).

2.6 *H. pylori* Eradication as a Strategy for Primary Prevention of Gastric Cancer in Asia

Because *H. pylori* infection accounts for two-thirds of gastric cancer cases globally, it would seem logical to prevent gastric cancer by eradicating this bacterium in the community. Public health measures such as improvements in sanitation and water supply and better housing to reduce overcrowding, while not specifically targeting *H. pylori*, have resulted in the reduction of this infection in many countries over the period following World War II. While the development of a vaccine for mass vaccination would appear a logical step, this approach has proved to be elusive owing to difficulties encountered in vaccine development (see Blanchard and Nedrud, Chapter 9, this volume).

Antimicrobial eradication of *H. pylori* infection in patients with gastric cancer has been examined as a possible approach to reduce the recurrence rate of early gastric

cancer in patients (see Mégraud, Chapter 4, this volume). The first study to provide evidence that *H. pylori* eradication had a direct impact on gastric recurrence was reported by Uemura *et al.* (1997). In this non-randomized trial, patients with early gastric cancer treated by endoscopic resection were offered *H. pylori* eradication therapy. During a 3-year follow-up, metachronous gastric cancer developed in 9% of the untreated group, as compared with 0% in patients treated with *H. pylori* eradication therapy (Uemura *et al.*, 1997). Since then, several randomized trials have been conducted in China and Japan.

At a consensus meeting convened to discuss strategies to prevent gastric cancer in the Asia Pacific region, a group of experts recommended a test-and-treat approach for *H. pylori* infection in asymptomatic adults residing in high gastric cancer risk areas and communities, to prevent gastric cancer (Fock *et al.*, 2008). This recommendation was based on a meta-analysis of randomized controlled studies of *H. pylori* eradication conducted in Asia that showed a reduced gastric cancer incidence (odds ratio = 0.56; 95 % CI 0.4, 0.8) following *H. pylori* eradication (Fock *et al.*, 2008; Moayyedi *et al.*, 2008). The consensus report emphasized that this practice should not replace the current practice of gastric cancer surveillance in two East Asian countries (namely Japan and Korea) as well as in Matsu, Taiwan. Based on a number of studies that have suggested that eradication of *H. pylori* may be either ineffective or less effective in people with precancerous lesions such as intestinal metaplasia and atrophic gastritis, the group further recommended that the age to commence this screen-and-treat strategy was approximately 10 years, prior to the rise in the ASR of gastric cancer. In addition to preventing gastric cancer, eradication of *H. pylori* in the adult population should also reduce the rate of peptic ulcer disease and its associated complications. Furthermore, data would suggest that a reduction in dyspeptic symptoms and functional dyspepsia would be likely to occur (Ford *et al.*, 2005).

There are some concerns, however, that such an approach may increase the risk of other diseases, which might outweigh any potential benefits of a population screen-and-

treat strategy (see Mitchell *et al.*, Chapter 5, this volume). While there have been a number of studies suggesting that *H. pylori* eradication increases the risk of gastro-oesophageal reflux, the preponderance of evidence based on meta-analyses of *H. pylori* eradication and post hoc analyses of peptic ulcer trials indicates that *H. pylori* does not lead to the development of erosive oesophagitis or new symptomatic reflux (Malfertheiner *et al.*, 2002; Vakil *et al.*, 2006). Furthermore, studies within the general population support the view that *H. pylori* is unlikely to increase significantly the risk of reflux symptoms or Barrett's oesophagus and oesophageal adenocarcinoma (Moayyedi *et al.*, 2001; Moayyedi and Talley, 2006).

A further concern is that antibiotic resistance to *H. pylori* in the community could further increase; however, given that most treatments are for 1 week, the impact is likely to be limited.

2.7 Treatment Regimens for *H. pylori* Infection in Asia

In Asia, the first-line therapy for *H. pylori* infection is a proton pump inhibitor (PPI)-based triple therapy using a combination of amoxicillin and clarithromycin for 7 days. While metronidazole has been an acceptable alternative to amoxicillin with similar eradication rates, it has not been as widely used in Asia where the amoxicillin-containing combinations are preferred due to high metronidazole resistance rates in the region, and regulatory restrictions exist in some areas such as Japan. In patients who have penicillin allergy, metronidazole is issued in place of amoxicillin, or a bismuth-based quadruple therapy is used. In parts of Asia there are increasing rates of primary resistance to clarithromycin. For example, in Japan it has been reported that resistance to clarithromycin increased from 19 to 28% between 2002 and 2005 (Kobayashi *et al.*, 2007). In Korea, primary resistance increased to 14% in 2003 and in China to 18% in 2002 (Kim *et al.*, 2004; Cheng and Hu, 2005). Some data indicate that primary resistance to clarithromycin reduces treatment efficacy by 66% in the PPI, amoxicillin and clarithromycin

combination. Metronidazole resistance reduces the efficacy of the PPI, amoxicillin and metronidazole combination by 30%, and the PPI, clarithromycin and metronidazole combination by 18% (Fischbach and Evans, 2007). Bismuth-based quadruple therapy is an effective alternative to PPI-based triple therapy, but this regimen is not available in some Asian countries. At the second Asia Pacific consensus on the management of *H. pylori* infection, the group recommended the following salvage therapy following failed PPI-based triple therapy: (i) a standard triple therapy that has not been used previously; (ii) bismuth-based quadruple therapy; (iii) levofloxacin-based triple therapy; or (iv) rifabutin-based triple therapy. The choice of salvage therapy depends on the local pattern of antibiotic resistance, drug availability and the local prevalence of tuberculosis in the context of rifabutin use. The group considered that, at that time, there were insufficient data to recommend sequential therapy as an alternative first-line therapy in Asia. Sequential therapy consists of a 5-day treatment of a PPI and one antibiotic (usually amoxicillin) followed by a 5-day treatment with the same PPI and two antibiotics (usually clarithromycin and metronidazole). This strategy has generated much interest, as data suggest that eradication rates were as good, if not better, than those achieved with triple therapy.

2.8 Conclusions

In conclusion, the role of *H. pylori* in the pathogenesis of peptic ulcer disease was first discovered 25 years ago in Australasia, and its role in gastric carcinogenesis was recognized by the IARC in 1994. Since then, and with the development of eradication therapy, *H. pylori*-positive peptic ulcer disease has seen a decline in Asia. Gastric cancer too has declined in incidence, although it is still a major health issue in Asia. It remains to be seen if *H. pylori* eradication through a population search-and-treat strategy, combined with public health measures to improve sanitation and living standards, could eliminate this dreaded disease with a high mortality from Asia.

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3 Gastric Adenocarcinoma

P. CORREA* AND M.B. PIAZUELO

3.1 Introduction

Gastric adenocarcinoma has been considered an infectious disease since 1994, when the International Agency for Research on Cancer categorized *Helicobacter pylori* infection as a class I human carcinogen (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994). The pathogenesis of gastric adenocarcinoma represents a prototype for bacteria-induced and inflammation-driven malignancies. While *H. pylori* infection plays a primary role, other environmental and host factors modulate its outcome. Gastric cancer is a major cause of global morbidity and mortality. It is the fourth most common cancer worldwide, after cancers of the lung, breast and colorectum. In 2002, there were approximately 934,000 new cases of gastric cancer, accounting for 9% of all new cancers, and 700,000 deaths, making it second only to lung cancer as a cause of cancer death (Table 3.1) (Parkin *et al.*, 2005).

Although the incidence of gastric cancer is declining in many countries (Jemal *et al.*, 2008), the global burden is rising. Approximately two-thirds of the cases occur in developing countries, with a wide variation in incidence rates worldwide. The highest incidence rates are reported in Eastern Asia,

Central and Eastern Europe, and Central and South America (Parkin *et al.*, 2005). Most cases of gastric adenocarcinoma are diagnosed when the tumour has invaded the muscularis propria. Overall, the 5-year survival rate for gastric cancer is under 20%. In Japan, where gastric cancer is the most common cause of cancer-related deaths, mass screening programmes for gastric cancer are well developed. As a result, more than 70% of gastric cancers are diagnosed at an early stage, providing a 5-year survival rate of over 90% (Miyahara *et al.*, 2007). During recent decades, the relative proportion of carcinomas originating in the gastric cardia has increased (Blot *et al.*, 1991; Devesa *et al.*, 1998; Newnham *et al.*, 2003). However, this matter is controversial due to the difficulty of distinguishing tumours originating in the distal oesophagus, gastro-oesophageal junction and gastric cardia. El-Serag *et al.* (2002) reported that in the USA the incidence of adenocarcinoma of the cardia peaked in 1991 and has subsequently declined steadily. Approximately 90% of stomach cancers are adenocarcinomas (Coleman *et al.*, 1993). The model for development of the most frequent type of gastric adenocarcinoma (intestinal type) consists of the sequential changes in the gastric mucosa shown in Fig. 3.1 (Correa *et al.*, 1975). This model is discussed below.

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Table 3.1. New cases and deaths from cancer worldwide in 2002.
(From Parkin *et al.*, 2005.)

	New cases	Deaths
Lung	1,352,132	1,178,918
Stomach	933,937	700,349
Liver	626,162	598,321
Colon and rectum	1,023,152	528,978
Breast	1,151,298	410,712
Oesophagus	462,117	385,892
Cervix and uteri	493,243	273,505
Pancreas	232,306	227,023
Leukaemia	300,522	222,506
Prostate	679,023	221,002
Non-Hodgkin lymphoma	300,571	171,820
Bladder	356,557	145,009
All sites but skin	10,862,496	6,723,887

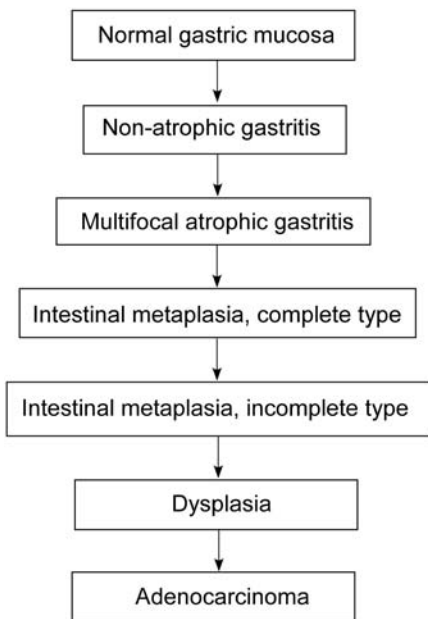


Fig. 3.1. Gastric precancerous multistep model.
(Adapted from Correa *et al.*, 1975.)

3.2 Aetiology

The aetiology of gastric adenocarcinoma eluded scientists for centuries. During most of the 20th century, the prevalent hypotheses

were focused on chemical carcinogens, especially those possibly ingested in the diet. After numerous attempts at identifying chemical carcinogens in foods, especially fish and meat, no consistent findings were reported in populations at high risk throughout the world. A search for contaminants in food provided limited information which was not generally applicable to most high-risk populations. One dietary item associated with increased cancer risk across populations is the excessive intake of salt (Joossens *et al.*, 1996; Tsugane, 2005; Tsugane and Sasazuki, 2007). The role of salt intake in cancer aetiology is in general not considered to be that of a primary (genotoxic) carcinogen, but more probably that of an adjuvant that may increase cancer risk by indirect mechanisms such as enhancing cell replication (Charnley and Tannenbaum, 1985). In addition, co-culture of gastric epithelial cells with *H. pylori* grown under high salt conditions has shown an increase in tyrosine-phosphorylated cytotoxin-associated gene A protein (CagA) and in secretion of the pro-inflammatory cytokine interleukin (IL)-8 by the epithelial cells compared with co-culture of the cells and *H. pylori* grown under low salt conditions (Loh *et al.*, 2007). Another recognized role of diet in gastric carcinoma is the protective effect of adequate intake of fresh

fruits and vegetables (González *et al.*, 2006; Larsson *et al.*, 2006). Again it is not considered to have a primary role, but rather one of ameliorating the effects of another (as yet unknown) carcinogen.

That rather hopeless panorama changed drastically when Warren and Marshall published their seminal letter in *The Lancet* in 1983 (Warren and Marshall, 1983). Although the main focus of these letters addressed the effects of *H. pylori* infection on non-neoplastic outcomes such as peptic ulcers, a mention was made, in passing, that the infection could play a role in gastric cancer causation. The debate that followed was mostly settled by the International Agency for Research on Cancer report (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994). Although several viral infections and one parasitic infection play a role in cancer causation, up to now *H. pylori* is the only bacterium directly implicated in cancer causation in man. Furthermore, *H. pylori* infection is the single most important cause of infection-associated cancer globally, contributing to 5.5% of the global cancer burden, approximately 25% of all infection-associated cancers, and 63% of all stomach cancer (Parkin, 2006). The long-term decline in gastric cancer mortality in developed countries has resulted, in part, from interrupting *H. pylori* transmission through improved basic sanitation, housing and socio-economic status. *H. pylori* infection plays a primary role, but its effects are modulated by other factors, mainly related to environmental forces and host genetic susceptibility (see Sutton *et al.*, Chapter 7, this volume). Approximately one-half of the world's population is infected with this bacterium, but only a very small fraction of infected people will develop cancer. Gastric cancer incidence rates differ considerably worldwide, from about 70 per 100,000 per annum in high-risk countries, to less than five per 100,000 per annum in low-risk populations (Ferlay *et al.*, 2004).

The model of gastric cancer causation is outlined in Fig. 3.2 (Correa *et al.*, 2004). In this model, the primary neoplastic stimulus is provided by the *H. pylori* infection, which can be intense with more virulent bacteria and mild with the less virulent strains. The

outcome of the infection is modulated by the genetic susceptibility of the host, mostly documented for the inflammatory cytokine polymorphisms; in particular, polymorphisms of the gene encoding IL-1 β (*IL1B*) have been associated with gastric cancer in some populations but not in others (El-Omar *et al.*, 2000; Lee *et al.*, 2003; Machado *et al.* 2003; Alpizar-Alpizar *et al.*, 2005; Sicinski *et al.*, 2006). Two meta-analyses concluded that the *IL1B*-511T genotype was associated with gastric cancer susceptibility in Caucasian populations, but not in Asians (Camargo *et al.*, 2006; Loh *et al.*, 2009). IL-1 β is a proinflammatory cytokine induced by *H. pylori* infection and is a powerful inhibitor of gastric acid secretion. It promotes hypochlorhydria, favouring further colonization of *H. pylori* and a more severe gastritis. Over decades, gastric atrophy and adenocarcinoma may develop (El-Omar, 2001). In addition, there is evidence showing that, for subjects with the *IL1B*-511T genotype and infected with more virulent *H. pylori* strains, the gastric cancer risk is 87 times greater than for subjects with genotype *IL1B*-511CC and infected with less virulent *H. pylori* strains (Figueiredo *et al.*, 2002).

Environmental influences are of several kinds. Socio-economic conditions may determine the timing and severity of the first infection, usually during childhood in developing countries (Goodman and Correa, 1995, 2000; Camargo *et al.*, 2004). Co-infections with parasites, more prevalent in certain lower socio-economic strata, may modulate the immune reaction to the bacterial infection towards an anti-inflammatory T-helper (Th) type 2 response (Whary and Fox, 2004; Whary *et al.*, 2005), which possibly lowers the risk of a neoplastic outcome.

A well-established additional risk factor for gastric cancer is cigarette smoking. A significant dose-dependent association between tobacco smoke and gastric cancer risk has been demonstrated (González *et al.*, 2003; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2004; Nishino *et al.*, 2006). In the European Prospective Investigation into Cancer and Nutrition cohort, it was estimated that 17.6% of gastric cancer in this European population was attributable to cigarette smoking (González *et al.*, 2003).

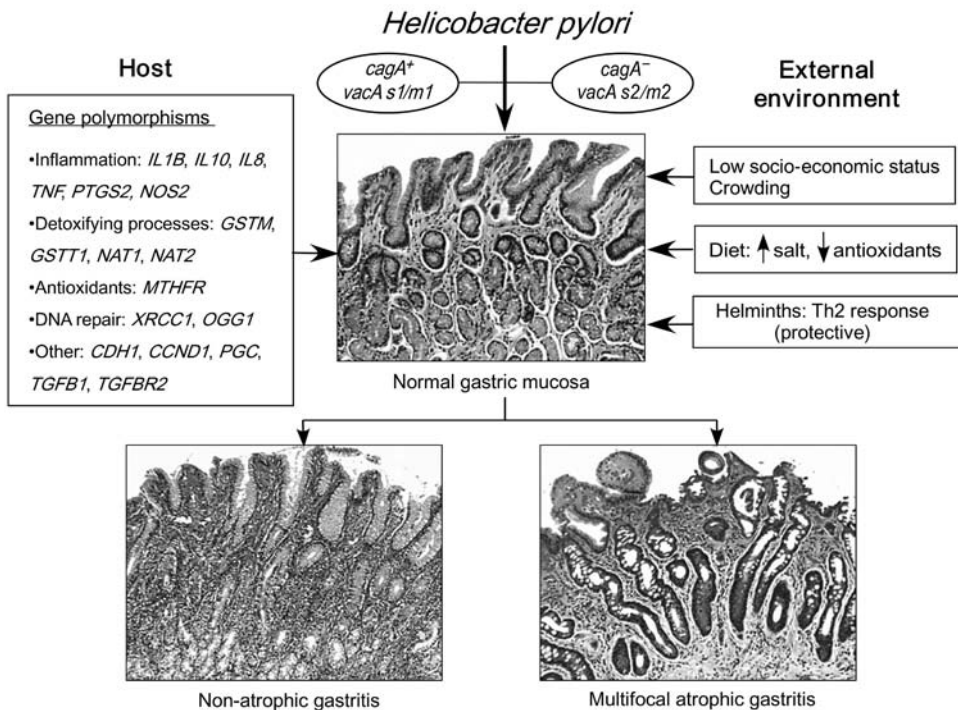


Fig. 3.2. Schematic representation of bacterial, human and environmental factors that may interact and influence the clinical outcome of *Helicobacter pylori* infection. Abbreviations/gene names: *cagA*, cytotoxin-associated gene A; *CCND1*, cyclin D1; *CDH1*, E-cadherin; *GSTM*, glutathione S-transferase μ ; *GSTT1*, glutathione S-transferase θ 1; *IL1B*, *IL10* and *IL8*, interleukins-1 β , -10 and -8; *MTHFR*, 5,10-methylenetetrahydrofolate reductase; *NAT1* and *NAT2*, N-acetyl transferases 1 and 2; *NOS2*, inducible nitric oxide synthase; *OGG1*, 7,8-dihydro-8-oxoguanine-DNA glycosylase; *PGC*, pepsinogen C; *PTGS2*, cyclooxygenase 2; *TGFB1*, transforming growth factor- β 1; *TGFB2*, transforming growth factor- β 2; *TNF*, tumour necrosis factor; *vacA*, vacuolating cytotoxin; *XRCC1*, X-ray repair cross-complementing protein 1. (Adapted from Correa *et al.*, 2004, with permission.)

Another infectious agent associated with gastric cancer is Epstein-Barr virus (EBV). EBV has been also classified as a class I oncogenic pathogen by the World Health Organization (WHO) and has been strongly linked to the development of nasopharyngeal carcinoma, Burkitt's lymphoma and Hodgkin's disease. For gastric carcinogenesis, the pathogenic role of EBV remains to be established. Multiple studies around the world have identified the presence of EBV in the malignant cells of approximately 9% of cases (Lee *et al.*, 2009). EBV-associated adenocarcinomas are predominant in men and in younger individuals (Truong *et al.*, 2009), develop more

frequently in the proximal stomach (cardia or corpus) and generally show the diffuse histological type. EBV is closely associated with remnant (post-gastrectomy) cancer and highly prevalent in the uncommon lymphoepithelial carcinoma. There is no association between EBV-positive adenocarcinomas and *H. pylori* infection (Lee *et al.*, 2009).

The interaction of the factors mentioned above, as outlined in Fig. 3.2, may determine if the gastric inflammation follows the path of non-atrophic gastritis, which is not associated with elevated risk of gastric cancer, or that of multifocal atrophic gastritis, the initial step in the precancerous process.

3.3 Pathology

3.3.1 Macroscopy

Anatomically, gastric adenocarcinomas are classified as cardia or non-cardia (distal). Non-cardia adenocarcinomas most commonly arise in the antrum–corpus junction of the lesser curvature, the incisura angularis, and may disseminate to the antrum or the corpus, following the topographic distribution observed for precancerous lesions (Correa *et al.*, 1970). The macroscopic appearance of early gastric cancer (described below) follows the classification established by the Japanese Endoscopic Society (Japanese Gastric Cancer Association, 1998). Advanced gastric carcinomas are grossly classified according to the Borrmann classification: type I for well-circumscribed polypoid or fungating lesions, type II for polypoid tumours with marked central ulceration, type III for the ulcerated tumours with infiltrative margins and type IV for diffusely infiltrating (linitis plastica) tumours (Lewin and Appelman, 1996).

3.3.2 Early gastric cancer

Early gastric carcinomas are defined as invasive adenocarcinomas in which the invasion is limited to the mucosa or submucosa, with or without lymph node metastasis (Lewin and Appelman, 1996). Early carcinomas may be multiple, synchronous or metachronous. Penetration of the muscularis mucosa is the criterion for sub-classifying early gastric cancer into intramucosal and submucosal types. Improvement in diagnostic techniques worldwide and implementation of screening programmes have resulted in more frequent diagnoses of gastric cancer at this stage. Gastric cancers can be classified endoscopically according to the growth pattern. The Japanese classification defines the following types: I, protruded or polypoid; II, superficial; IIa, elevated; IIb, flat; IIc, depressed; III, excavated (Japanese Gastric Cancer Association, 1998). In eastern Asia, up to 70% of all gastric cancers are diagnosed as early gastric cancers

(due to mass population screening), whereas in the western hemisphere, the proportion of early gastric cancers is only about 15% (Siewert, 2005; Oda *et al.*, 2006). In Japan, the 5-year survival rate following resection of early gastric cancer reaches 90–100% (Yamamoto and Kita, 2005).

3.3.3 Adenocarcinoma of the gastric cardia

Because of its borderline location between the oesophagus and stomach, many discrepancies exist in the literature regarding the cause and classification of these tumours. According to internationally accepted criteria, three entities are included under the term adenocarcinoma of the oesophagogastric junction (AEG): adenocarcinoma of the distal oesophagus (AEG I), true carcinoma of the cardia (AEG II) and sub-cardial gastric carcinoma (AEG III) (Siewert and Stein, 1998). The association between *H. pylori* and gastric cancer is stronger for neoplasias involving the distal stomach or non-cardia than for those involving the proximal stomach or cardia (Lochhead and El-Omar, 2008). In addition, a stronger association exists for cardia adenocarcinomas and EBV than for distal tumours (Lee *et al.*, 2009).

3.3.4 Histopathology

Gastric adenocarcinomas display high histological variability. They may be composed of tubular, acinar or papillary structures, or consist of a mixture of discohesive, isolated cells with variable morphologies, sometimes in combination with glandular, trabecular or alveolar solid structures. Mucin production is variable and pools of extracellular mucin may be present. Several classification systems have been proposed, but the most frequently used are the WHO and Laurén classifications. The WHO classification (Hamilton and Aaltonen, 2000) includes the following categories: papillary, tubular, signet-ring cell, mucinous and undifferentiated. The prevailing histological classification, which originated in the studies published by Laurén and Jarvi, characterizes

two main types, intestinal and diffuse, based on their display of features resembling intestinal mucosa (Jarvi and Laurén, 1951; Laurén, 1965). The two types follow distinct precancerous processes and have different clinical and epidemiological implications.

Intestinal-type adenocarcinoma

Intestinal-type gastric cancer is believed to develop through a sequence of steps involving recognized precancerous lesions. Chronic infection with *H. pylori* is the main recognized aetiological factor. The intestinal type is most common in men and in the elderly, and has a better prognosis than the diffuse type. Non-cardia gastric adenocarcinoma is most commonly of the intestinal type.

PRECANCEROUS PROCESS. The widely accepted model for development of gastric adenocarcinoma of the intestinal type consists of the following sequential changes in the gastric mucosa: chronic active gastritis, atrophy, intestinal metaplasia and dysplasia (Fig. 3.1) (Correa *et al.*, 1975; Correa, 1992). This process usually takes decades after persistent infection with *H. pylori*. Upon colonization of the gastric mucosa, *H. pylori* induces an immune response that attracts abundant polymorphonuclear neutrophils and mononuclear inflammatory cells to the lamina propria. The well-orchestrated inflammatory response is mediated by cytokines, chemokines and other mediators, which facilitate chemoattraction of haematopoietic cells, recruit effector cells and determine the natural history of the infection (Peek and Crabtree, 2006). Unless treated and eradicated, the infection becomes chronic, leading initially to non-atrophic chronic gastritis. The localization of *H. pylori* colonization within the stomach is mostly determined by local acid production. Initially, *H. pylori* colonizes the antral mucosa, probably because the acid secretion in the corpus inhibits colonization. Acid suppression, either by use of pharmacological agents or surgical intervention (vagotomy), leads to the extension of *H. pylori* colonization to the corpus and fundus. The same effect in colonization may also be seen in genetic or other medical conditions with reduced acid output (Lee *et al.*, 1995).

In some patients, the infection may result in ulcer formation. Subjects who develop duodenal ulcers are not at increased risk of gastric cancer (Uemura *et al.*, 2001). In contrast, subjects with gastric ulcers typically have multifocal atrophic gastritis and high risk of gastric cancer (Hansson *et al.*, 1996; Uemura *et al.*, 2001). In *H. pylori*-associated gastritis, alterations in the cell cycle have been documented (Peek *et al.*, 1997, 2000; Moss *et al.*, 2001; Crabtree *et al.*, 2004). Imbalance between proliferation and apoptosis may lead to progressive loss (atrophy) of gastric glands. The development of atrophy starts in small foci in the mucosa, so-called multifocal atrophic gastritis. As atrophy increases, foci coalesce covering more extensive areas of the mucosa, and the lamina propria may show proliferation of fibrocollagenous tissue.

Some of the glands may then be replaced by epithelium with intestinal morphology, a stage called intestinal metaplasia. The classification of intestinal metaplasia most widely used consists of complete and incomplete types, based on the preservation or lack, respectively, of digestive enzymes such as sucrase, trehalase and alkaline phosphatase (Matsukura *et al.*, 1980). Jass and Filipe (1981) proposed the following classification, according to the phenotypic characteristics. Type I (complete) is characterized by the presence of absorptive enterocytes, Paneth cells and goblet cells secreting sialomucins, and corresponds to the small intestine phenotype (Fig. 3.3A). Types II and III (incomplete type) are characterized by more irregular glands, with cells containing multiple mucus vacuoles of different sizes, without a brush border and lacking absorptive enterocytes. Metaplastic cells secrete sialomucins and/or sulfomucins: neutral and acid sialomucins in type II and sulfomucins in type III. Studies have shown that type I does not increase the risk of malignant transformation, whereas type III is associated with an increased risk of developing adenocarcinoma (Rokkas *et al.*, 1991; Filipe *et al.*, 1994).

A small proportion of subjects with intestinal metaplasia will develop dysplasia (also called intraepithelial neoplasia), which is subclassified as low grade and high grade, according to the degree of cytological and

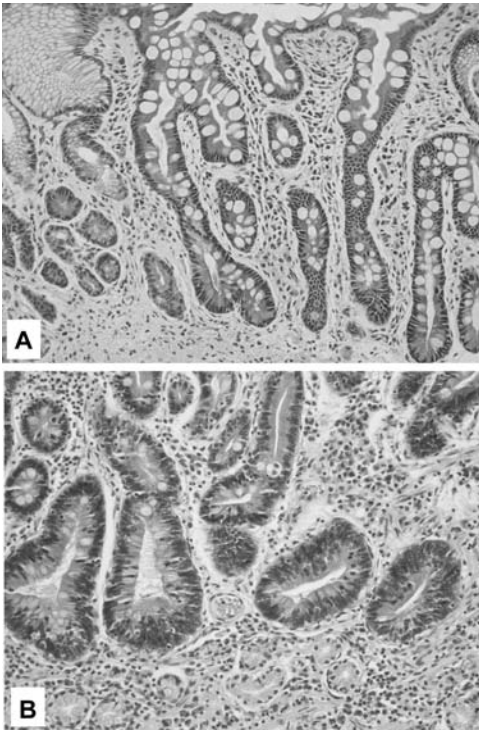


Fig. 3.3. Microphotographs of gastric precancerous lesions. (A) Intestinal metaplasia, complete type: glands with alternating goblet cells and absorptive enterocytes. (B) Low-grade dysplasia originating in intestinal metaplasia: nuclei are enlarged, hyperchromatic and crowded, but maintain the polarity.

architectural alterations. In low-grade dysplasia, epithelial nuclei are elongated (sometimes with a cigar-like appearance), present slight pseudostratification and maintain polarity with respect to the basement membrane. The glands may show some irregularity in shape and size (Fig. 3.3B). In high-grade dysplasia (a term equivalent to carcinoma *in situ*), there is severe cellular and architectural atypia. Cigar-like, pseudostratified nuclei alternate with rounded nuclei that show loss of polarity. Glandular structures may be markedly irregular and often show branching and folding (Rugge *et al.*, 2000). Differences between Western and Japanese pathologists exist in this category. For Western pathologists, invasion to the lamina propria is the hallmark of carcinoma and is the criterion considered by

the authors in this chapter. For Japanese pathologists, the presence of neoplastic epithelium, even in the absence of proven invasion, is grounds for a diagnosis of carcinoma. The Padova classification provides equivalent terms to accommodate both Western and Japanese classifications (Rugge *et al.*, 2000). Histologically, intestinal-type adenocarcinomas are composed of cohesive tumoural epithelial cells forming acinar or tubular structures often resembling intestinal epithelium, infiltrating the gastric wall (Fig. 3.4A). Sometimes, the tumoural cells form papillary structures or clusters. The tumours exhibit a variable degree of differentiation.

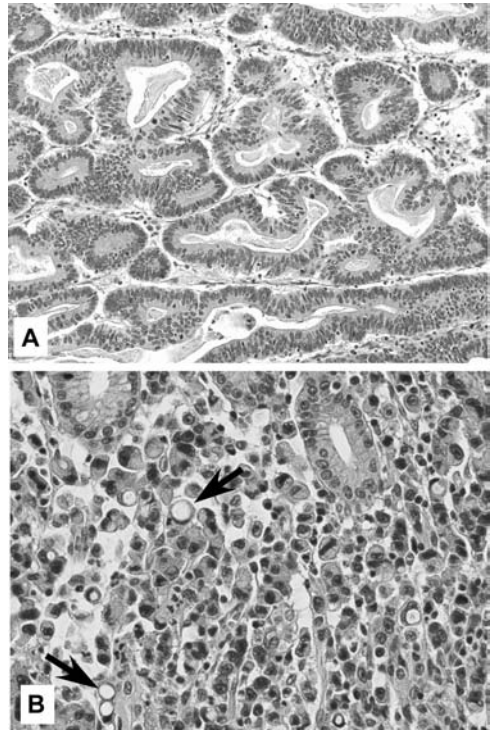


Fig. 3.4. Microphotographs of gastric adenocarcinoma. (A) Intestinal type: cohesive tumour cells forming irregular tubular structures that resemble the intestinal epithelium. (B) Diffuse type: non-cohesive tumour cells with large, irregular and hyperchromatic nuclei infiltrate diffusely into the lamina propria. There are scattered signet-ring cells containing intracytoplasmic mucin that push the nuclei to the periphery (arrows).

Well-differentiated adenocarcinomas display well-formed glands, closely resembling metaplastic intestinal epithelium. Moderately differentiated adenocarcinomas exhibit intermediate characteristics between well differentiated and poorly differentiated. Poorly differentiated adenocarcinomas are composed of highly irregular glands that are recognized with difficulty, or cells arranged in small or large clusters with mucin secretion. In general, intestinal-type adenocarcinomas are associated with intestinal metaplasia of the surrounding mucosa.

Diffuse-type adenocarcinoma

Diffuse-type adenocarcinoma is relatively more frequent in women and younger patients and in populations at low risk of gastric cancer, and occurs more commonly in the body of the stomach. Its development does not follow the sequential steps outlined above for the intestinal type. Genetic predisposition seems to play an important role. Although environmental agents seem to play a less important role than genetic factors, *H. pylori* infection has been also associated with the development of diffuse-type adenocarcinoma (Parsonnet *et al.*, 1997; Rugge *et al.*, 1999). Microscopically, diffuse-type gastric adenocarcinomas are composed of poorly differentiated tumour cells lacking cohesion that diffusely infiltrate the gastric wall. In some cases, signet-ring cells containing intracytoplasmic mucin and nuclei flattened against the side of the cell are observed (Fig. 3.4B). In general, diffuse-type adenocarcinomas show a great propensity to spread locally and have poorer prognosis than the intestinal-type tumours. Within this group, a well-characterized syndrome, hereditary diffuse gastric cancer, is an autosomal dominant disorder that accounts for less than 1% of all cases of gastric cancer. It was initially described in three New Zealand Maori families with early-onset diffuse gastric cancer (Guilford *et al.*, 1998). Since then, at least 151 families have been reported in different countries (Kaurah *et al.*, 2007; Norton *et al.*, 2007; Carneiro *et al.*, 2008). This syndrome is associated with germline mutations in the gene encoding E-cadherin (*CDH1*) in 30–40%

of the families. Although uncommon, this disease constitutes an important health issue due to its severity, high penetrance, early age at presentation and the unavailability of effective screening tools (Lynch *et al.*, 2008).

3.4 The *H. pylori*/Gastric Cancer Enigmas

Although *H. pylori* infection has been recognized as a class I carcinogen, incongruence between infection prevalence and gastric cancer incidence rates has been reported. Holcombe (1992) called attention to the high prevalence of infection in the face of low cancer rates, which he called the 'African enigma'. Similar observations have been made in other geographic areas. Miwa *et al.* (2002) compared the infection prevalence rates with the cancer incidence rates in Asia and called attention to the fact that in some countries (mostly northern, like Japan and Korea) both rates are high, while in others (mostly southern, like Thailand and Bangladesh) infection prevalence rates are high but cancer rates are low. In larger countries, like India and China, intra-country contrasts in subpopulations may reflect the two situations. In addition, the immune response to *H. pylori* infection, in terms of immunoglobulin (Ig) G subclass, has been compared between the population of Soweto, South Africa, and populations of Germany and Australia (Mitchell *et al.*, 2002). The IgG1/IgG2 ratio was significantly lower in Germans and Australians than in Sowetans, reflecting a Th1 immune response bias in the former and a Th2 bias in the latter. Obviously, the Germans and Australians represent wealthy ('developed') societies, while the Sowetans represent lower socio-economic ('developing') societies. The north-south geographical gradient reported in Asia is also applicable to the comparison between Germans and Sowetans. Climatic conditions are known to influence helminthic infections, which have an extra-corporal component in their life cycle. Warm climates with abundant rainfall favour helminthic life cycles (Birrie *et al.*, 1994). Dietary patterns are usually different in

populations with contrasting gastric cancer risks. In Colombia, South America, high-altitude inhabitants of the Andes Mountains have a very high prevalence of infection and very high gastric cancer incidence rates. By contrast, inhabitants of the Pacific coast, who also have a very high prevalence of infection, have very low gastric cancer rates (Correa *et al.*, 1976). The diet in the mountains is rich in starchy foods like potatoes, while coastal dwellers consume abundantly fresh fish. A survey of intestinal parasites reported a very much higher prevalence, especially of helminths, in the coastal compared with the mountain inhabitants. Blood levels of IgE, a marker of Th2 response, were much higher in the coastal than in the mountain inhabitants (Whary *et al.* 2005). Gastric mucosa biopsies from both populations revealed that eosinophilic infiltration (a marker of allergic or Th2 response) was much more prominent in inhabitants of the coast (Piazuelo *et al.*, 2008). A Th2 bias in immunological response has been reported in Chinese patients infected with *Schistosoma japonicum*. In those subjects, IgG1/IgG2 ratios were 5.88, higher than the 4.0 ratio observed in patients without the parasite (Du *et al.*, 2006). Experimental evidence has been provided by Fox *et al.* (2000) in mice co-infected with *Helicobacter felis* and the nematode *Heligmosomoides polygyrus*. Their study showed that the immune reaction to the bacterial infection was biased towards a Th2 response by the nematode co-infection, which was associated with a considerable reduction in *Helicobacter*-induced gastric atrophy, a known premalignant lesion. Taken together, the available data indicate that the predominant anti-inflammatory Th2 response to *H. pylori*, probably brought about by helminthic infections, may prevent the progression of the gastric precancerous process to more advanced lesions such as intestinal metaplasia and dysplasia.

Variations in the virulence potential of different *H. pylori* strains have also been studied as a possible explanation for the discrepancy between cancer rates and infection prevalence (Yamaoka *et al.*, 2008). Several virulence-associated genes have been identified. A major focus of enquiry has been the *cag* pathogenicity island (PAI), an approxi-

mately 40 kb locus containing 31 genes, inserted in the glutamate racemase gene. The marker of the island is the cytotoxin-associated gene *cagA* (Covacci *et al.*, 1993; Tummuru *et al.*, 1993; Censini *et al.*, 1996). Other genes in the island code for a type IV secretion system, a syringe-like structure that injects CagA and probably other proteins into the cytoplasm of the gastric epithelial cells (see Backert *et al.*, Chapter 11, this volume). The geographic distribution of different *H. pylori* genotypes led Covacci *et al.* (1999) to suggest co-evolution of man and *H. pylori* species. According to their hypothesis, *H. pylori* travelled along with man during major migrations. On the basis of nucleotide sequences, Covacci *et al.* (1999) were able to differentiate Asian from European and African strains. The genetic geography of *H. pylori* coincides with human populations and supports the hypothesis that *H. pylori* was already established in the human stomach at least 100,000 years ago, before intercontinental migrations. The antiquity of human infection was corroborated using carbon dating of South American mummies, 800–3000 years old, found colonized with *Helicobacter* species (Allison *et al.*, 1999).

Several studies have shown that *cagA*-positive bacteria lead to more inflammation and more severe outcomes, including peptic ulcer and gastric carcinoma (Blaser *et al.*, 1995; Peek *et al.*, 1995; Parsonnet *et al.*, 1997). It has been suggested that *cagA*-positive genotypes may partially explain the African and related enigmas (Yamaoka *et al.*, 2008). In Latin America, *cagA* positivity may partially explain the enigmas. The prevalence of *cagA*-positive strains in Colombian communities with a high risk for gastric cancer was shown to be higher than in low-risk communities: 90.4% versus 81.1% (Bravo *et al.*, 2002). Patients from a high-risk area in Costa Rica were reported to have a higher proportion of *cagA*-positive strains than patients from a low-risk area: 78.8% versus 53.6% (Con *et al.*, 2006). This difference is not found in most Asian countries, where the prevalence of *cagA* positivity is almost 100% (Hatakeyama, 2009).

A similar paradox is the dichotomy outlined in Fig. 3.2, namely non-atrophic gastritis, which does not increase gastric

cancer risk, versus multifocal atrophic gastritis–intestinal metaplasia, which is clearly a marker of a precancerous process. The paradox is clearly demonstrated in patients with duodenal ulcer, causally associated with virulent *H. pylori* genotypes: most duodenal ulcer patients carry *cagA*-positive, *vacA s1/m1* bacteria. Although patients with duodenal ulcer may have antral gastritis for decades, the lesion remains non-atrophic in nature. Such patients are not at increased gastric cancer risk when compared with the general population (Hansson *et al.*, 1996).

The CagA protein of *H. pylori* isolates from different populations exhibit diverse sequence polymorphisms in their C-terminal regions containing EPIYA (glutamic acid–proline–isoleucine–tyrosine–alanine) motifs (see Backert *et al.*, Chapter 11, this volume). According to the amino acid sequences surrounding those motifs, the segments are designated EPIYA-A, EPIYA-B, EPIYA-C and EPIYA-D. Most isolates contain EPIYA-A and EPIYA-B motifs. The EPIYA-C motifs, most typically present in one to three copies, characterize the denominated Western CagA, present in isolates from European, American and Australian patients. The EPIYA-D motifs are characteristic of strains from East Asian patients (Hatakeyama, 2009). EPIYA-C and EPIYA-D motifs contain major tyrosine phosphorylation sites, and are associated with cell damage. Increasing numbers of phosphorylation sites lead to more severe gastric lesions (Naito *et al.*, 2006; Sicinski *et al.*, 2010), but East Asian CagA has shown more oncogenic potential than Western CagA (Hatakeyama, 2009). The possible role of EPIYA motifs in gastric carcinogenesis has been investigated in Malaysia and Singapore, where three distinct ethnic groups (Chinese, Indian and Malay) share the same geographic environment (Schmidt *et al.*, 2009). In this population, Chinese have higher gastric cancer rates than Indians. While EPIYA-C was found to predominate in the Indian population with a higher proportion of strains having four or more motifs, EPIYA-D predominated in the Chinese. Interestingly, in the Malays who have a low cancer rate and a lower prevalence of *H. pylori* infection, EPIYA-C and EPIYA-D motifs were found to have a similar prevalence (Schmidt *et al.*, 2009).

Another major virulence factor is the vacuolating cytotoxin VacA, a secreted protein toxin that is responsible for gastric epithelial vacuolar degeneration and interference with cell membrane function (Cover and Blaser, 1992; Cover, 1996). Several alleles have been identified that are associated with bacterial virulence in the signal (s), medial (m) or intermediate (i) domains. The combination of s- and m-region alleles determines the production of the cytotoxin and is associated with the pathogenicity of the bacterium (see Backert *et al.*, Chapter 11, this volume). In general, *s1* and *m1* variants are associated with more severe gastric lesions, including gastric cancer, than *s2* and *m2* strains (Atherton *et al.*, 1995; Atherton *et al.*, 1997; Cover and Blanke, 2005). In Colombia, the prevalence of *vacA s1* and *vacA m1* genotypes in isolates from communities with high gastric cancer risk was significantly higher than in communities with low risk: 93.2% versus 83.7% for *s1* genotypes and 83.3% versus 70.2% for *m1* genotypes (Bravo *et al.*, 2002). In addition to *cagA* and *vacA*, *H. pylori* expresses many other putative virulence factors, such as outer inflammatory protein A (*oipA*), blood group antigen binding adhesin A (*babA*) and adherence-associated proteins A and B (*alpA/B*). Structures of these genes also vary among populations (see Lindén *et al.*, Chapter 12, this volume). Polymorphisms of *oipA* and *babA* have also been linked to cancer risk (Yamaoka *et al.*, 2008). It has been proposed that the combination of high-virulence polymorphisms of *cagA*, *vacA*, *oipA*, *babA* and *alpA/B* may largely explain the African and related enigmas (Yamaoka *et al.*, 2008). Populations of Asian extraction tend to carry more virulent *H. pylori* strains than those of African extraction. Other bacterial factors may influence infection outcome and potential carcinogenesis. Tumour necrosis factor- α (TNF α)-inducing protein (Tip α), secreted by *H. pylori*, is a potent inducer of TNF α and various other chemokine genes. *H. pylori* isolates from gastric cancer patients have been shown to secrete significantly larger amounts of Tip α compared with those from chronic gastritis patients (Suganuma *et al.*, 2008).

The genetic identity of *H. pylori* strains has been analysed by multilocus sequence

typing (MLST) of housekeeping genes. The geographic sources of *H. pylori* reflect major events in human settlement history. MLST of 370 strains from 27 geographical, ethnic and/or linguistic human groupings assigned the strains to four main clusters: hpEurope, hpEastAsia, hpAfrica1 and hpAfrica2 (Falush *et al.*, 2003). Further analyses split hpEastAsia into hspMaori and hspAmerind subpopulations. An expanded data set including 769 *H. pylori* isolates from 51 ethnic sources identified two new populations designated hpAsia2 and hpNEAfrica (Linz *et al.*, 2007). Before Columbus discovered the New World, strains in the Americas were assumed to be all hspAmerind, and in Oceania hspMaori. After Columbus, human migrations from Europe and Africa changed the original predominant genotypes. In the Americas, the original hspAmerind strains became rare in Native American populations. hpEurope strains became more common, probably replacing or modifying the original strains. Similarly, in Oceania, hpEurope strains became more frequent. Before the MLST technology, a study of isolates from native Peruvians described the relatedness of Latin American and Spanish strains, suggesting that *H. pylori* had been brought to the New World by European conquerors and colonists about 500 years ago (Kersulyte *et al.*, 2000). Re-analysis of the native Peruvian strains with MLST reported that 20% of the isolates had hspAmerind markers (Devi *et al.*, 2006). A correlation between high gastric cancer rates and hpEastAsian genotypes has been noted, contrasting with low cancer rates in Africa, where most strains are hpAfrica (Yamaoka *et al.*, 2008). MLST technology has been also applied to *H. pylori* isolates from the three ethnic groups residing in Malaysia, which have different rates of gastric cancer: Chinese (with the highest rate), Indian and Malay (Tay *et al.*, 2009). Phylogenetic analysis assigned the isolates to previously identified *H. pylori* ancestral populations, hpEastAsia, hpAsia2 and hpEurope. Chinese isolates predominantly fell into hpEastAsia populations. Malays carried isolates related to those of Indian origin, apparently acquired relatively recently. Malaysian Indian/Malay isolates were found to differ from the Ladakh isolates

from India (Linz *et al.*, 2007) and revealed a new subpopulation, hspIndia, within hpAsia2 (Tay *et al.*, 2009). These studies contribute substantially to our understanding of the ancestral origins of Asian populations, and their innate *H. pylori* flora.

3.5 Mechanisms of Carcinogenesis

The mechanisms by which *H. pylori* induces gastric cancer are not well understood. The intense inflammatory infiltrate induced by more virulent strains has been proposed as an explanation for the higher cancer rates. It has been suggested that higher cell replication may immortalize mutations or other DNA alterations that may arise during the repeated cycles of cell division. The gastric precancerous process in its initial stage is characterized by non-atrophic gastritis. However, the stage that apparently marks the entrance into the precancerous process is gastric atrophy (gland loss). Such loss is generally multifocal, initiated around the incisura angularis and the corpus-antrum junction. As the precancerous process advances, intestinal metaplasia sets in and the inflammatory infiltrate tends to gradually become less prominent. It would then appear that a separate set of events takes place after the initial stage of inflammation subsides. Some evidence suggests that inflammation, per se, is not a sufficient cause of neoplastic transformation. In subjects with duodenal ulcer, severe antral gastritis persists for decades if the *H. pylori* infection is not eradicated. However, duodenal ulcer patients are not at higher cancer risk than the general population. Comparisons of the histopathological parameters of the gastric mucosa in populations with contrasting cancer risks do not reveal more severe inflammation in subjects from the high-risk area (Piazuelo *et al.*, 2008). However, a significantly higher prevalence of intestinal metaplasia is observed in subjects from high-risk areas (Correa *et al.*, 1970; Bravo *et al.*, 2002).

The link between genomic characteristics and functional mechanisms which can be associated with carcinogenesis has not been established. Oxidative and nitrosative stress

has been proposed as the relevant pathway to *H. pylori*-related gastric carcinogenesis (Mannick *et al.*, 1996; Pignatelli *et al.*, 1998, 2001; Xu *et al.*, 2004). Several enzymes induced by *H. pylori* infection may contribute: (i) the inducible nitric oxide synthase (iNOS) enzyme results in expression of nitric oxide, capable of inducing genetic damage to epithelial cell DNA; (ii) arginase is an enzyme that plays a role in the polyamine metabolism by inducing L-ornithine, a step in the putrescine→spermidine→spermine cycle; (iii) ornithine decarboxylase stimulates the polyamine cycle progression; and (iv) spermine oxidase activates spermidine→spermine transformation, a reversible step that may induce H₂O₂ expression, capable of inducing DNA damage (Xu *et al.*, 2004).

A recent study addressed the issue of oxidative and nitrosative damage in *H. pylori* isolates from Colombian populations with contrasting gastric cancer risks (Asim *et al.*, 2008). Bacterial strains from subjects from a high cancer risk population induced significantly higher levels of iNOS in macrophages, a clear indicator of excessive nitrosative stress. The same isolates from the high-risk population induced significantly higher expression of spermine oxidase and H₂O₂, clearly indicating excessive oxidative stress. All the strains tested in that study were *cagA*-positive, *vacA* *s1/m1*, indicating that *H. pylori* may partially be responsible for gastric carcinogenesis, and contribute to the cancer/infection enigmas by mechanisms related to oxidative and nitrosative stress not linked to these known virulence genes.

The causal role of oxidative damage is supported by chemoprevention trials using antioxidant agents. Supplementing the diet with ascorbic acid and β-carotene for 6 years resulted in a significant regression of precancerous lesions in Colombia (Correa *et al.*, 2000; Mera *et al.*, 2005). That trial also showed the same effects after curing the *H. pylori* infection. However, combining these two interventions did not have an additive effect in the regression of precancerous lesions. Such results can be interpreted as indicating that the role of *H. pylori* infection in carcinogenesis is driven by oxidative damage which could be avoided by curing the infection or by

providing an adequate supply of anti-oxidants.

Evidence suggests that *H. pylori* influences stomach carcinogenesis through the development of chronic multifocal atrophic gastritis (Ohata *et al.*, 2004), which may disseminate proximally to the fundic mucosa and is usually accompanied by intestinal metaplasia in advanced stages. Although multifocal atrophic gastritis and intestinal metaplasia are histopathological diagnoses, reduction of the functional fundic gland mucosa may be assessed by serum pepsinogen (PG) levels. Serum PG levels are considered a reliable marker for the extent of gastric atrophy/metaplasia (Miki, 2006). Ohata *et al.* (2004) followed a cohort of 4655 asymptomatic male subjects in Japan for 7.7 years. At baseline, *H. pylori* infection was evaluated by serum specific antibodies, and the presence of gastric mucosal atrophy was documented by serum PG levels. Chronic atrophic gastritis (CAG) was diagnosed if serum PGI levels were below 70 µg/l and the PGI/PGII ratio was less than 3.0. Forty-five new cases of gastric cancer were detected during the period of observation. No cancer developed in subjects without *H. pylori* infection or CAG. A stepwise increase in gastric cancer risk was observed from *H. pylori*⁺/CAG⁻ subjects to *H. pylori*⁺/CAG⁺ subjects and finally to *H. pylori*⁻/CAG⁺ subjects. It appears that the early stages of the process are driven by *H. pylori* infection but the advanced stages are driven by gastric atrophy (Table 3.2).

The highest hazard ratio and incidence of gastric cancer are observed in the last group, representing subjects who cleared their infection and had advanced gastric atrophy. Apparently, the *H. pylori*-induced damage persists after the bacterium is no longer present in the gastric mucosa, as observed in intestinal metaplasia, which no longer provides a favourable niche for *H. pylori* colonization.

3.6 Cancer Control

The prognosis of gastric cancer is largely determined by the stage of tissue invasion at the time of diagnosis. If detected while the

Table 3.2. Incidence rate and hazard ratio of gastric cancer according to serological diagnosis of *Helicobacter pylori* infection and chronic atrophic gastritis. (From Ohata *et al.*, 2004.)

<i>H. pylori</i>	Chronic atrophic gastritis	Hazard ratio	Gastric cancer incidence per 100,000 person-years
Negative	Negative	1.00 (referent)	0
Positive	Negative	7.13	107
Positive	Positive	14.85	238
Negative	Positive	61.85	871

tumour cells are confined to the mucosa and submucosa (early carcinoma), surgical resection, either endoscopically or by laparotomy, results in a 5-year survival rate above 90% (Yamamoto and Kita, 2005). Detection at such an early (mostly asymptomatic) stage requires extensive screening programmes in the population, so far available almost exclusively in Japan. In most Western countries, most cases are detected after the tumour cells have invaded the muscularis propria. In the USA, two-thirds are diagnosed at this stage (Jemal *et al.*, 2008). Five-year survival rates at this stage are below 20% in most populations. The inescapable conclusion from the Western experience is that prevention is the only hope of controlling the disease.

The key to effective prevention is the endoscopic identification of advanced precancerous lesions, namely dysplasia. In Japan and some other countries, the word ‘adenoma’ is equivalent to dysplasia (Rugge *et al.*, 2000). The Japanese model of prevention and early diagnosis is very expensive. It requires an extensive infrastructure of advanced endoscopy services and outstanding expertise in the diagnosis of minimal lesions, not available in most other countries. It is also combined with special equipment and expertise in double-contrast barium X-rays and photo-fluorography. Some attempts to set up such programmes in Latin America have been conducted in specific subpopulations in Venezuela and Costa Rica. Those efforts have resulted in the identification of a limited number of early-stage carcinomas that have been successfully resected surgically. The impact on the general population, however, has been very limited (Oliver, 1994; Pisani *et al.*, 1994; Rosero-Bixby and Sierra, 2007).

The strategies for prevention vary according to the cancer risk. In many Western countries the incidence is so low that screening and early detection programmes are not cost-effective. No organized screening and early detection programmes exist in most high-risk countries, with the exception of Japan and to some degree South Korea (Choi *et al.*, 2009). In high-risk countries with limited economic resources, there is a need to develop, pilot (test) and implement prevention strategies that are cost-effective and viable. Some investigators advocate elimination of *H. pylori* infection in the general population. In the absence of a vaccine, bacterial eradication can be achieved with antibiotics in approximately 90% of cases. However, treating millions of persons with antibiotics is problematic not only in terms of the cost, but also in making other pathogenic bacteria resistant to antibiotics, a potentially serious health threat that is becoming increasingly recognized in several countries. Some investigators have reported that unwanted effects may become apparent when *H. pylori* infection is eradicated. The complex of reflux oesophagitis, Barrett’s oesophagus and adenocarcinoma of the lower oesophagus is a prime example of possible unexpected complications of eradication of *H. pylori* infection (Blaser, 1999, 2005). Other possible consequences might be bronchial asthma and other allergic and autoimmune diseases, which may result from the absence of some minor infections and their effect in modulating the immune response to other antigens (the so-called hygiene hypothesis) (Strachan, 1989; Zaccane *et al.*, 2006; see Mitchell *et al.*, Chapter 5, this volume).

The classic epidemiological model of prevention consists of identifying the subjects

at the highest risk of developing the disease. In the case of *H. pylori*, even in the very high-risk communities, only approximately 50 of every 100,000 persons are expected to develop gastric cancer in a year. The challenge is to identify them before they develop invasive disease. As discussed above, the cancer risk is determined by host genetic susceptibility factors, bacterial genotypes and immune modulation of the response to *H. pylori* infection. What is lacking at the present time is a strategy to identify such individuals in the community. Although the techniques needed to assess genetic susceptibility, bacterial genotypes and immune response to the infection are known, no attempts have been made to develop cost-effective high-throughput methods to apply to the general population in high-risk countries.

As discussed above, serum PGs are specific indicators of gastric atrophy, a well-known marker of elevated cancer risk. Some studies have reported the use of serum PG levels in cancer patients and controls. While very low PGI levels and low PGI/PGII ratios are very specific indicators of invasive gastric cancer, these markers may not be sensitive indicators of cancer risk. It has been estimated that approximately one-half of the invasive cancers are not associated with low serum PG markers. The reason for this is that PGI is exclusively expressed in oxyntic mucosa and is drastically reduced when the oxyntic mucosa becomes atrophic and metaplastic. However, antrum-restricted atrophy and metaplasia, not reflected in serum PG levels, may be associated with high cancer risk. The real promise of serum PG levels is the identification of subjects with cancer precursors, before they develop invasive disease (Miki, 2006). *H. pylori*-induced atrophy classically starts in the incisura angularis and the antrum–corpus junction. The area covered by multifocal atrophy advances with time and extends to the neighbouring antral and corpus mucosa. The latter situation leads to lower PGI levels and PGI/PGII ratio. A sound strategy for prevention in high-risk countries would be to detect subjects with low serum PG markers. Once identified, a battery of host,

bacterial and immune markers as well as endoscopic monitoring could be effective in detecting advanced precancerous lesions. Their progression to invasive carcinomas could be avoided by endoscopic monitoring and appropriate interventions. Such a strategy could be reinforced by curing *H. pylori* infection and providing an adequate supply of antioxidants.

3.7 Epilogue

The natural history of *H. pylori* infection and gastric cancer is changing drastically as the 21st century advances. The steady decline in the frequency of both conditions will probably continue and become more accentuated. However, the health burden of gastric adenocarcinoma will continue to be considerable for decades to come. Special attention should be given to addressing the issue of whether the gradual decrease in *H. pylori* infection and gastric cancer incidence will be followed by increases in other conditions such as oesophageal adenocarcinoma, bronchial asthma and autoimmune diseases. As of now, the health burden of gastric adenocarcinoma is much greater than that of the conditions just mentioned. However, it is not clear whether this imbalance will persist in future decades.

The role of infection and chronic inflammation in the causation and progression of major health burdens is being increasingly recognized. Such is the case for neoplasias with aetiologies much less clearly understood than those of gastric carcinoma, in which an infectious agent has been identified. Carcinomas of the colon, pancreas, breast, prostate and probably other organs may be influenced by infections and chronic inflammation. As an example, clinical trials utilizing anti-inflammatory drugs such as aspirin and cyclooxygenase-2 inhibitors have reported promising results in the prevention and progression of cancer. The present scientific interest in solving the enigmas of the aetiology and pathogenesis of gastric cancer may throw light on the general subject of infection/inflammation-induced carcinogenesis.

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4 Antimicrobial Resistance and Approaches to Treatment

F. MÉGRAUD

4.1 Introduction

The discovery of *Helicobacter pylori* in 1982 was a tremendous breakthrough in relation to the treatment of gastroduodenal diseases (Marshall and Warren, 1984). For the first time it was possible to treat a cause and, this cause being infectious, to easily cure the disease. However, as for any bacterial agent, resistance has developed and in many places we are now faced with multi-resistant bacteria for which antimicrobial susceptibility testing is mandatory. This chapter presents the basis of *H. pylori* treatment, the currently recommended treatment regimes, and the problems linked to antimicrobial resistance.

4.2 Basis for *H. pylori* Treatment

4.2.1 Rationale to use antisecretory drugs

The antibiotics to be used to treat *H. pylori* infection have to be chosen from among the numerous drugs naturally active against the bacterium. Indeed, *H. pylori* is intrinsically resistant to only a few compounds.

However, the particularity of *H. pylori* is to live in the specialized niche of the gastric mucus, outside the interior milieu, and which, unlike most of our body, is not at a neutral pH but rather presents a pH gradient. This gradient ranges from pH 1.82 to 5.5 in the corpus

and from pH 3.5 to 5.4 in the antrum (Quigley and Turnberg, 1987). However, given the important production of urease by *H. pylori* and consequently of ammonia in the immediate environment of the bacterium, the pH is higher when *H. pylori* is present (Marshall *et al.*, 1990).

These conditions impose important restrictions for antimicrobial use. The first concern is chemical stability: clarithromycin, for example, has been shown to degrade rapidly at gastric pH (Erah *et al.*, 1997). Second, most antibiotics have decreased activity when the pH is decreased (Table 4.1). For this reason, the first attempts using an antibiotic alone were unsuccessful and it became mandatory to add an antisecretory drug, i.e. a drug that diminishes gastric acid secretion, to the antibiotics. The first to be used was ranitidine, a histamine H₂-receptor antagonist (Hentschel *et al.*, 1993), but when the more potent antisecretory drugs – the proton pump inhibitors (PPIs) – emerged, they quickly became the standard (Gisbert *et al.*, 2003). The PPIs omeprazole, lansoprazole, pantoprazole, rabeprazole and esomeprazole can all be used for this purpose (Vergara *et al.*, 2003). These drugs also have an anti-*H. pylori* activity per se (Megraud *et al.*, 1991; Suerbaum *et al.*, 1991), but this occurs at concentrations which are most likely unachievable in the gastric mucosa and therefore this activity is only theoretical. However, these drugs have

Table 4.1. Distribution of the minimal inhibitory concentrations (MIC₉₀) of various antibiotics against wild-type *Helicobacter pylori* at various pH.

Agent	MIC ₉₀ (mg/l)		
	pH 7.5	pH 6.0	pH 5.5
Penicillin	0.03	0.5	0.5
Ampicillin	0.06	0.25	0.5
Cephalexin	2	16	32
Erythromycin	0.06	2	8
Clarithromycin	0.03	0.06	0.25
Ciprofloxacin	0.12	0.5	2
Tetracycline	0.12	0.25	0.5
Nitrofurantoin	1	2	2
Metronidazole	2	2	2
Bismuth subcitrate	16	8	–

an added value for the treatment: they lead to pain relief, which is a major benefit for the patient.

Metronidazole has the property of being a pro-drug, which is reduced by nitroreductases to an active compound inside the bacterium. This reduction depends on the redox potential of the bacterial cell environment. Along these lines, it has been shown *in vitro* that an anaerobic pre-incubation of the agar plates decreases the minimal inhibitory concentration (MIC) of metronidazole as compared with agar plates that have been left in contact with air (Cederbrant *et al.*, 1992). Therefore, redox potential rather than pH appears to be an important factor of activity when metronidazole is used; however, this cannot be controlled *in vivo*.

4.2.2 Rationale for the use of absorbable antibiotics

The possibility of a direct topical action of a drug on bacteria in the stomach following oral administration was first considered to be important for the drug's activity. Indeed, this is true for drugs like bismuth salts which have an 'antiseptic-like' activity, i.e. a short contact leads to the destruction of the bacterium (Armstrong *et al.*, 1987). However, this does not appear to be true for antibiotics whose activity requires prolonged contact with the

bacterium in order to be active, and for these a systemic diffusion is required.

Orally delivered antibiotics are absorbed in the intestine, circulate in the bloodstream and then finally diffuse into the gastric mucus through the epithelial layer. Antibiotics that are not absorbed have never been shown to be effective for *H. pylori* eradication. However, all antibiotics do not have the same characteristics in this respect. To date, diffusion has mainly been studied *in vivo* for amoxicillin, clarithromycin and metronidazole. By increasing the pH, the intragastric concentration of amoxicillin increases, while that of metronidazole decreases and clarithromycin is not affected (Goddard *et al.*, 1996).

The difference between amoxicillin and clarithromycin has also been studied in an *in vitro* model using epithelial cells, with contrasting results (Matysiak-Budnik *et al.*, 2002). Unlike amoxicillin, clarithromycin is able to accumulate intracellularly where a high concentration can be reached, conferring several advantages. The first advantage is that clarithromycin can act on the few bacteria which have penetrated intracellularly. Even if *H. pylori* is not an intracellular pathogen, recent data have pointed out the occurrence of intracellular bacteria and their importance in the natural history of infection (Dubois and Boren, 2007). Second, the slow release of clarithromycin into the mucus allows a sufficient concentration of the

antibiotic over time to kill the bacteria. In contrast, amoxicillin is not able to penetrate intracellularly but instead diffuses between the epithelial cells, which is effective only when blood concentrations of this antibiotic are high. Given this, a good approach would be to administer amoxicillin several times daily, or even better intravenously, in order to increase its activity, but due to practical problems this approach is not used.

The antibiotics more recently recommended for *H. pylori* eradication have not been studied from a pharmacological point of view. However, it is well known that fluoroquinolones (levofloxacin) and rifampicins (rifabutin) are able to diffuse intracellularly. Furazolidone is like metronidazole, a small molecule that easily crosses the epithelial barriers.

4.2.3 Rationale for the use of a combination of antibiotics

The limitations of antibiotic efficacy mentioned previously led to the use of a combination of two antibiotics. The rationale for this approach is: (i) to increase efficacy by an additive (or eventually synergistic) effect; and (ii) to avoid the emergence of resistance. Resistance to antibiotics in *H. pylori*, similar to the case of *Mycobacterium tuberculosis*, arises essentially via mutations (Table 4.2).

The probability of observing mutations for two antibiotics in a given bacterial cell is the product of the probability of having it occur for each antibiotic separately. For example, if the probability of mutations is 10^{-7} for clarithromycin and 10^{-5} for metronidazole, it will be 10^{-12} for both, i.e. a frequency so rare that it should not occur.

4.3 Currently Recommended Treatment

4.3.1 First-line therapy

The combinational use of a PPI with two antibiotics was proposed in 1993 in Italy by Bazzoli *et al.* (1993; clarithromycin and metronidazole) and in France by Lamouliatte *et al.* (1993; clarithromycin and amoxicillin). These treatments, known as PPI-based triple therapies, were recommended as first-line treatments at the first Maastricht Conference held in 1995 (Anonymous, 1997) and then reconfirmed at other consensus conferences held around the world, including the Maastricht III Conference (Malfertheiner *et al.*, 2007). The only controversy arising from these consensus meetings relates to the length of treatment regimens, which ranges from 7 to 14 days. A recent meta-analysis by Fuccio *et al.* (2007) was in favour of 7 days.

Given that amoxicillin resistance seldom occurs, the combination of clarithromycin and amoxicillin was originally preferred, despite the subsequent observation that the combination of clarithromycin and metronidazole is slightly more effective on susceptible strains. An alternative was also to use amoxicillin and metronidazole. All of these treatments reached an eradication rate of more than 80%, the threshold that was defined as being acceptable at consensus conferences (Anonymous, 1997).

There is a large body of data published on these various combinations. In particular, the causes of failure have been documented (Table 4.3). Pre-treatment with a PPI is not a risk factor for failure (Janssen *et al.*, 2005). Instead, antimicrobial resistance, which concerns essentially clarithromycin, while

Table 4.2. Genes involved by point mutations or other genetic events leading to antibiotic resistance in *Helicobacter pylori*.

Antibiotics	Gene(s) concerned	Reference(s)
Macrolides	<i>rrn</i> 23S	Versalovic <i>et al.</i> (1996)
Fluoroquinolones	<i>gyrA</i>	Moore <i>et al.</i> (1995)
Tetracyclines	<i>rrn</i> 16S	Gerrits <i>et al.</i> (2002a); Trieber and Taylor (2002)
Rifampicins	<i>rpoB</i>	Heep <i>et al.</i> (1999)
Amoxicillin	<i>pbp1</i>	Gerrits <i>et al.</i> (2002b)
Metronidazole	<i>rdxA</i> , <i>frxA</i>	Goodwin <i>et al.</i> (1998)

Table 4.3. Factors implicated in the failure of proton pump inhibitor-based triple therapies to eradicate *Helicobacter pylori*.

Factor	Reference(s)
Resistance to antibiotics	Mégraud (2004); Fischbach and Evans (2007)
Lack of compliance	Wermeille <i>et al.</i> (2002)
High gastric acidity	Villoria <i>et al.</i> (2008); Zhao <i>et al.</i> (2008)
High bacterial load	Moshkowitz <i>et al.</i> (1995)
<i>cagA</i> -negative status	Suzuki <i>et al.</i> (2006)
Presence of intracellular bacteria	Lai <i>et al.</i> (2006)
Altered immunity	Borody <i>et al.</i> (2002)

cagA, cytotoxin-associated gene A.

not the explanation for all cases, appears to be the main reason for failure. Details on resistance prevalence, impact on treatment success and modes of detection are presented below. At the last Maastricht Conference in 2007, the recommendation was made to perform susceptibility testing for clarithromycin, or not to use this antibiotic if the prevalence of resistance in a specific country/region was in the range of 15–20% or higher (Malfertheiner *et al.*, 2007).

Alternative first-line treatments are now emerging, especially the use of PPI, amoxicillin and a fluoroquinolone such as levofloxacin. This combination, first used by Cammarota *et al.* (2000) in Italy, resulted in eradication rates between 92 and 95% and has been recommended as the first choice of salvage therapy. However, similar to clarithromycin, resistance to levofloxacin easily occurs and jeopardizes the success of treatment. Another alternative treatment that has been claimed not to be influenced by antimicrobial resistance is the so-called ‘sequential therapy’. The concept is to first treat with a PPI and amoxicillin for 5 days and then replace amoxicillin with clarithromycin plus metronidazole for

the following 5 days. The rationale is to decrease the bacterial load during the first 5 days using amoxicillin, then to eradicate the remaining bacteria by the combination of clarithromycin and metronidazole (Zullo *et al.*, 2003). Decreasing the bacterial load reduces the chance of mutations to almost nil, given that mutations occur at a low frequency. Another postulated mechanism to explain the efficacy of the sequential therapy is the impact of amoxicillin on the bacterial cell wall and then on efflux pumps, which would prevent the binding of clarithromycin to the ribosome (Zullo *et al.*, 2007a). However, the role of efflux pumps in macrolide resistance is usually not considered a major one.

This sequential therapy has proved to be successful (Gisbert *et al.*, 2010). However, antimicrobial susceptibility was not tested in all studies and the number of cases where efficacy is shown on resistant strains is small (Table 4.4) and deserves further study.

Performing antimicrobial susceptibility testing is the best way to ensure successful treatment of a patient and should be carried out whenever possible for clarithromycin and also for levofloxacin, since new tools to

Table 4.4. Impact of *Helicobacter pylori* clarithromycin resistance on the success of sequential treatment (number of patients in whom *H. pylori* was eradicated/number treated).

	Standard therapy		Sequential therapy		Reference
	Susceptible	Resistant	Susceptible	Resistant	
		3/11		15/19	Zullo <i>et al.</i> (2003)
	51/54	7/16	58/59	18/22	De Francesco <i>et al.</i> (2006b)
	86/91	8/28	108/114	8/13	Vaira <i>et al.</i> (2007)
	8/8	4/7	10/12	1/1	Kalach <i>et al.</i> (2008)
Total	145/153	22/62	176/185	42/55	
%	94.7	35.4	95.1	76.3	

determine antibiotic susceptibility are now available (see Section 4.4.3).

Lastly, the sole treatment that appears to be only modestly influenced by resistance is bismuth-based quadruple therapy. This treatment can be advocated as a first-line treatment in areas of high antibiotic resistance, as was the recommendation at the Maastricht III Conference (Malfertheiner *et al.*, 2007).

4.3.2 Second-line therapy

In individuals in whom initial eradication therapy has failed, unless there is the possibility of showing their efficacy by testing clarithromycin and levofloxacin susceptibility, subsequent treatments using these drugs should be avoided. The PPI-based triple therapies to be considered are indicated in Table 4.5. The amoxicillin–metronidazole combination has been used in a large study in France (Lamouliatte *et al.*, 2003) among others, but led to less than 80% eradication as a second-line treatment. The tetracycline–metronidazole combination appears more effective, but few records exist (Realdi *et al.*, 1999). The amoxicillin–rifabutin combination should be used as little as possible because of the fear of rifampicin resistance in *M. tuberculosis*.

Bismuth-based triple and, even better, quadruple therapies are of great interest in this context. Indeed, the Maastricht Conferences always recommended bismuth-based quadruple therapy as a second-line treatment. Given the potential adverse events due to bismuth salts a systematic review was performed, including 35 randomized

controlled trials with 4763 patients (Ford *et al.*, 2008). No serious adverse event was reported in these trials. The total number of adverse events observed with bismuth-based therapies was indeed not significantly different, with the exception of dark stools, from those observed with control therapies comprising antisecretory-based triple therapies or association of antibiotics: odds ratio (OR) = 1.01 (95% confidence interval (CI) 0.87, 1.16). In addition, there was no increase in the withdrawal of therapy when bismuth salts were administered (Ford *et al.*, 2008).

Non-bismuth quadruple therapies (including three antibiotics and a PPI) have also been used. A meta-analysis reporting on five randomized trials found that pooled estimates of these trials showed a superiority of quadruple therapy over triple therapy: OR = 2.86 (95% CI 1.73, 4.73) in intention-to-treat analysis (Essa *et al.*, 2009).

4.3.3 Specific problems

Specific problems may arise in some countries because certain drugs are not available. Such is the case in France, for example, where bismuth salts and tetracycline are currently not on the market. If, in addition, the doctor is faced with a patient supposedly allergic to β -lactams who is infected with a strain resistant to clarithromycin and levofloxacin, it may become impossible to find an adequate treatment. This point highlights the need to consider the treatment of *H. pylori* infection seriously and therefore to apply susceptibility testing as often as possible.

Table 4.5. Therapies used as second-line treatment for *Helicobacter pylori* eradication in cases of clarithromycin and levofloxacin resistance.

Therapy	Regimen	Duration
PPI-based	PPI–amoxicillin–metronidazole	14 days
	PPI–tetracycline–metronidazole	14 days
	PPI–amoxicillin–rifabutin	10 days
Bismuth-based	Bismuth–tetracycline–metronidazole	14 days
	Bismuth–tetracycline–furazolidone	14 days
	Bismuth–tetracycline–metronidazole–PPI	7–14 days
Dual therapies	PPI–amoxicillin	14 days (high dosage)

PPI, proton pump inhibitor.

4.3.4 Adjuvant therapy

Antisecretory drugs are already an adjuvant therapy, i.e. an additional drug that has limited effects by itself but improves the efficacy of the other drugs given at the same time. In addition, attempts have been made to improve eradication rates by other means.

The most interesting is the use of probiotics. A meta-analysis of trials (14 trials, 1671 patients) where probiotics were added to triple therapy regimens found a beneficial effect of this supplementation. The pooled eradication rate in this meta-analysis was 83.6% (95% CI 80.5, 86.7) with probiotics versus 74.8% (95% CI 71.1, 78.5) without, by intention-to-treat analysis (Tong *et al.*, 2007). While some probiotics are able to decrease the load of bacteria, it appears that their beneficial effect is essentially the result of decreasing adverse events (from 38.5 to 24.7%) and thereby probably facilitating better compliance.

Another adjuvant therapy comprises the addition of lactoferrin. A meta-analysis conducted on nine randomized trials representing 1343 patients found a beneficial effect, with the pooled eradication rate with lactoferrin being 86.5% (95% CI 84.0, 89.1) versus 74.4% (95% CI 71.1, 77.7) without lactoferrin, by intention-to-treat analysis (Zou *et al.*, 2009). The positive effect of lactoferrin on adverse events was particularly notable regarding nausea. The same beneficial effects have been noted in another meta-analysis (Sachdeva and Nagpal, 2009).

Vegetable products may also be interesting for this purpose. Cranberry juice, for example, has been reported to improve eradication in a subgroup of patients, but further studies are needed (Shmueli *et al.*, 2007).

4.4 Antimicrobial Resistance

4.4.1 Prevalence

Antibiotics with high resistance rates: clarithromycin, levofloxacin, metronidazole

The prevalence of antimicrobial resistance is by essence an evolving phenomenon in time and space. Therefore only recent data are of interest and they vary from place to place.

We are essentially interested in the so-called primary resistance, i.e. the *H. pylori* resistance found in patients not having been previously treated for *H. pylori* infection. This primary resistance is most likely the consequence of treatments administered to the patient for other types of infection. Examples are respiratory infections for clarithromycin, urinary tract and respiratory infections for levofloxacin, and gynaecological and dental infections for metronidazole. In such cases the antibiotic used as a monotherapy may have selected resistant *H. pylori* mutants, but was not able to lead to eradication. Another possibility, which probably occurs only in a minority of cases, is the transmission of resistant organisms to a new host. Since *H. pylori* is essentially transmitted from parents to children, and clarithromycin has been mainly administered to children for less than two decades, the transmission of clarithromycin-resistant strains is considered to be rare. The situation is different for resistance to fluoroquinolones, as these antibiotics are not used to treat children. In Portugal, a country in which the new fluoroquinolones have been widely used (in adults) for almost 20 years, fluoroquinolone resistance is now being observed in children (4.5%) (Lopes *et al.*, 2005). Such transmission of clarithromycin-resistant organisms will also occur in the future, when yesterday's children become adults and have children themselves.

Another point to highlight when considering the prevalence of *H. pylori* resistance is how representative were the cases studied. An interesting design was applied in a Swedish study which selected a random sample of 3000 subjects in the population and contacted them by mail. Seventy-four per cent responded and a random sample of one-third of these respondents was offered an endoscopy. The *H. pylori* strains obtained (333), which were definitely representative of the population as a whole, were then tested for antimicrobial susceptibility (Storskrubb *et al.*, 2006). Interestingly, the authors found only 1.5% of the strains resistant to clarithromycin and 16.2% resistant to metronidazole.

It may be argued that it is more pertinent to test the strains of patients seeking medical attention and this has been done many times.

In order to get meaningful prevalence values with small confidence intervals, it is important to test large numbers of strains and therefore to perform multicentre studies. An important Japanese study carried out between 2002 and 2005 gathered 3707 strains (from 36 centres) and showed a clarithromycin resistance rate of 22.7% (Kobayashi *et al.*, 2007).

In Europe, multicentre studies have also been conducted. The first in 1991 (involving 12 centres from 11 countries) surveyed only metronidazole resistance (Anonymous, 1992), while subsequent studies tested more antibiotics: in 1998 (22 centres from 17 countries) (Glupczynski *et al.*, 2001) and in 2008–2009 (31 centres from 17 countries). Despite the fact that the centres involved were not exactly the same and that the protocols used evolved between the studies, information was collected on the development of *H. pylori* resistance over time. For example, clarithromycin resistance that was 9.9% in 1998 has doubled over the last decade.

There is a marked difference between northern and southern European countries. In northern countries where there is a strict policy for antibiotics resulting in more restrictive use, the resistance rates are much lower (4.2% in the north and 18.4% in the south for clarithromycin in 1998).

A European multicentre study conducted recently over a 4-year period in 17 paediatric centres from 14 countries showed a resistance rate to clarithromycin among 1233 patients of 24% (Koletzko *et al.*, 2006).

A further source of information on resistance corresponds to the data generated by clinical trials where strains were cultured and tested, and those from monocentre studies. The problem with monocentre studies, however, is that these are usually referral centres, which gather difficult-to-treat patients, hence giving higher resistance rates.

The results of recent studies are presented in Table 4.6.

Antibiotics with low resistance rates: amoxicillin, tetracycline, rifabutin

In most of the studies performed, resistance to these three antibiotics was nil or less than 1% with a few exceptions for tetracycline

(Korea: 5.3%, Bulgaria: 4.4%) (Kim *et al.*, 2001; Boyanova *et al.*, 2008). Indeed, concerning amoxicillin, the prevalence rates observed in some studies must be treated with caution if the strains were not studied in detail.

Rifabutin resistance can be found in patients having received treatment for tuberculosis (Suzuki, S. *et al.*, 2009).

Multiple resistance

H. pylori strains resistant to two or more antibiotics are still not very common in patients receiving a first treatment (<10%). After failure of a clarithromycin-based triple therapy, the risk of finding a clarithromycin-resistant strain is approximately 65%. It is in the same range after failure of a levofloxacin-based triple therapy (Perna *et al.*, 2007; Nishizawa *et al.*, 2008). After several treatment attempts, it was possible to find strains resistant to three, four and even five antibiotics in 15% of the cases, for example in Germany (Wueppenhorst *et al.*, 2009).

Results concerning resistance to metronidazole have shown low reproducibility (Glupczynski *et al.*, 2002) and limited clinical relevance on an individual basis (see next section).

4.4.2 Clinical impact of antibiotic resistance

Data on the clinical impact of antibiotic resistance exist essentially for clarithromycin and metronidazole, with contrasting figures. Indeed, for clarithromycin, they can explain the majority of treatment failures. The most recent study published in this respect is the meta-analysis of Fischbach and Evans (2007) (Table 4.7). For the triple therapy PPI–clarithromycin–amoxicillin, there was a reduction in *H. pylori* eradication of 66% (95% CI 58.2, 74.2) when the strain was resistant to clarithromycin, versus susceptible strains. This result is in the range of those previously published by Megraud (2004), which indicated a 70% decrease in eradication from 87.8% to 18.3%.

For the triple therapy PPI–clarithromycin–metronidazole, the decrease in efficacy was

Table 4.6. Prevalence of *Helicobacter pylori* resistance in adults, worldwide. Data are from studies including more than 100 strains isolated totally or partially since the year 2000.

Country	Year(s)	Study	Method	No. of strains	Clari ^R (%)	Metro ^R (%)	Tetra ^R (%)	Amox ^R (%)	FQ ^R (%)	Reference
<i>Europe</i>										
Belgium	2002	MonoC	DD	164	3	31	0	0	NP	Aguemon <i>et al.</i> (2005)
Belgium	2003–2004	MultiC	E test	488	NP	NP	NP	NP	16.8	Bogaerts <i>et al.</i> (2006)
Bulgaria	2005–2007	MultiC	AD	613	17.8	25	4.4	0.9	7.7	Boyanova <i>et al.</i> (2008)
Croatia	2001	MonoC	E test	196	8	33	NP	0	NP	Bago <i>et al.</i> (2002)
Croatia	2002–2005	MonoC	AD	592	8.2	32.9	NP	NP	NP	Filipeć Kanizaj <i>et al.</i> (2009)
Finland	2000–2002	MultiC	E test	292	2	38	NP	NP	NP	Koivisto <i>et al.</i> (2004)
France	2004–2005	MonoC	AD	128	NP	NP	NP	NP	17.2	Cattoir <i>et al.</i> (2007)
Germany	2001–2005	MultiC	E test	126	9.5	42	NP	0	9.6	Glocker <i>et al.</i> (2007a)
Hungary	2004–2006	MonoC	FISH	238	17.3	NP	NP	NP	NP	Buzas <i>et al.</i> (2007)
Italy	2004–2006	MultiC	E test	255	16.9	29.4	NP	NP	19.1	Zullo <i>et al.</i> (2007b)
Italy	1998–2002	MonoC	AD	406	23.4	36.7	NP	0.2	NP	Toracchio and Marzio (2003)
Italy	2004–2005	MonoC	RT-PCR	178	21.3	NP	NP	NP	NP	De Francesco <i>et al.</i> (2007)
Italy	2007	MultiC	E test	109	18	27	0	0	3	Romano <i>et al.</i> (2008)
Italy	2004–2005	MultiC	RT-PCR	232	26.7	NP	NP	NP	NP	De Francesco <i>et al.</i> (2006a)
Poland	2001–2004	MultiC	E test	130	15	42	0	0	NP	Dzierzanowska-Fangrat <i>et al.</i> (2005)
Sweden	1998–2001	MultiC	AD	333	1.5	16.2	0.3	0	NP	Storskrubb <i>et al.</i> (2006)
Netherlands	1997–2002	MonoC	DD	1125	1	14.4	NP	NP	NP	Janssen <i>et al.</i> (2006)
UK (Wales)	2000–2003	MonoC	E test	363	7	24	0.3	0	NP	Elviss <i>et al.</i> (2004b)
UK (Wales)	2000–2005	MonoC	E test	664	8.4	36.3	<0.5	0	NP	Chisholm <i>et al.</i> (2007)
UK (England)	2000–2005	MonoC	E test	646	12.7	28.6	<0.5	0	NP	Chisholm <i>et al.</i> (2007)
<i>North America</i>										
USA (Alaska)	1999–2003	MultiC	AD	352	31	44	0	2	NP	Bruce <i>et al.</i> (2006)
USA	2000–2001	MultiC	AD	106	12.2	33.9	NP	NP	NP	Laine <i>et al.</i> (2003)
USA	1998–2002	MultiC	AD	347	12.9	25.1	0	0.9	NP	Duck <i>et al.</i> (2004)
<i>Middle East</i>										
Iran	2001–2002	BiC	DD	120	16.7	57.5	0	1.6	NP	Mohammadi <i>et al.</i> (2005)
Israel	2000–2001	MonoC	E test	110	8.2	38.2	0	0.9	NP	Samra <i>et al.</i> (2002)
Turkey	Unknown	MonoC	RT-PCR	110	48.2	NP	NP	NP	NP	Onder <i>et al.</i> (2007)

Far East

Hong Kong	2003–2004	MonoC	E test	102	7.8	39.2	NP	0	NP	Gu <i>et al.</i> (2006)
Hong Kong	2004–2005	MonoC	AD	191	NP	NP	NP	NP	11.5	Lee <i>et al.</i> (2008)
Japan	2001–2004	MonoC	E test	507	NP	NP	NP	NP	15	Miyachi <i>et al.</i> (2006)
Japan	2002–2005	MultiC	AD	3707	22.7	2.7	NP	0.08	NP	Kobayashi <i>et al.</i> (2007)
Korea	2001–2005	MonoC	E test	144	16.7	34.7	NP	11.8	NP	Bang <i>et al.</i> (2007)
Singapore	2002	MonoC	AD	120	NP	31.7	NP	NP	NP	Lui <i>et al.</i> (2003)
Taiwan	1998–2007	MonoC	AD	210	9.5	27.6	0.5	1	5.7	Hung <i>et al.</i> (2009)
Taiwan	2001–2004	MonoC	E test	134	6.7	25.4	NP	NP	NP	Poon <i>et al.</i> (2009)
Taiwan	2004–2005	MonoC	E test	133	13.5	51.9	0	0.7	NP	Hu <i>et al.</i> (2007)

Africa

Kenya	2003–2004	MonoC		114	6.4	100	1.9	4.6	NP	Lwai-Lume <i>et al.</i> (2005)

Abbreviations: AD, agar dilution; Amox, amoxicillin; Clari, clarithromycin; DD, disc diffusion; FISH, fluorescence *in situ* hybridization; FQ, fluoroquinolones; Metro, metronidazole; MonoC, monocentre; MultiC, multicentre; NP, not performed; PCR, polymerase chain reaction; R, resistant; RT, real time; Tetra, tetracycline.

Table 4.7. Decreased efficacy of *Helicobacter pylori* eradication treatment according to antimicrobial resistance. The antibiotic for which *H. pylori* resistance was detected is underlined. (Adapted from Fischbach and Evans, 2007.)

Treatment	No. of studies	No. of arms	No. of patients	Decreased efficacy	
				%	95% CI
PPI– <u>Clari</u> –Amox	24	64	2556	66	58.2, 74.2
PPI– <u>Clari</u> –Metro	34	99	3128	35	25.4, 45.4
PPI–Clari– <u>Metro</u>	34	99	3128	18	13.4, 24.0
PPI–Amox– <u>Metro</u>	24	57	1945	30	21.8, 38.2
Bi–Tetra– <u>Metro</u>	14	31	711	26	14.2, 37.8
Bi–Tetra– <u>Metro</u> –PPI	16	30	899	14	5.4, 22.6

Abbreviations: Amox, amoxicillin; Bi, bismuth; Clari, clarithromycin; Metro, metronidazole; PPI, proton pump inhibitor; Tetra, tetracycline.

35% (95% CI 25.4, 45.4) when the strain was resistant to clarithromycin, versus susceptible strains. In contrast, for the same triple therapy (PPI–clarithromycin–metronidazole), when the strain was resistant to metronidazole instead of clarithromycin, the reduction in efficacy was 18% (95% CI 13.4, 24.0). The combination of PPI–amoxicillin–metronidazole was also studied and showed a 30% (95% CI 21.8, 38.2) reduction in efficacy. The impact of metronidazole resistance appears to be even lower when the regime contains a bismuth salt. For the bismuth-based triple therapy bismuth–tetracycline–metronidazole, a decrease in efficacy of 26% (95% CI 14.2, 37.8) was observed. However, for the bismuth-based quadruple therapy where a PPI was added, the decrease in efficacy observed was only 14% (95% CI 5.4, 22.6) (see Table 4.7) (Fischbach and Evans, 2007).

Concerning the resistance to fluoroquinolones, only two studies were found in the literature. In one study the triple therapy PPI–amoxicillin–levofloxacin, administered to 33 patients for 10 days, led to eradication in 75% of those harbouring susceptible strains, but in only 33.3% of those with resistant strains (Perna *et al.*, 2007). In another trial using gatifloxacin instead of levofloxacin over 7 days, 100% of susceptible strains were eradicated, but only one-third of resistant strains (Nishizawa *et al.*, 2008). Resistance to the other antibiotics used may also have an impact but the clinical trials performed either did not test for resistance or did not include resistant strains to these antibiotics.

4.4.3 Detection of antibiotic resistance

Phenotypic methods

As for any bacterium, the standard method of agar diffusion can be applied to determine the antibiotic sensitivity of *H. pylori* strains. However, *H. pylori* being a fastidious organism, the E test (an agar diffusion method using a strip with an antimicrobial gradient which gives the exact MIC of the strain) appears to be more adapted and provides MIC values. The agar dilution method is, nevertheless, considered to be the reference method being the most accurate, and can be simplified by including in the agar the concentration of antibiotic considered to be the cut-off for resistance. This is commonly performed for metronidazole and has also been proposed with a four-sector agar plate in which one sector does not include any antibiotics and the three others include, for example, clarithromycin, amoxicillin and another antibiotic (Caristo *et al.*, 2008).

Cut-off values for resistance have been officially validated only for clarithromycin, by the National Committee for Clinical Laboratory Standards (NCCLS), now the Clinical and Laboratory Standards Institute (CLSI), as follows: susceptible, <0.25 mg/l; intermediary, 0.25–0.75 mg/l; and resistant, ≥1 mg/l.

The thresholds commonly used for the other antibiotics are: fluoroquinolones, 1 mg/l; rifabutin, 1 mg/l; tetracycline, 2 mg/l; amoxicillin, 1 mg/l; and metronidazole, >8 mg/l. For amoxicillin, MICs of 0.25 and 0.5 mg/l are considered to correspond to a decreased

susceptibility, but the clinical impact has not been studied.

Currently, the minimal antibiogram to be performed, i.e. the least number of antibiotics with high clinical relevance to test, includes only two antibiotics: clarithromycin and a fluoroquinolone (ciprofloxacin can be tested if levofloxacin is not available, but must never be used clinically due to its low efficacy *in vivo*). It can be performed either by agar diffusion or by E test. If agar diffusion is used, an inhibition diameter of 20 mm is applied as the threshold. An E test can be used as a control when the diameter is less than 20 mm.

Genotypic methods

Given that *H. pylori* resistance to antibiotics essentially occurs by point mutations, it is possible to detect these mutations by molecular methods. Numerous methods have been applied to the detection of clarithromycin resistance and some to the detection of fluoroquinolone and tetracycline resistance. Such methods have proved more difficult to develop for amoxicillin and metronidazole.

CLARITHROMYCIN. Mutations. Macrolides target the peptidyl transferase loop on domain V of 23S ribosomal RNA (*rrn*) and produce an arrest in peptide elongation. Resistance occurs in *H. pylori* by mutations, especially at positions 2142 (A2142G, A2142C) and 2143 (A2143G), which probably lead to a conformational modification of the target site and a decrease in fixation (Versalovic *et al.*, 1996; Occhialini *et al.*, 1997). These mutations lead to a cross-resistance between all macrolides. Despite the fact that *H. pylori* contains two rRNA operons, heterozygosity is rarely found.

Such mutations occur spontaneously and are thus selected when the bacteria are exposed to the antibiotic. *In vitro*, the frequency of these mutations ranges from 3.2×10^{-7} to 6.0×10^{-8} but could be higher *in vivo* due to oxidative stress (Kuwahara *et al.*, 2009). The most commonly found mutations do not appear to have a biological cost and appear to be stable, while other possible mutations are either deleterious to the bacteria or too costly to be maintained.

Detection. Numerous methods have been applied for clarithromycin resistance, either on strains or directly on biopsy specimens (Table 4.8). Those that are currently commercially available are discussed further below.

Mutation detection by real-time PCR. One of the most effective methods is real-time polymerase chain reaction (PCR). This method can be performed using the fluorescence resonance energy transfer (FRET) principle in a LightCyclerTM apparatus. First, primers have to be designed for the 23S rRNA genes in order to specifically detect *H. pylori*. Second, a specific biprobe must be designed inside the amplicon. It comprises: (i) a detection probe 5'-labelled with LC-Red 640 whose hybridization site includes the nucleotides where the mutation may occur; and (ii) a fixation probe 3'-labelled with fluorescein, which hybridizes three to five nucleotides upstream of the others. When the fixation probe is excited, there is an energy transfer to the detection probe because of its proximity, which results in emission of a signal. This allows the possibility of following amplicon synthesis in real time in order to detect *H. pylori* in a gastric biopsy. When the amplification is finished, a melting curve analysis (MCA) of the amplicons is conducted. Because of the mismatch, the melting temperature of the mutant is lower than that of the wild-type strain.

Different designs of this reaction have been published (Matsumura *et al.*, 2001; Oleastro *et al.*, 2003; Schabereiter-Gurtner *et al.*, 2004) and one is now commercially available (ClariRes Assay; Ingenetix, Vienna, Austria).

Advantages of real-time PCR are:

- rapid results (2 h);
- limited manipulations;
- no risk of contamination with amplicons; and
- application to specimens other than gastric biopsies.

Limitations of real-time PCR are:

- possible amplification of bacteria having a similar *rrn23S*, including *Helicobacter heilmannii* and possibly others not currently in databases;

Table 4.8. Genotypic methods used to detect macrolide resistance in *Helicobacter pylori*.

Technique	Reference(s)
<i>Using 23S rDNA amplification</i>	
Sequencing	
Restriction fragment length polymorphism (RFLP)	Versalovic <i>et al.</i> (1996); Menard <i>et al.</i> (2002)
Oligonucleotide ligation assay (OLA)	Stone <i>et al.</i> (1997)
DNA enzyme immunoassay (DEIA)	Pina <i>et al.</i> (1998); Marais <i>et al.</i> (1999)
Real-time polymerase chain reaction (PCR)	Gibson <i>et al.</i> (1999); Chisholm <i>et al.</i> (2001); Matsumura <i>et al.</i> (2001); Lascols <i>et al.</i> (2003); Oleastro <i>et al.</i> (2003)
Line probe assay (InnoLipa)	Van Doorn <i>et al.</i> (1999)
Genotype HelicoDR	Cambau <i>et al.</i> (2009)
Preferential homoduplex formation assay (PHFA)	Maeda <i>et al.</i> (2000)
3' mismatched PCR	Alarcon <i>et al.</i> (2000)
3' mismatched reverse PCR	Elviss <i>et al.</i> (2004a)
Pyrosequencing	Hjalmarsson <i>et al.</i> (2004); Moder <i>et al.</i> (2007)
PCR-based denaturation HPLC	Posteraro <i>et al.</i> (2006)
Allele-specific PCR	Furuta <i>et al.</i> (2007); Nishizawa <i>et al.</i> (2007)
Quadruplex real-time PCR	Burucoa <i>et al.</i> (2008)
Oligonucleotide microarray	Chen <i>et al.</i> (2008)
Dual-priming oligonucleotide (DPO)-PCR	Woo <i>et al.</i> (2009)
<i>Without using 23S rDNA amplification</i>	
Fluorescence <i>in situ</i> hybridization (FISH)	Trebesius <i>et al.</i> (2000)
Electrocatalytic detection	Lapierre <i>et al.</i> (2003)
Microelectronic chip array	Xing <i>et al.</i> (2005)

- detection of mutations only inside the chosen amplicon;
- difficulty in identifying the exact mutation (position 2642 or 2643) in some formats; and
- need for an expensive thermocycler.

Mutation detection by dual-priming oligonucleotide-based multiplex PCR. Another method is dual-priming oligonucleotide (DPO)-based multiplex PCR (Seeflex® ClaR-*H. pylori* ACE detection; Seegene, Seoul, Korea). The DPO primer system differs from a conventional system by introducing a poly(I) linker between two unequal segments of primer sequences. This addition increases the specificity sufficiently to discriminate between single-base changes by using a one-step PCR, thus allowing an accurate multiplex PCR. To determine *H. pylori* resistance to clarithromycin, two forward and two reverse DPO primers were designed against the 23S rRNA gene: HP-F, HP-R, A2142G-F and A2143G-R, respectively. This four-primer combination amplifies three fragments: a common *H. pylori* sequence, an A2142G mutant and an A2143G mutant in a multiplex

PCR. The mutant-specific DPO primers A2142G-F and A2143G-F were designed to contain either a single- or a two-base variation in the middle of the 3' segment to maximize the ability of DPO to discriminate between single-base changes resulting from disruptions in the annealing of the 3' segment.

When an *H. pylori* strain is a wild-type, neither A2142G-F nor A2143G-R anneals to the template, and PCR amplification occurs between HP-F and HP-R, resulting in the production of a 621 bp amplicon. In the case of the A2143G mutation, PCR amplification occurs simultaneously between HP-F and HP-R and between HP-F and A2143G-R, resulting in the production of a 621 bp and a 475 bp amplicon, respectively. Similarly, in the case of the A2142G mutation, PCR amplification occurs simultaneously between HP-F and HP-R and between A2142G-F and HP-R, resulting in the production of a 621 bp and a 193 bp amplicon, respectively. The detection of the A2142C mutation is not performed, as this mutation has rarely been identified (Woo *et al.* 2009). A specific apparatus has been designed to minimize post-PCR

manipulations. A procedure to minimize contamination with amplicons is available.

Advantages of DPO-based multiplex PCR are:

- rapid results (less than 5 h);
- no need for expensive apparatus; and
- limited risk of contamination.

Limitations of DPO-based multiplex PCR are:

- manipulation required;
- possible amplifications of bacteria having a similar *rrn23S*; and
- detection of only the mutations for which primers are designed (e.g. A2142C is not included).

Mutation detection by DNA-strip test. One of the first methods developed to detect *H. pylori* and the mutations associated with its resistance to macrolides was a DNA-strip test (Van Doorn *et al.*, 1999). A new test based on this technology is now commercially available which also includes the detection of the mutations associated with fluoroquinolone resistance (GenoType HelicoDR; Hain LifeSciences, Nehren, Germany). It consists of a multiplex PCR followed by a hybridization with biotin-labelled specific oligonucleotides immobilized on a strip and visualized by a streptavidin/alkaline phosphatase-mediated staining reaction (Cambau *et al.*, 2009).

Advantages of the DNA-strip test are:

- rapid results (less than 6 h); and
- no need for expensive apparatus.

Limitations of the DNA-strip test are:

- manipulation required;
- risk of contamination;
- possible amplification of bacteria having a similar *rrn23S*; and
- detection of only the mutations for which hybridization is carried out.

Mutation detection by fluorescence in situ hybridization. It is also possible to detect *H. pylori* and its resistance to clarithromycin without PCR by using a fluorescence *in situ* hybridization (FISH) method. The principle includes the use of specific probes, one targeting the 16S rRNA gene labelled with a Cy3

fluorochrome (red) allowing the specific detection of *H. pylori*, and three others targeting the mutated 23S rRNA labelled with fluorescein (green). These four probes are used simultaneously. When a susceptible organism is present, it hybridizes only with the first probe and *H. pylori* appears red. When a resistant organism is present, the 23S rDNA probe as well as one of the other probes hybridize and *H. pylori* appears yellow due to the overlapping of the red and green colours (Trebesius *et al.*, 2000).

In contrast to the other methods, this method must be used on slides and is adapted to formalin-fixed paraffin-embedded histological preparations. It is commercially available as the SeaFAST *H. pylori* Combi Kit (SeaPro Theranostics, International b.v., Lelystad, Netherlands).

Advantages of FISH are:

- rapid results;
- no need for special apparatus;
- no impact of PCR inhibition;
- bacteria are visualized; and
- test can be performed in a pathology laboratory.

Limitations of FISH are:

- manipulation required;
- possible difficulty in interpretation;
- results are observer-dependent; and
- detection of only the mutations for which probes are available.

When a mutation occurs, it concerns only a single organism within a whole population. However, the descendants of this organism will also have the mutation, so along with successive replications, the population will be enriched in resistant organisms. However, it is only when the antibiotic is administered that the resistant organisms will be selected and eventually constitute the whole population. Molecular methods appear to be more sensitive in the detection of resistant organisms than phenotypic methods, as they are able to detect resistant organisms at a lower proportion in a mixed population of susceptible and resistant organisms.

The correlation between the presence of resistant organisms and *H. pylori* eradication with clarithromycin-based triple therapy has

been performed essentially using phenotypic methods and shows good results. However, among the strains classified as susceptible, failure to eradicate can still occur on occasion. While this can be explained by causes other than antimicrobial resistance, it may also be due to resistant mutants present at a low level and not detected phenotypically. Such resistant mutants are prone to emerge once the clarithromycin-based treatment is administered.

Molecular methods also allow detection of *H. pylori* in faecal samples, as anything present in the stomach is eliminated in faeces. However, except in cases where there is a very short transit time, these bacteria do not remain viable in faeces (Parsonnet *et al.*, 1999).

The detection of specific antigens of *H. pylori* has been used for diagnosis, and the detection of specific DNA fragments is now possible, with the advantage of also providing information on clarithromycin susceptibility. A nested PCR targeting the 23S rDNA can be performed on faeces followed by restriction of the amplicons or sequencing (Rimbara *et al.*, 2005; Kawai *et al.*, 2008). The ClariRes real-time PCR assay can also be used for this purpose. Two published studies (Schabereiter-Gurtner *et al.*, 2004; Lottspeich *et al.*, 2007) showed a marked difference with regard to the sensitivity in detecting *H. pylori* in stools: 98% and 63%, respectively. This difference may relate to the maintenance of the samples prior to testing, or the need for duplicate testing of samples. However, the difficulty in detecting targets that are not present in high quantities in stool samples, e.g. *H. pylori*, has often been raised, due to the presence of inhibitors of the *Taq* polymerase in faeces. These inhibitors have been identified as complex polysaccharides of vegetable origin, which follow the DNA extraction procedure and are difficult to eliminate (Monteiro *et al.*, 1997). Therefore, special care must be taken in DNA preparation.

FLUOROQUINOLONES. Mutations. Fluoroquinolones target the A subunit of bacterial DNA gyrase, an enzyme that supercoils DNA. This enzyme is comprised of four subunits: two A subunits encoded by the *gyrA* gene and two B subunits encoded by the *gyrB* gene. Resistance to fluoroquinolones has been associated with

mutations in the so-called 'quinolone resistance-determining region' (QRDR) of the *gyrA* gene in *H. pylori* as in other bacteria (Moore *et al.*, 1995).

The amino acids in positions 87 and 91 of *gyrA* are very important, and a mutation in either may lead to a high level of resistance. However, it is possible to find resistant strains without a mutation in these positions (Tankovic *et al.*, 2003; Bogaerts *et al.*, 2006; Cattoir *et al.*, 2007).

While there is a cross-resistance between the different fluoroquinolones, the impact of such mutations on MICs appears to be less important for some of the newer fluoroquinolones, including sitafloxacin, garenoxacin and fleroxacin (Suzuki, H. *et al.*, 2009). In contrast to most fluoroquinolones, the activity of fleroxacin increases when the pH decreases.

Detection. The above mutation can be detected by designing a real-time PCR based on the QRDR of *gyrA*. The FRET-MCA principle has also been applied. However, the situation is complicated by the fact that there is a polymorphism, i.e. all of the mutations (eventually present) are not linked to resistance but generate peaks when the MCA is performed. Nevertheless, using two biprobes, Glocker and Kist (2004) were able to detect the relevant mutations in the triplets for aa87 and aa91 leading to resistance. The allele-specific PCR methodology has also been applied to resistance detection (Nishizawa *et al.*, 2007). However, only one kit (GenoType HelicoDR) is currently commercially available that allows the detection of both fluoroquinolone resistance and clarithromycin resistance. This is a DNA-strip test, the principle of which was presented above. Nine bands can be detected for *gyrA*, five corresponding to susceptible phenotypes which had to be included because of the polymorphism observed (N87 (AAC or AAT), T87 (AAC or ACT) and D91 (GAT)), and four corresponding to resistant phenotypes (N87K (AAA), D91W (AAT), D91G (GGT) and D91Y (TAT)). The sensitivity and specificity of this approach, based on 105 gastric biopsy specimens, were 87% and 98%, respectively (Cambau *et al.*, 2009).

TETRACYCLINES. Mutations. Tetracyclines target the 30S ribosomal subunit where they prevent the fixation of the aminoacyl-tRNA, resulting in the termination of protein synthesis and leading to a truncated peptide. Resistance is due to a mutation(s) in the nucleotide triplet in position 926–928 of the h1 loop of the 16S rRNA (AGA926–928TTC). There is some cross-resistance with minocycline and doxycycline (Gerrits *et al.*, 2002a; Trieber and Taylor, 2002).

Mutations in only one or two of the nucleotides at the same site can also be encountered. In this case the resistance level is lower (4 mg/l). The need for a triple mutation may explain the rarity of such strains (Dailidienė *et al.*, 2002; Gerrits *et al.*, 2002a; Nonaka *et al.*, 2005). However, some resistant strains where the above mutation(s) was not present have been described and the mechanism for this has been linked to efflux pumps (Wu *et al.*, 2005).

Detection. A PCR–restriction fragment length polymorphism (RFLP) method was the first approach used for detecting tetracycline resistance. The PCR amplifies part of the 16S rDNA and the amplicons are then submitted to the *Hinf*I restriction enzyme. Strains having triple mutations associated with a high resistance level exhibit three bands, while those susceptible or with reduced susceptibility have only two bands (Ribeiro *et al.*, 2004).

Two real-time PCRs have also been developed which can distinguish single, double or triple base-pair mutations directly on biopsy specimens (Glocker *et al.*, 2005; Lawson *et al.*, 2005). However, in one of the studies where 1000 isolates were screened, the test was able to detect only ten out of 18 strains that possessed reduced susceptibility (Lawson *et al.*, 2005).

RIFAMYCINS. Mutations. Rifamycins, including rifabutin, which is used in *H. pylori* treatment, target the β subunit of the DNA-dependent RNA polymerase subunit β encoded by the *rpoB* gene. Resistance is associated with mutations at codons 524, 525 and 585 of the *rpoB* gene (Heep *et al.*, 1999). Another mutation at

position 149 has also been described (Heep *et al.*, 2000). However, some resistant strains may harbour a mutation other than those described (Glocker *et al.*, 2007b; Chisholm and Owen, 2009). There is also cross-resistance between all compounds of this group.

Detection. The detection of rifamycin resistance has been performed only by PCR and sequencing. The rarity of this resistance and the presence of numerous silent mutations may be the reasons for the lack of development of a molecular test.

AMOXICILLIN. Mutations. Amoxicillin, along with other β -lactams, inhibits peptidoglycan synthesis. The first amoxicillin-resistant *H. pylori* (MIC 8 mg/l) was described in 1996 in the Netherlands. This resistance was linked to a mutation in the gene coding for penicillin binding protein (PBP)-1A (Ser414Arg), which leads to a decreased affinity for β -lactams (Gerrits *et al.*, 2002b). Further studies have shown that other mutations could also be involved.

While β -lactamase-producing *H. pylori* strains had never been isolated in the past, a recent publication reports on such a strain (MIC 256 mg/l) which had a type *bla* (TEM1) β -lactamase (Tseng *et al.*, 2009). The possible spread of this strain is alarming, in view of the limited armamentarium we have to eradicate *H. pylori*.

Detection. No molecular test has been developed to detect the mutation in *pbp1A*.

METRONIDAZOLE. Mutations. Metronidazole must be reduced inside the bacterial cell to be active and induce DNA alterations. This reduction is due to the nitroreductase encoded by *rdxA* (Goodwin *et al.*, 1998) and possibly the flavin oxidoreductase encoded by *frxA* and other enzymes (Mendz and Megraud, 2002; Marais *et al.*, 2003). As stated in Section 4.2.1, this reduction depends on the redox potential in the intracellular environment (Kaakoush *et al.*, 2009). The presence of mutations either in or upstream of the *rdxA* and *frxA* genes has been associated with

metronidazole resistance. Efflux mechanisms could also be involved (Van Amsterdam *et al.*, 2005).

Detection. The link between mutations and metronidazole resistance is not strong enough to develop a molecular test. An original approach proposed was detection of the protein RdxA by immunoblotting. Using a rabbit anti-RdxA antiserum, a 24 kDa band can be observed in susceptible strains but not in resistant strains (Latham *et al.*, 2002). However, this technique has never been used further.

4.5 Conclusions

The difficulty of treating patients infected with *H. pylori* is now common in many parts of the world, essentially due to an increase in antimicrobial resistance. This chapter is a plea to consider *H. pylori* infection as any infectious disease and to carry out susceptibility testing before prescribing a treatment. The aim of this approach is to achieve a greater than 90% success rate and minimize the selection of resistance, as advocated by Graham and Shiotani (2008). The comparison of regimens that include antibiotics for which *H. pylori* resistance is known to be high, without breaking down the results according to susceptibility testing, has limited value.

There is no excuse for failing to perform susceptibility testing, because *H. pylori* treatment is not an emergency and commercial kits are readily available for quickly testing susceptibility to the most relevant antibiotics (clarithromycin, levofloxacin). Metronidazole remains the only antibiotic for which testing is not currently recommended and this drug can be used in many different combinations, including with bismuth salts, which appears to be quite successful as a quadruple therapy.

New drugs are desperately needed, but few appear to be in the pipeline. New combinations must be tested. In this respect the sequential treatment is promising even if its impact on resistant strains is still not well documented.

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5 Extragastric Manifestations of *Helicobacter pylori* Infection

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5.1 Introduction

The initial recognition by Marshall and Warren that *Helicobacter pylori* was the principal aetiological agent of gastritis proved to be a seminal finding that led to the later recognition that *H. pylori* also played a major role in peptic ulcer disease, gastric cancer and B-cell mucosa-associated lymphoid tissue (MALT) lymphoma (Marshall and Warren, 1984; Marshall *et al.*, 1985; International Agency for Research on Cancer, 1994; McColl *et al.*, 1997; Wotherspoon, 1998). This knowledge has resulted in the introduction of therapies targeted against *H. pylori* that not only have been shown to prevent ulcer recurrence and lead to regression of low-grade MALT lymphoma, but also have the potential, if subjects are treated early, to prevent progression to gastric cancer (Graham *et al.*, 1992; Isaacson *et al.*, 1999; Du and Isaacson, 2002; see Mégraud, Chapter 4, this volume).

In addition to studies investigating the role of *H. pylori* in gastroduodenal disease, since the late 1980s an increasing number of studies have focused on the role of *H. pylori* infection in a range of extragastric diseases. The ability of *H. pylori* to induce chronic gastric inflammation that in many cases is lifelong makes the possibility that this bacterium could lead to extragastric disease,

through induction of systemic effects, a feasible hypothesis.

To date, associations between *H. pylori* infection and a range of cardiovascular, respiratory, hepatobiliary, dermatological, neurological, autoimmune, allergic and haematological diseases, as well as growth and weight disorders and retardation, have been investigated. However, there is by no means consensus with regard to the findings of such studies. As discussed in an excellent review by Goodman *et al.* (2006), many studies examining the association between *H. pylori* infection and extragastric manifestations have major limitations in regard to experimental design and statistical power. In particular, many studies include a limited number of subjects, involve biases in the selection of controls and often fail to recognize confounders such as age, ethnic background and socioeconomic status. Given that acquisition of *H. pylori* infection is strongly linked to these factors, failure to match patients and controls for such factors is highly likely to confound these studies (Mitchell, 2001; Goodman *et al.*, 2006). In addition, the biological plausibility of a number of the investigated association studies is questionable.

Given the plethora of extragastric diseases investigated in relation to their possible association with *H. pylori* infection, it is not feasible

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to cover all of these in this chapter. Thus, extra-gastric diseases for which there is currently strong or mounting evidence for a role of *H. pylori* infection are discussed in depth.

5.2 Idiopathic Thrombocytopenic Purpura

Perhaps the most compelling and mysterious association of *H. pylori* infection and extra-gastric disease is that with ITP. While the term 'ITP' initially arose from 'idiopathic thrombocytic purpura', reflecting the unknown aetiology of the condition at the time, with increased understanding of the pathogenic mechanisms behind this condition, ITP is now increasingly referred to as 'immune or immune-mediated thrombocytopenia' (Rodeghiero *et al.*, 2009). This often mild pathological condition is characterized by the presence of low platelet counts. Individuals with ITP are frequently asymptomatic or may develop bruising (purpura). However, in a subset of patients in whom platelet counts are particularly low, severe bleeding can result, with significant morbidity and mortality.

This condition can be subdivided into primary and secondary ITP, with secondary ITP including those resulting from an identified underlying disease, drug exposure or infection. For example, ITP is believed to develop as a secondary outcome of a wide range of conditions, including autoimmune diseases such as systemic lupus erythematosus and lymphoproliferative diseases such as chronic lymphocytic leukaemia, as well as chronic infections including HIV (Cines *et al.*, 2009).

The precise mechanism behind ITP is still unclear, but appears related to the production of platelet-reactive autoantibodies. In individuals with ITP, platelets coated with these autoantibodies are removed more quickly than they can be generated, resulting in a deficiency. These autoantibodies can react with a number of platelet surface glycoproteins, including GPIIb-IIIa, GPIb-IX and GPIa-IIa (McMillan, 2000).

In 1998, Gasbarrini and colleagues investigated whether this condition could also be

caused by chronic *H. pylori* infection. Of 18 ITP patients examined, 11 were found to be positive for *H. pylori*. These infected patients were treated with an *H. pylori* triple therapy eradication regime, then blood platelet and anti-platelet autoantibody levels were assessed 2 and 4 months later (Gasbarrini *et al.*, 1998). While there was no change in these parameters in ITP patients who were *H. pylori*-negative, in infected patients in whom *H. pylori* was eradicated a significant increase in platelet numbers was observed. Further, anti-platelet autoantibodies disappeared in six out of eight successfully treated patients. In contrast, however, in three patients in whom therapy failed to eradicate *H. pylori*, there was no increase in platelets, nor a change in autoantibody levels. This last point is very important, as overall these data indicate that the resolution of ITP in treated patients was strongly associated with successful eradication of *H. pylori* infection and not due to the incidental treatment of another, unidentified bacterial infection – although this possibility cannot be completely discounted.

This initial observation has been supported by the majority of similar, subsequent trials (Veneri *et al.*, 2002; Suvajdzic *et al.*, 2006; Campuzano-Maya, 2007), although some studies did not find the same effect (Ahn *et al.*, 2006; Estrada-Gomez *et al.*, 2007). Further, in a prospective study in which *H. pylori*-negative ITP patients were treated with an *H. pylori* eradication regime, no effects on their platelet counts were observed, providing important support for the role of *H. pylori* eradication per se, rather than other effects of the medication on ITP being responsible for resolution of this condition (Asahi *et al.*, 2006). For a recent systematic review on studies that have evaluated ITP following *H. pylori* eradication, see Stasi *et al.* (2009).

While evidence based on observational and treatment studies would support the role of *H. pylori* in ITP, the big question that remains is how *H. pylori* infection could induce autoantibodies against platelets. While this remains unknown, it has been proposed that it could possibly be related to the infection inducing cross-reactive antibodies. For example, one study has reported that antibodies eluted from the surface of platelets

obtained from some ITP patients react with the *H. pylori* virulence factor cytotoxin-associated gene A protein (CagA), although as this also occurred for samples from three *H. pylori*-uninfected individuals, the significance is unclear (Takahashi *et al.*, 2004). The best explanation for the pathogenic mechanism behind ITP and *H. pylori* infection has thus far been provided by Asahi *et al.* (2008), who found that *H. pylori* infection of ITP patients was associated with a change in the balance of Fc γ receptors on their macrophages. This receptor imbalance is believed to result in increased macrophage activation, which was redressed following *H. pylori* eradication and shown to precede an improvement in platelet parameters in these ITP patients. The authors proposed that activation of macrophages in *H. pylori*-infected ITP patients played two roles in this autoimmune condition: in platelet destruction and by supporting the generation of autoimmune antibodies (Asahi *et al.*, 2008).

Therefore, in contrast to many other diseases with which *H. pylori* has been associated, there is strong evidence indicating that infection with *H. pylori* is a key factor in the development of ITP. While the precise mechanism behind this association requires further investigation, the 2006 Maastricht III Consensus Report recommended that *H. pylori* infection should be sought for and treated in patients with ITP (Malfertheiner *et al.*, 2007).

5.3 Iron-deficiency Anaemia

Iron is crucial for the production of haemoglobin (Hb), an essential transporter of oxygen. Most of the body's iron is stored in Hb inside red blood cells, excluding approximately 30% that is stored as ferritin and haemosiderin in the bone marrow, spleen and liver. Iron deficiency can result from a number of mechanisms. For example, if the body loses iron in relatively high quantities, this can, over time, result in depletion of its iron stores. Iron deficiency can also develop when iron intake is low or metabolism of iron is inefficient. The metabolic uptake and use of iron is limited by several factors that include its low

solubility and the ability of chelators to impair iron absorption. Gastric acidity has been shown to assist in the conversion of iron to absorbable forms and thus high gastric pH can impede absorption of iron by the stomach. Additionally, some disorders disrupt the integrity of the enteric mucosa, which can result in the inhibition of iron uptake (Gasche *et al.*, 2004).

Iron-deficiency anaemia (IDA) develops due to a lack of metabolic iron, a common causal factor in human subjects being chronic blood loss. It is estimated that this condition affects roughly 15% of the population worldwide (Clark, 2008).

5.3.1 Mechanisms by which *H. pylori* infection may produce iron deficiency

In the 1990s, researchers began investigating the involvement of *H. pylori* infection in IDA. As opposed to some conditions with which *H. pylori* infection has been correlated, associations with IDA are biologically plausible. One proposed mechanism may be blood loss as a result of infection-driven gastric erosion, development of atrophy or peptic ulcer disease (Milman *et al.*, 1998; Muhsen and Cohen, 2008), although not all research supports this possibility (Carnicer *et al.*, 1997; Marignani *et al.*, 1997; Barabino *et al.*, 1999; Konno *et al.*, 2000; Ashorn *et al.*, 2001; Sugiyama *et al.*, 2002; Kostaki *et al.*, 2003; Yoshimura *et al.*, 2003). For example, Dufour *et al.* (1993) observed that, following eradication of *H. pylori*, resolution of IDA occurred in patients diagnosed with gastritis who at the time of endoscopy had no bleeding lesions and no blood in their faecal stream. Another example is a case series by Annibale *et al.* (1999) on 30 patients with a long history of IDA, in whom *H. pylori*-associated asymptomatic gastritis was the only pathological finding detected in their gastrointestinal tract. Following *H. pylori* eradication therapy and discontinuation of iron replacement therapy, 75% of patients recovered from IDA ($P < 0.001$) and ferritin values (mean $\pm$ standard error) increased from $5.7 \pm 0.7 \mu\text{g/l}$ to $24.5 \pm 5.2 \mu\text{g/l}$ after 6 months. Significantly, after 12 months the percentage of patients who had

recovered from IDA increased to 91.7% (Annibale *et al.*, 1999).

Another putative mechanism is a reduction in the host's ability to absorb iron (Lee, 2007). A low gastric pH and sufficient quantities of ascorbic acid have been shown to be required for efficient absorption of iron (Muhsen and Cohen, 2008). *H. pylori* increases stomach pH by buffering the secreted acid and damaging the epithelial layer due to atrophy. In addition, several studies have observed a reversible negative impact by *H. pylori* on gastric ascorbic acid (Ruiz *et al.*, 1994; Zhang *et al.*, 1998). The effect of *H. pylori* on iron absorption is supported by the findings of Ciacci *et al.* (2004) who analysed iron absorption and ferritin levels in 55 individuals without occult blood in their stools. This showed that the levels (mean $\pm$ standard deviation) of ferritin (ng/ml) and iron absorption (delta iron, μ g/dl)

were significantly lower in *H. pylori*-positive versus *H. pylori*-negative subjects: 59.3 ± 22.2 versus 76.5 ± 23.7 ($P = 0.008$) and 86.8 ± 42.7 versus 132.5 ± 55.7 ($P = 0.001$), respectively. When the results were divided by gender, only the *H. pylori*-positive women retained significantly lower iron absorption rates and ferritin levels (Ciacci *et al.*, 2004). Iron absorption improved significantly, although only in females, after clearance of *H. pylori* infection, with iron absorption in individual patients after *H. pylori* eradication being on average significantly higher (95% confidence interval (CI) 17.022, 32.746; $P < 0.001$) than before treatment (Ciacci *et al.*, 2004).

A third possible mechanism is the enhanced capacity of the bacterium to scavenge iron in the gastric mucosa (Choe *et al.*, 2003; Lee, 2007) (Fig. 5.1). Park *et al.* (2006) performed proteomic analyses on *H. pylori*

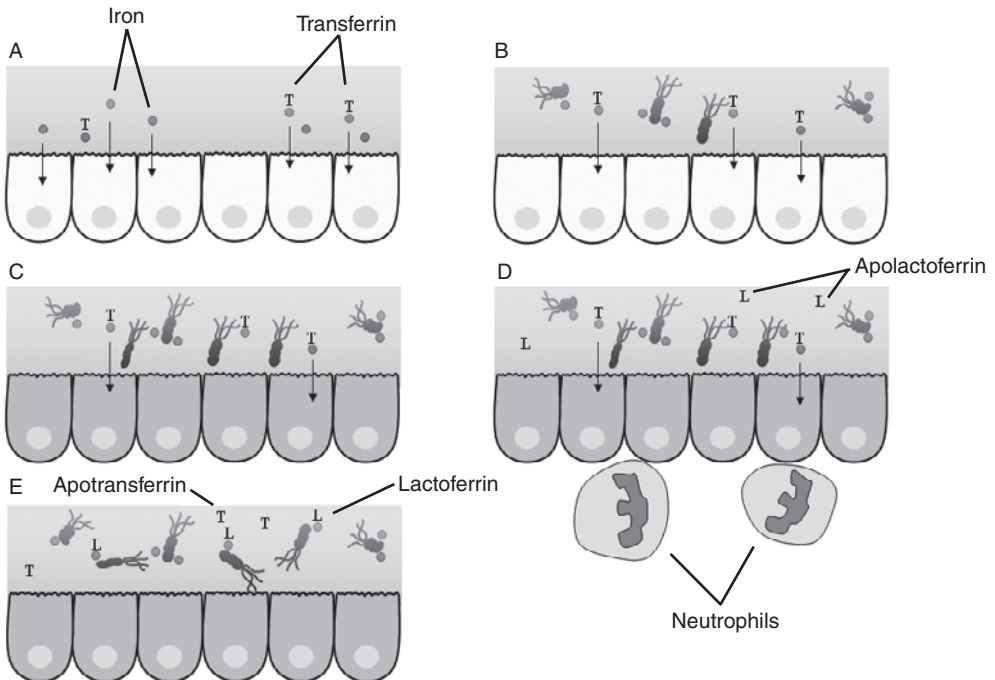


Fig. 5.1. Enhanced capacity of *Helicobacter pylori* to scavenge iron in the gastric mucosa. Iron is present in its free form and bound to transferrin within the mucous layer (A). Infection with *H. pylori* results in the inflammation of the epithelial layer (B, C) and in neutrophil migration to the site of infection (D). Neutrophils secrete lactoferrin, which has the capacity to capture iron from transferrin (D). Through its ability to target lactoferrin via receptors, *H. pylori* infection may decrease the amount of iron available for uptake (E).

strains isolated from patients with and without IDA, demonstrating that these strains could be distinguished by their protein expression patterns and that a number of proteins had higher levels of expression in IDA strains. Furthermore, it was shown that strains recovered from patients with IDA possessed enhanced iron uptake abilities and iron-dependent rapid growth compared with those from patients without IDA (Yokota *et al.*, 2008). Recently, iron-repressible outer membrane proteins (IROMP) were identified under iron-restricted conditions but not under iron-rich conditions in *H. pylori* strains associated with IDA (Lee *et al.*, 2009). In the iron-depleted state, specific *H. pylori* strains associated with IDA demonstrated an advantage in iron acquisition due to a higher expression of IROMP. These results suggest that some strains possess an advantage in iron acquisition, thus providing a possible explanation as to why some patients infected with *H. pylori* are more likely to develop IDA.

5.3.2 Evidence for and against a role for *H. pylori* infection in iron deficiency

Since the late 1980s many studies have examined the association between *H. pylori* infection and development of IDA. Having reviewed the literature, Goodman *et al.* (2006) concluded that 'overall the evidence shows a positive association, and plausible mechanisms by which the bacterium could cause, perpetuate, or worsen IDA have been proposed, but support for a causal relationship is not strong'. While at the time Goodman *et al.* were not convinced that *H. pylori* was a cause of IDA, they clearly did not rule out this possibility. In 2008, Muhsen and Cohen performed comprehensive literature searches and subsequent analyses on the relationship between *H. pylori* infection and iron stores. Their analyses indicated that evidence supporting a role for *H. pylori* in IDA was not strong, being based on case reports, observational epidemiological studies and a very limited number of intervention trials (Muhsen and Cohen, 2008). Their review identified 15 observational epidemiological studies that reported supportive findings linking *H. pylori*

and decreased iron stores (Yip *et al.*, 1997; Milman *et al.*, 1998; Peach *et al.*, 1998; Collett *et al.*, 1999; Choe *et al.*, 2000, 2001; Parkinson *et al.*, 2000; Berg *et al.*, 2001; Seo *et al.*, 2002; Kaffes *et al.*, 2003; Nahon *et al.*, 2003; Weyermann *et al.*, 2005; Yang *et al.*, 2005; Baggett *et al.*, 2006; Cardenas *et al.*, 2006a). However, due to shortcomings in the design of many of these studies they could not conclude whether the infection per se caused changes in iron levels (Muhsen and Cohen, 2008). These authors found a limited number of trials that attempted to resolve IDA using anti-*H. pylori* therapy; of these, most had shortcomings in either design or number of subjects, and of those that were well-designed conflicting results were reported. Nevertheless, based on the results of three well-designed studies (Choe *et al.*, 2001; Baggett *et al.*, 2006; Cardenas *et al.*, 2006a), Muhsen and Cohen (2008) calculated that there was a 2.8-fold (95% CI 1.9, 4.2) increased risk of developing IDA in *H. pylori*-infected patients. Additionally, based on the results of four studies (Milman *et al.*, 1998; Parkinson *et al.*, 2000; Baggett *et al.*, 2006; Cardenas *et al.*, 2006a), they determined that the risk of developing iron deficiency alone was approximately 40% higher in *H. pylori*-infected patients.

However, the number of studies supporting a role for *H. pylori* in IDA would have been higher if Muhsen and Cohen (2008) had not missed two studies performed in 2007. In one study, 29 male IDA patients were given a triple therapy regimen for eradication of *H. pylori*. Following treatment, all patients had normal Hb levels within 4–69 months, which was also accompanied by a significant decrease in *H. pylori* immunoglobulin (Ig) G antibodies (Hershko *et al.*, 2007). Interestingly, four of the patients achieved normal Hb levels without receiving any oral iron treatment (Hershko *et al.*, 2007). In a second study consisting of 70 patients aged 4–16 years who had undergone upper endoscopy for recurrent gastrointestinal complaints during a 2-year period, significantly lower levels of serum iron and ferritin were observed in *H. pylori*-positive patients than in those without infection ($P < 0.001$) (Suoglu *et al.*, 2007). IDA was observed in 20 (57.1%) and six (17.1%) patients in the *H. pylori*-positive and -negative

groups, respectively ($P < 0.01$). These authors found *H. pylori* infection to be the only variable that had a significant effect on IDA (odds ratio (OR) = 6.444; 95% CI 2.134, 19.453; $P = 0.001$) (Suoglu *et al.*, 2007).

Further studies published since 2008 have continued to provide controversial results, with some supporting and some opposing a role for *H. pylori* in the development of IDA. For example, a case-control study on 325 adult patients found that gastric atrophy was strongly associated with IDA, and that this was likely to be causative in some patients and contributory in others (Kaye *et al.*, 2008). In that study, 40 of 156 (25.6%) of patients with IDA were shown to have significant body atrophy compared with only seven of 169 (4.1%) controls ($P < 0.001$). Additionally, a statistically significant association was found between *H. pylori* infection and IDA in 117 women with uncomplicated pregnancy, all 18 women with IDA being *H. pylori*-positive as compared with only 50% of women without IDA (Mulayim *et al.*, 2008).

In a paediatric study, Cherian *et al.* (2008) found no association between *H. pylori* infection or helminthic infection and the presence of IDA in 181 African refugee children less than 16 years of age. In contrast, iron deficiency was corrected following antibiotic eradication of *H. pylori* infection in three children who had symptomatic IDA with no obvious clinical cause (Cardamone *et al.*, 2008). However, in a study involving 200 *H. pylori*-infected Bangladeshi children, Sarker *et al.* (2008) found that iron supplementation cure rates of IDA were comparable between patients who received *H. pylori* treatment and those who did not. In a further study, Muhsen *et al.* (2009) examined the association between *H. pylori* seropositivity and low ferritin levels in sera from 509 Israeli Arab children. Low ferritin levels were found in a significantly higher proportion of *H. pylori*-seropositive (14.5%) than -seronegative (8.6%) children. Interestingly, a higher frequency of low ferritin levels was detected among children with *H. pylori*-CagA seropositivity (Muhsen *et al.*, 2009). Additionally, while Fayed *et al.* (2008) reported a high prevalence of *H. pylori* infection in 25 patients <18 years of age with refractory IDA, no significant influence of *H. pylori*

infection was observed with regard to the severity of IDA.

In a study conducted in six Latin American countries (Argentina, Brazil, Bolivia, Cuba, Mexico and Venezuela) and following adjustment of the statistical analyses to include confounding factors, no association was found between *H. pylori* infection and IDA (Santos *et al.*, 2009). Recently, Gessner and colleagues revisited their previous results, which had suggested that treatment of *H. pylori* infection did not reduce the prevalence of iron deficiency or anaemia among Alaskan native children and, based on a study by Cardenas *et al.* (2006b), they hypothesized that 14 months was not sufficient time for the resolution of *H. pylori*-associated gastric problems (Gessner *et al.*, 2006). Their new study showed that 40 months after resolution of *H. pylori* infection the prevalence of iron deficiency and anaemia was reduced, suggesting that for some children *H. pylori* likely plays a role in haematological outcomes (Fagan *et al.*, 2009). Gessner (2009) also investigated reasons for the high incidence of IDA in Alaskan native individuals (50,964 children and 30,154 pregnant or postpartum women) and concluded that the increased risk observed supported the role of a region-specific environmental factor, but that nutritional iron deficiency or *H. pylori* infection was unlikely to be the sole or major aetiology of this high anaemia prevalence.

5.3.3 Conclusion

Although overall there is evidence to support a positive association between *H. pylori* infection and IDA, and plausible mechanisms have been proposed for this association, data indicating otherwise are also available; moreover, support for a direct causal link is minimal due to inadequate study design and the presence of a multitude of confounding factors in many studies. Although the association between *H. pylori* and IDA requires further investigation, the 2006 Maastricht III Consensus Report recommended that *H. pylori* infection should be sought for and treated in patients with unexplained IDA (Malfertheiner *et al.*, 2007).

5.4 Cardiovascular Disease

Cardiovascular disease remains the principal cause of death in the USA, Europe and much of Asia despite interventions such as lifestyle changes and the use of a range of pharmacological approaches to lower plasma cholesterol concentrations (Ross, 1999).

Coronary heart disease (CHD) commonly results from the accumulation of atheromatous plaques within the walls of the coronary arteries that supply the myocardium of the heart with oxygen and nutrients. As a result of this accumulation, a reduced blood supply to the heart muscle occurs, which in turn can lead to ischaemic heart disease (IHD). Although for many years the process of atherogenesis was considered to be due to the accumulation of lipids within the artery wall, current evidence suggests this is a multifactorial process in which chronic inflammation plays a pathophysiological role in the evolution, progression and destabilization of atherosclerosis (Ross, 1999; Shah, 2002). The possibility that chronic inflammation resulting from infection could play a role in atherosclerosis was first raised in the late 1800s (Shah, 2002). Indeed in 1911, Frothingham stated 'The sclerosis of old age may simply be a summation of lesions arising from infections or metabolic toxins' (Frothingham, 1911). Given the postulated role of chronic infections in the genesis and development of vessel wall injury and atheromatous plaques, the associations between a range of chronic infective agents (including viruses and bacteria) and cardiovascular disease have been investigated since the late 1970s.

5.4.1 *H. pylori* and cardiovascular disease

In 1994 Mendall and colleagues first investigated the specific association between *H. pylori* infection and CHD. In this case-control pilot study, the *H. pylori* serostatus of 111 consecutive patients with documented CHD was compared with that in 74 controls, all of whom were white men aged 45–65 years. After adjustment for age, cardiovascular risk factors, current social class and features of their childhood environment known to be risk factors for

H. pylori infection, a weak positive association was shown to exist between *H. pylori* seropositivity and CHD (OR=1.9) (Mendall *et al.*, 1994). Following this report, at least 20 epidemiological studies were conducted between 1994 and 1997 in which the association between *H. pylori* antibody levels and either CHD or stroke was investigated. A review of these studies by Danesh *et al.* (1997) showed that many comprised small numbers of subjects and failed to adjust for potential confounding factors, including selection bias in relation to controls and socio-economic status. While a positive association between *H. pylori* and CHD was observed in many studies, Danesh *et al.* (1997) noted that, in studies where attempts were made to adjust for potential confounding factors and where controls were sampled from approximately the same population as the cases, the association tended to be weaker.

In 1998, Pasceri *et al.* investigated for the first time the association between both *H. pylori* and CagA status and IHD in 88 patients with IHD and 88 age- and sex-matched controls from a similar social background. Following adjustment for age, sex, major cardiovascular risk factors and social class, this showed a significant association between *H. pylori* seropositivity and IHD (OR = 2.8; 95% CI 1.3, 7.4; $P < 0.001$). Further investigation showed that the prevalence of CagA-positive strains in IHD patients (43%) was significantly higher than in the controls (17%; adjusted OR = 3.8; 95% CI 1.6, 9.1; $P < 0.001$). Conversely, the prevalence of CagA-negative strains was not significantly different in patients and controls, a finding that led these authors to suggest that the association between *H. pylori* infection and IHD may be related to more virulent *H. pylori* strains (Pasceri *et al.*, 1998).

While several subsequent case-control and cohort studies further assessed the possible association between CagA status and vascular disease, these studies resulted in conflicting results (Koenig *et al.*, 1999; Gunn *et al.*, 2000; Figura *et al.*, 2002; Mayr *et al.*, 2003). In an attempt to clarify this issue, Pasceri *et al.* (2006) conducted a meta-analysis on ten retrospective case-control studies (1527 cases/1661 controls) and three prospective cohort studies (701 cases/1439 controls) that examined the association between CagA status and IHD,

plus four retrospective case-control studies (513 cases/590 controls subjects) that examined the association between CagA status and cerebral ischaemia. While in the case-control studies a positive association was observed between IHD and CagA-positive strains (OR=1.87; 95% CI 1.46, 2.40), no significant association was found with CagA-negative strains (OR = 1.15; 95% CI 0.83, 1.60) (Pasceri *et al.*, 2006). Interestingly, in the three prospective cohort studies, the association of CagA-positive strains with IHD was weaker, but still significant (OR = 1.26; 95% CI 1.05, 1.51). In the four retrospective case-control studies, CagA-positive strains were shown to be significantly associated with cerebral ischaemia (OR = 2.43; 95% CI 1.89, 3.13); however, again no association was observed for CagA-negative strains. Based on the results of the three prospective cohort nested case-control studies in which a weak association was found between CagA-positive *H. pylori* and the occurrence of fatal and non-fatal myocardial infarction in follow-up, they concluded that while these findings 'virtually ruled out any major relationship between CagA status and IHD they did not rule out a minor role in IHD' (Pasceri *et al.*, 2006).

In a more recent systematic meta-analysis including studies of both Chinese and Caucasian subjects and restricted to case-control studies published between 1990 and July 2007, Zhang *et al.* (2008) examined the role of CagA-positive *H. pylori* strains and the risk of ischaemic stroke and CHD. In total, 26 case-control studies (11 on ischaemic stroke and 15 on coronary artery disease) were included in the final meta-analysis. All of these studies had been adjusted for major confounding factors including age, gender, diabetes, hypertension, smoking, social class, body mass index, hyperlipidaemia, peptic diseases and infection with other pathogenic microorganisms. Meta-analysis of the relationship between CagA seropositivity and ischaemic stroke showed an overall OR of 2.68 (95% CI 2.20, 3.27). When the total population was subdivided into Caucasian and Chinese populations, no significant difference was observed between the two groups. Examination of the association between CagA seropositivity and CHD in the total popu-

lation showed a positive association (OR = 2.11; 95% CI 1.70, 2.62) with no significant difference between Chinese and Caucasian populations being observed. Zhang *et al.* (2008) concluded that, although their study supported an association between *H. pylori* CagA-positive strains and susceptibility to ischaemic stroke and CHD, further prospective studies and studies examining the effect of the eradication of CagA-positive strains of *H. pylori* were required.

A further meta-analysis of nine studies (4241 subjects), comprising six retrospective case-control studies and three nested prospective case-control studies that had assessed the association between CagA seropositivity and acute coronary events, was published by Franceschi *et al.* (2009). This found that CagA seropositivity was significantly, although weakly, associated with the occurrence of acute coronary events (OR = 1.34; 95% CI 1.15, 1.58; $P = 0.0003$) (Franceschi *et al.*, 2009), a finding consistent with that of Pasceri *et al.* (2006).

5.4.2 Effect of *H. pylori* on intimal-medial thickness

Given the known positive association between cardiovascular risk factors and carotid artery intimal-medial thickness (O'Leary *et al.*, 1991, 1992), a number of studies have examined the relationship between *H. pylori* and intimal-medial thickness. While the majority of studies found no association between intimal-medial thickness and *H. pylori* positivity *per se* (Espinola-Klein *et al.*, 2000), a number have reported a link with CagA-positive strains. For example, in a large prospective study which investigated the relationship between CagA status and progression of carotid atherosclerosis, Mayr *et al.* (2003) showed that infection with CagA-positive strains was associated with a greater increase of carotid intimal-medial thickness during 5-year follow-up, compared with CagA-negative and *H. pylori*-negative subjects. While Dimedi *et al.* (2004) also reported an association between CagA-positive *H. pylori* strains and increased intimal-medial thickness in patients with atherosclerotic stroke, Markus *et al.* (2002) found neither *H. pylori* nor CagA-

positive strains to be a major risk factor for early arteriosclerosis as assessed by carotid artery intimal-medial thickness.

5.4.3 Potential effect of *H. pylori* on risk factors for ischaemic heart disease

To date, a large number of studies have focused upon the effect of *H. pylori* on a range of risk factors for atherosclerosis including plasma levels of lipids, fibrinogen, C-reactive protein (CRP), total cholesterol and cytokines. In 1998, a meta-analysis of 18 epidemiological studies examined the possible link between *H. pylori* and vascular risk factors including systolic blood pressure, diastolic blood pressure, body mass index, plasma viscosity, white cell count, and concentrations of total cholesterol, high-density lipoprotein cholesterol, triacylglycerols, fibrinogen, blood glucose and CRP. However, no strong correlations were found between *H. pylori* seropositivity and any of these vascular risk factors (Danesh and Peto, 1998). Since then, numerous studies have further investigated the possible association between *H. pylori* and a range of risk factors for IHD with, as previously, conflicting results. A recent review by Manolakis *et al.* (2007) provides a good coverage of this topic. In a recent study, Siddiqui (2009) examined the effect of *H. pylori* eradication on blood levels of CRP, homocysteine, folate and vitamin B₁₂ in 46 healthy African Americans over 40 years of age with *H. pylori* CagA positivity. In subjects in whom eradication was successful ($n = 43$), eradication induced no significant change in homocysteine, folate or B₁₂ levels; however, CRP levels decreased significantly 2 months after eradication ($P = 0.02$) (Siddiqui, 2009). These findings, however, are by no means consistent (Manolakis *et al.*, 2007).

5.4.4 *H. pylori* and the possibility of a direct role in atherosclerosis

A substantial number of studies have investigated the possibility of a direct role for *H. pylori* in atherosclerosis. For example, Kaplan

et al. (2006) investigated the presence of *H. pylori* DNA in atherosclerotic plaque specimens from the carotid arteries of 52 patients who had undergone carotid endarterectomy and in macroscopically healthy regions of the ascending aorta of 52 control patients who had undergone coronary artery bypass grafting. This showed a significantly higher detection rate of *H. pylori* DNA in atherosclerotic plaques (17.3%) as compared with none in controls ($P = 0.003$) (Kaplan *et al.*, 2006). Consistent with these findings, Arias *et al.* (2006) detected *H. pylori* DNA in 83% of atherosclerotic carotid tissue samples, 64% of which were CagA-positive, while Farsak *et al.* (2000) detected *H. pylori* DNA in 37% of endarterectomy specimens as compared with none in controls ($P < 0.001$). Interestingly Ameriso *et al.* (2001) not only detected *H. pylori* DNA in 20/38 atherosclerotic plaques but also provided immunohistochemical evidence of *H. pylori* infection in 50% of positive plaques.

However, many other studies have failed to detect *H. pylori* in atherosclerotic plaques. For example, Blasi *et al.* (1996) failed to detect *H. pylori* DNA in plaque specimens collected from 47 *H. pylori*-seropositive patients, while Danesh *et al.* (1999) found *H. pylori* DNA in only 1/39 atheromatous specimens collected from patients at carotid surgery and Weiss *et al.* (2006) were unable to detect *H. pylori*-specific DNA in atherosclerotic plaques from 36 patients undergoing carotid endarterectomy for symptomatic carotid artery stenosis. More recently Reszka *et al.* (2008) reported a similar frequency of *H. pylori* DNA to be present in aortic wall samples from patients with stable coronary artery disease (80%) and controls (85%).

5.4.5 *H. pylori* and molecular mimicry

CagA antibodies

To determine the link between CagA-positive strains and atherosclerosis, Franceschi *et al.* (2002) investigated whether anti-CagA antibodies cross-reacted with antigens of normal and atherosclerotic arteries. Immunohistochemical examination of eight umbilical

cord sections, 14 atherosclerotic artery sections and ten gastrointestinal tract sections showed that polyclonal anti-CagA antibodies cross-reacted with antigens of both normal and atherosclerotic blood vessels, suggesting that molecular mimicry may exist between CagA and vascular wall peptides (Franceschi *et al.*, 2002). Based in part on these findings, Franceschi *et al.* (2009) recently explored the role of CagA-positive *H. pylori* strains in the pathogenesis of coronary instability. The study showed that, in patients with unstable angina, anti-CagA antibody titres were significantly higher than in patients with stable angina ($P < 0.02$), normal coronary arteries ($P < 0.01$) or in 50 age- and sex-matched healthy controls ($P < 0.02$). Significantly, in all specimens obtained from patients with both stable and unstable angina, anti-CagA antibodies recognized antigens localized inside coronary atherosclerotic plaques. Based on these findings Franceschi *et al.* (2009) concluded that 'in a subset of patients with unstable angina, an intense immune response against CagA-positive *H. pylori* strains might play a critical role in precipitating coronary instability mediated by antigen mimicry between CagA antigen and a protein contained in coronary atherosclerotic plaques'.

Heat-shock proteins

Heat-shock proteins (HSP) are a class of functionally related proteins whose expression is increased when cells are exposed to environmental stress (Bosch *et al.*, 1988). The fact that HSP are highly conserved in virtually all eukaryotes and prokaryotes led to the hypothesis that immune reactants against microbial HSP may cross-react with homologous host proteins in a form of 'molecular mimicry' (Kaufmann, 1990). HSP are known to be expressed/and or secreted by a range of pathogens, including *Chlamydia pneumoniae* and *H. pylori*, and by endogenous cells within atherosclerotic plaques (Ayada *et al.*, 2007). Given that *H. pylori* infection has been shown to result in IgG antibodies against HSP60 and to induce Th-1 polarization of T lymphocytes, it has been posited that this may promote the development of atherosclerosis through the interaction of *H. pylori* immune reactants and

host HSP (Ayada *et al.*, 2007). Studies on the association between HSP and atherosclerosis, and the possible mechanisms by which this may occur, are covered in two recent reviews by Manolakis *et al.* (2007) and Ayada *et al.* (2009).

5.4.6 Conclusion

Despite the large number of studies investigating the possible link between *H. pylori* and cardiovascular disease, the relationship between *H. pylori* and cardiovascular disease remains in dispute. Similarly, the pathogenic mechanism by which this bacterium might contribute to atherosclerosis in the coronary arteries and thus induce IHD also remains contentious.

5.5 Asthma

5.5.1 Associations between *H. pylori* and asthma

Since the late 1960s a sharp increase in the global prevalence of asthma and allergy has occurred, particularly in children (Peat *et al.*, 1994; Omran and Russell, 1996; Howarth, 1998; Eder *et al.*, 2006). Asthma is considered one of the most common chronic diseases of the Western world (Codolo *et al.*, 2008), with approximately 300 million people worldwide currently reported to have this condition (Braman, 2006). Given that asthma is associated with a high degree of morbidity and mortality (Braman, 2006), considerable research has been invested into determining the aetiology of this chronic condition, and in particular why such dramatic increases have occurred in the developed world.

One hypothesis posited to explain the increasing incidence of asthma is the 'hygiene hypothesis', which suggests that a lack of exposure to microorganisms in the early years of life predisposes to later development of allergic diseases, including asthma (Strachan, 1989). The scientific rationale for this hypothesis is based on the view that exposure to specific chronic microbial infections, at an

early age, results in the host developing an immune phenotype that reduces their susceptibility to allergic disease. That is, under-exposure to infectious agents has the potential to lead to immature and pro-allergenic immune responses (Strachan, 1989; Liu, 2007).

A considerable number of studies investigating the aetiology of allergic disease have focused upon exposure to a range of micro-organisms and parasites that cause chronic infections in man (Matricardi *et al.*, 2000). Given that *H. pylori* chronically infects >50% of the world's population, is acquired during childhood (Mitchell, 2001) and, if left untreated, causes lifelong inflammation, this makes *H. pylori* a potential candidate. Moreover, the observation that the increase in incidence of asthma and related allergic diseases over recent decades correlates with a decline in *H. pylori* prevalence in developed countries over a similar timeframe, led researchers to investigate the possible relationship between *H. pylori* and allergic disease.

In an early study investigating the possible association between anti-*H. pylori* and anti-CagA IgG antibody titres and food allergy, atopic asthma and inflammatory bowel disease ($n = 30$), Corrado *et al.* (1998) found significantly higher anti-*H. pylori* IgG titres in children with food allergy as compared with the other two groups. In contrast, the anti-CagA IgG titres did not differ significantly between the patients. As a result Corrado *et al.* (1998) hypothesized that virulence factors other than CagA may be involved in the pathogenesis of *H. pylori* infection in paediatric patients with food allergy.

In a later study, Matricardi and colleagues (2000) investigated markers of exposure to food-borne and orofaecal microbes (*Toxoplasma gondii*, *H. pylori* and hepatitis A virus) in 240 atopic and non-atopic Italian male cadets, aged 17–24 years. Exposure to both *T. gondii* and hepatitis A virus was inversely associated with atopy. A similar trend was observed with *H. pylori* but this did not reach significance, although the adjusted odds of atopy were shown to decrease with a gradient of exposure to *H. pylori*, *T. gondii* and hepatitis A virus (Matricardi *et al.*, 2000). A

nested case-control study conducted in the same year by Bodner *et al.* (2000) showed no association between exposure to hepatitis A, *T. gondii* or *H. pylori* and the development of wheezing symptoms or atopic status in adulthood. Similarly a study by Tsang *et al.* (2000) of 90 asthmatic Chinese patients with mild intermittent asthma, and 97 age-, sex- and occupation-matched healthy control subjects, found no significant difference in *H. pylori* seroprevalence in asthmatic patients with mild intermittent asthma and controls.

A further investigation of *H. pylori* and allergy was undertaken by Kosunen *et al.* (2002), who determined the prevalence of allergen-specific IgE antibodies to the four most common inhalant allergens causing allergic reactions in Finland (birch, timothy grass, cats and dogs) in two cross-sectional adult populations, using sera samples collected in 1973 and 1994 for which *H. pylori* serostatus was known. Between 1973 and 1994 a rise in specific IgE antibodies to the above allergens had occurred, with this increase being mainly limited to *H. pylori*-seronegative subjects. Interestingly, a particularly strong effect was noted in the 15–24-year age group, where the prevalence of IgE antibodies rose from 7.9% in 1973 to 29.7% in 1994 in *H. pylori*-seronegative subjects, as compared with 15.4% in 1973 to 20.0% in 1994 in *H. pylori*-seropositive subjects (Kosunen *et al.*, 2002).

The first major study to show a strong association between *H. pylori* and asthma was conducted by Chen and Blaser (2007), who investigated whether acquisition of *H. pylori* was associated with a reduced subsequent risk of asthma and allergy using data drawn from 7663 adults who had been part of the Third National Health and Nutrition Examination Survey (NHANES III), conducted in the USA between 1988 and 1991. In this survey, all interviewed subjects were questioned as to whether they had ever been diagnosed with asthma or hay fever, the age at which they were first diagnosed, and whether they still had asthma or hay fever. Furthermore, information on a range of allergy symptoms in the previous year as well as potential exposures that may have elicited allergic symptoms was obtained. In all

subjects >20 years of age, *H. pylori* and CagA status was determined and, based on these results, subjects were categorized into three groups: (i) *H. pylori*- and CagA-positive; (ii) *H. pylori*-positive but CagA-negative; and (iii) *H. pylori*- and CagA-negative. In addition, immediate hypersensitivity reactions to ten allergens were investigated.

Overall, this showed that no association existed between the presence of *cagA*-positive or *cagA*-negative strains of *H. pylori* infection and current asthma status (Chen and Blaser, 2007). However, individuals who carried *cagA*-positive *H. pylori* strains had a decreased risk of ever having had asthma or rhinitis compared with those not infected with *H. pylori* (OR = 0.79; 95% CI 0.63, 0.99). When subjects with asthma were grouped into those diagnosed at <15 or >15 years of age, an even stronger inverse association was observed between *H. pylori cagA* positivity in the younger age group (OR = 0.63; 95% CI 0.43, 0.93) compared with those with adult onset (OR = 0.97; 95% CI 0.72, 1.32). Interestingly, a similar protective association was seen for rhinitis and skin sensitization due to moulds and pollens. Furthermore, infection with either *cagA*-positive or -negative strains of *H. pylori* was associated with a reduction in allergy symptoms, these associations being more commonly observed in younger individuals (Chen and Blaser, 2007). In contrast, an earlier study by Jun *et al.* (2005) failed to observe a significant difference in either anti-*H. pylori* or anti-*H. pylori* CagA IgG levels between patients with asthma and controls.

In 2008 Reibman *et al.* confirmed the inverse association between *H. pylori cagA*-positive strains and asthma reported by Chen and Blaser (2007). In this case-control study conducted in an ethnically diverse urban population resident in the USA, the relationship between asthma and both *H. pylori* and CagA status was examined in 318 adult patients (18–64 years old) diagnosed with asthma, and 208 controls. Although a trend towards an association between CagA status and asthma was observed, the crude odds for asthma associated with either *H. pylori*-positive/CagA-negative or CagA-positive status did not reach significance (Reibman *et al.*, 2008). However, when the data were

adjusted for confounding factors including age, race, Hispanic ethnicity, income and the genetic relatedness among some of the subjects, a significant inverse association was observed between CagA status and asthma (OR = 0.57; 95% CI 0.36, 0.89). Further analysis showed that CagA positivity was associated with an older age of asthma onset (Reibman *et al.*, 2008).

In a second study Chen and Blaser (2008) investigated whether *H. pylori* colonization during early life was associated with the risk of childhood asthma. This cross-sectional study used data from 3327 participants aged <20 years, enrolled in the NHANES III study (1999–2000), to assess the association between *H. pylori* infection and childhood asthma. While an inverse association was found between *H. pylori* positivity and either current asthma or ever having had asthma in children aged 3–19 years, this did not reach statistical significance. In contrast, when the age of asthma onset was taken into consideration, a significant inverse association was observed in children in whom asthma onset had occurred at <5 years (OR = 0.58; 95% CI 0.38, 0.88). Stratification of the subjects by age further showed that, in children aged 3–13 years, *H. pylori* positivity was inversely associated with current asthma (OR = 0.41; 95% CI 0.24, 0.69) or ever having had asthma (OR = 0.49; 95% CI 0.30, 0.80) (Chen and Blaser, 2008).

However, a recent study by Dowd *et al.* (2009), which examined the association between the burden of common chronic infections (including *H. pylori*, cytomegalovirus, herpes simplex virus 1, hepatitis A and B virus) with asthma or chronic respiratory conditions in American children aged 6 years and older, failed to show a protective effect of infection by any of these organisms. Indeed, a higher burden of infection was associated with an increased likelihood of asthma, a finding clearly at odds with the hygiene hypothesis (Dowd *et al.*, 2009).

5.5.2 Potential mechanisms

While, based on epidemiological studies, there is increasing evidence of an inverse association between *H. pylori* infection and

asthma, until recently a plausible biological explanation for this association had not been forthcoming. While some researchers suggested that the immune response to *H. pylori* may lead to changes in the immune environment that may protect against the development of asthma, it was not until 2006 that Amedei *et al.* (2006) provided evidence that, *in vitro*, the typical T-helper type 2 (Th2) responses observed in allergic asthmatic patients could be redirected towards a Th1 response by the *H. pylori* virulence factor, neutrophil-activating protein (HP-NAP). In that study, addition of HP-NAP to allergen-induced T-cell lines obtained from allergic asthmatic patients resulted in a substantial increase in interferon- γ (IFN γ)-producing T cells and a decrease in cells secreting interleukin (IL)-4. Based on these studies, Amedei *et al.* (2006) concluded that HP-NAP was able to inhibit the development of allergen-specific Th2 responses and to stimulate the production of IL-12 and IL-23 via the Toll-like receptor-2 (TLR2) pathway.

In a follow-up *in vivo* study, Codolo *et al.* (2008) used an ovalbumin (OVA)-induced mouse model of asthma to investigate whether HP-NAP might provide a novel therapeutic approach for redirecting Th2 to Th1 responses. Mice were first primed intraperitoneally (IP) with OVA, and then Th2 responses induced in their lungs by repeated OVA aerosol challenges. To determine the effect of HP-NAP on asthma development, mice were injected IP with either phosphate-buffered saline (PBS) or HP-NAP, simultaneously with the initial OVA sensitization. Eighteen days following initial priming and subsequent challenge with OVA, differential white blood cell counts were measured in lung washings (bronchoalveolar lavage (BAL)) collected from mice initially treated with OVA alone or OVA+PBS or OVA+HP-NAP. As expected, administration of OVA resulted in the recruitment and activation of eosinophils in the bronchial airways of the mice and a subsequent increase in serum IgE levels (Codolo *et al.*, 2008). However mice co-administered HP-NAP and OVA had significantly reduced numbers of BAL and airway eosinophils ($P < 0.01$), and OVA-induced airway eosinophilia was prevented.

In contrast, the numbers of macrophages, neutrophils and lymphocytes in systemic HP-NAP-treated mice were similar to those of OVA-treated mice. Moreover, mucosal administration of HP-NAP also suppressed the development of OVA-induced asthma (Codolo *et al.*, 2008). Finally they compared the potential immunomodulating activity of TLR2 and HP-NAP by measuring the Th2 cytokines, IL-4, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF), in the BAL of wild-type and TLR2^{-/-} mice. While a significant reduction in IL-4, IL-5 and GM-CSF was observed in BAL from wild-type mice following systemic and mucosal treatment with HP-NAP, no such reductions were observed in the TLR2^{-/-} mice, suggesting that TLR2 expression is required for the beneficial effects of HP-NAP. These findings suggest that both systemic and mucosal administration of HP-NAP may be effective in preventing allergic asthma (Codolo *et al.*, 2008; D'Elia *et al.*, 2009).

In a recent paper, Cam *et al.* (2009) investigated the relationship between atopy and *H. pylori* infection in healthy schoolchildren followed for a period of 6 years. All children were tested using skin prick tests for reactivity to a range of allergens, including *Dermatophagoides pteronyssinus* (Der p1 – house dust mite), and *H. pylori* status was determined by ¹³C-urea breath test. The immunological interaction between atopy and *H. pylori* infection was then determined in ten *H. pylori*-positive atopic children, nine *H. pylori*-positive non-atopic children, seven *H. pylori*-negative atopic children and eight *H. pylori*-negative non-atopic children. Immunological interactions were based on IFN γ , IL-4, IL-10 and transforming growth factor- β (TGF β) production by peripheral blood monocyctic cells (PBMC) obtained from these four groups following stimulation with Der p1 or *H. pylori* antigen. This study showed that *H. pylori*-induced IFN γ production by PBMC was significantly higher in *H. pylori*-positive children; however, when atopy was simultaneously present in *H. pylori*-infected cases, *H. pylori*-induced IFN γ production was reduced. In contrast IL-4 production was highest in non-infected atopic children, but significantly suppressed when *H. pylori*

infection was concurrently present in atopic children. IL-10 production was not modified by either *H. pylori* infection or the presence of atopy. Production of TGF β was suppressed in atopic children irrespective of the presence of *H. pylori* infection. Based on these findings Cam *et al.* (2009) postulated, '*H. pylori* infection that primarily involves a Th1 immune response may suppress the Th2 pathway which is mainly associated with atopy'.

5.6 *H. pylori* and Inflammatory Bowel Disease

Inflammatory bowel disease (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), are idiopathic diseases of the gastrointestinal tract (Podolsky, 1991). Since the mid-1990s, an increasing number of studies have investigated the association between *H. pylori* infection and IBD. The rationale behind such studies relates to the observed correlation between the increased prevalence of IBD (Logan, 1998; Loftus, 2004; Gisbert, 2008; Thia *et al.*, 2008) and the decreased prevalence of *H. pylori* (see Malaty, Chapter 1, this volume), as well as to recent studies suggesting that *H. pylori* colonization may lead to modulation of the immune response.

In the first study to examine the possible association between *H. pylori* infection and IBD, El-Omar and colleagues (1994) compared the *H. pylori* IgG status of 110 patients with IBD with that of 100 age- and sex-matched controls. Overall, 22% of IBD patients were shown to be *H. pylori*-positive compared with 52% of control patients. While this was an interesting result, post hoc analysis suggested that the lower prevalence of *H. pylori* infection in the IBD group may have been related to current or previous use of sulfasalazine (El-Omar *et al.*, 1994). Although this latter finding was supported by a number of later studies (Pearce *et al.*, 2000), others found no relationship between sulfasalazine use and the prevalence of *H. pylori* infection (Halme *et al.*, 1996; Vare *et al.*, 2001; Luther *et al.*, 2009; Song *et al.*, 2009).

Since El-Omar *et al.*'s initial study in 1994, an estimated 350+ papers or abstracts have reported on the possible link between *H.*

pylori and IBD (Luther *et al.*, 2009). For example, Vare *et al.* (2001) compared the seroprevalence of *H. pylori* in 94 patients with CD, 185 with UC and 17 with intermediate colitis to that in 70 healthy age- and sex-matched controls. This showed an inverse association to exist between *H. pylori* prevalence and the presence of IBD, the overall prevalence in IBD patients (24%) being significantly lower than that in controls (37%). Interestingly the prevalence of *H. pylori* in CD patients (13%) was significantly lower than that in UC (30%), suggesting that *H. pylori* may be more likely to protect against CD. This stronger association between *H. pylori* and CD has been confirmed by many other studies (Halme *et al.*, 1996; Wagtmans *et al.*, 1997; Duggan *et al.*, 1998; Pearce *et al.*, 2000; Feeney *et al.*, 2002; Song *et al.*, 2009).

Unfortunately, as discussed previously in relation to other extragastric diseases, many studies investigating the possible association between *H. pylori* infection and IBD have significant limitations. Not only do they relate to study design, the number of subjects investigated and failure to adjust for confounding factors, but also in many studies the criteria laid down to establish the diagnosis of IBD are poor.

In 2009 an excellent systematic review and meta-analysis of published studies investigating the association between *H. pylori* and IBD was published, which has helped to clarify, to some degree, the possible association between *H. pylori* and IBD. In this review, Luther and colleagues (2009) conducted a literature search (Medline, EMBASE, bibliographies and meeting abstracts) and identified 369 articles on this topic. Following exclusion of a large number of these studies due to poor design, small sample size, poor or no controls, as well as suboptimal criteria for diagnosis of IBD or determination of *H. pylori* status, 29 studies were reviewed in detail. Of 23 studies that fitted their final inclusion criteria, 13 showed a statistically significant inverse correlation between *H. pylori* infection and IBD, the relative risk (RR) for these studies being less than 1. In an attempt to explain the observed heterogeneity in the 23 studies, multiple subgroup analyses were conducted, the data being divided according to the

methods of *H. pylori* and IBD diagnosis, study location (eastern versus western hemisphere) and study population age (paediatric versus adult). While this subgrouping failed to explain the observed diversity in study outcomes, division of the dataset according to IBD type (CD or UC) showed a statistically significant reduction in the RR of *H. pylori* infection in CD patients diagnosed by *H. pylori* non-serological methods (RR = 0.71; 95% CI 0.58, 0.87) (Luther *et al.*, 2009). These authors concluded that, although *H. pylori* infection may potentially have a protective effect for IBD development, and in particular CD, a number of factors might have impacted on their results, including the possibility that participants had been treated for *H. pylori* infection prior to entering the study, thus resulting in a falsely low *H. pylori* infection rate.

Given these limitations, they suggested that future studies should ideally be conducted at the time of IBD diagnosis, and that diagnosis of IBD should be based upon review of endoscopic and histological findings. In addition, they recommended that diagnosis of *H. pylori* infection should be based upon urea breath testing, rapid urease testing of gastric biopsies or histology, given that serological assays cannot determine whether a patient is currently infected with *H. pylori*. Given the possible protective benefits of *H. pylori* *cagA*-positive strains against other autoimmune diseases, they also suggested that *CagA* status should be investigated in future studies.

To date, there are limited data on the potential mechanism by which *H. pylori* may protect against IBD, although a number of studies have suggested that regulatory T cells (Tregs) may play an important role (see Robinson and Atherton, Chapter 6, this volume). Given that adoptive transfer of Tregs has been shown to prevent the development of and treat experimental colitis in a range of animal models (Read *et al.*, 2000; Mottet *et al.*, 2003), it may be that Tregs produced in response to *H. pylori* infection have a role in preventing the development of colitis. Clearly further studies investigating the potential mechanism by which *H. pylori* may protect against IBD are required.

5.7 Hepatobiliary and Pancreatic Diseases

Studies on the association between *Helicobacter* infection and hepatobiliary and pancreatic diseases have mainly focused on entero-hepatic *Helicobacter* species. Of the work performed on *H. pylori*, detection of DNA in samples collected from diseased patients has been most frequently employed. Indeed, to date, only one study has reported the isolation of *H. pylori* from hepatobiliary samples, in this case from human liver (De Magalhaes Queiroz and Santos, 2001). This bacterial strain had the same morphology as *H. pylori*, was catalase-, oxidase- and urease-positive, and had >99% homology with *H. pylori* (De Magalhaes Queiroz and Santos, 2001).

5.7.1 *H. pylori* and hepatobiliary disease

Several studies have reported the detection of *H. pylori* DNA in bile samples from patients suffering from a variety of hepatobiliary diseases (Lin *et al.*, 1995; Myung *et al.*, 2000). Interestingly in one study, the authors measured a significantly lower bile pH in patients who were positive for *H. pylori*, but could not find a pathogenic role for *H. pylori* in the formation of hepatolithiasis (Myung *et al.*, 2000). In a study by Nilsson *et al.* (2000) in which 24 patients with either primary sclerosing cholangitis or primary biliary cirrhosis were examined, *H. pylori* DNA was detected in a significantly higher number of patients than in controls with normal livers ($P < 0.05$).

In 2002, Fukuda *et al.* proposed that '*Helicobacter* species may play a role in the pathogenesis of hepatobiliary cancer through an acceleration of biliary cell kinetics'. These authors reported a statistically significant difference between the detection of *Helicobacter* DNA in patients with and without hepatobiliary cancer ($P = 0.03$). In this study, however, of ten *Helicobacter* DNA-positive patients, only one had *H. pylori* DNA and that patient was also positive for DNA from another *Helicobacter* species (Fukuda *et al.*, 2002), a finding that suggests that *H. pylori* per se was not associated with hepatobiliary cancer.

Further support for this conclusion comes from a study of 122 patients with and without non-malignant gallbladder diseases, which failed to find a significant difference in the detection rate of *H. pylori* DNA between patients and controls (Chen *et al.*, 2003). In addition, no differences in blood biochemistry and liver function tests were observed between patients positive or negative for *Helicobacter* DNA. Chen *et al.* (2003) concluded from their study that *H. pylori* DNA is commonly present in the human gallbladder, and whether or not *H. pylori* plays a significant role in hepatobiliary diseases remains unclear.

Further studies have expanded the literature on *H. pylori* DNA detection in patients with hepatobiliary diseases (Huang *et al.*, 2004; Stalke *et al.*, 2005; Krasinskas *et al.*, 2007; Pirouz *et al.*, 2009), with two showing striking differences between patients and controls. For example, while *Helicobacter* 16S rDNA (of *H. pylori*- and *H. pullorum*-like organisms) was detected in, respectively, 61 and 68% of liver samples from patients with hepatitis C virus-positive cirrhosis with and without hepatocellular carcinoma (HCC), only 4.2% of liver samples obtained from controls and 3.5% of patients with non-cirrhotic chronic hepatitis C were shown to be positive (Rocha *et al.*, 2005). In addition, Li *et al.* (2006) examined liver samples from 34 patients with HCC and 20 patients without primary liver carcinoma for *H. pylori* DNA. Remarkably, in this study, 65% of HCC samples tested positive for *H. pylori* DNA compared with none of the controls (Li *et al.*, 2006).

Several mechanisms have been proposed to explain how *H. pylori* infection may lead to hepatobiliary diseases. To better understand the possible pathogenicity of this bacterium within the hepatobiliary system, a number of mouse colonization studies have been performed. For example, one study conducted in *Helicobacter*-free C57L/J mice examined whether or not *H. pylori* could promote gallstone formation, but found no evidence that *H. pylori* plays a role in this process (Maurer *et al.*, 2006). However, a case of primary biliary cirrhosis in a C57BL/6 mouse infected with *H. pylori* has been reported, with the authors suggesting that an increase in vacuolating toxin resulting from *H. pylori* infection may

play a role in the development of cirrhosis (Goo *et al.*, 2008). More recently, Huang *et al.* (2009) studied the pathological changes in the livers and gallbladders of C57BL/6 mice infected with *H. pylori*. Of 20 mice inoculated with *H. pylori* three developed mild-to-moderate hepatitis but, more importantly, *H. pylori* was observed morphologically in four liver specimens and six gallbladders from infected mice. This indicates that *H. pylori* inoculated orally may reach the hepatobiliary system and cause inflammation (Huang *et al.*, 2009).

The degradation of urea by *H. pylori* results in the formation of high levels of ammonia, which has been associated with the degree of encephalopathy (Sethar *et al.*, 2004). Several studies have therefore focused on the role of ammonia present in patients' blood and hepatic encephalopathy. For example, Wang *et al.* (2006) assessed the involvement of ammonia produced as a result of *H. pylori* infection in hepatic encephalopathy status and demonstrated that the bacterium was an important source of ammonia. The authors concluded that these findings support a possible role for the bacterium in the development of hepatic encephalopathy in cirrhotic patients (Wang *et al.*, 2006).

5.7.2 *H. pylori* and pancreatic disease

Studies investigating the relationship between *H. pylori* infection and pancreatic diseases have yielded contradictory results. For example, Kountouras *et al.* (2005) proposed that the organism might trigger autoimmune pancreatitis (AIP) through induction of autoimmunity and apoptosis. This hypothesis was derived from the finding that *H. pylori* is capable of mimicking host factors to induce irregularities in T-cell apoptosis, which ultimately contribute to extragastric tissue destruction (Kountouras *et al.*, 2005). However, Chang *et al.* (2009) recently compared the *H. pylori* status of 40 patients with AIP and 113 patients with non-autoimmune chronic pancreatitis (NACP) and found that, although frequencies of gastric ulcers were significantly higher in patients with AIP than in those with NACP, *H. pylori* status could not explain the pathogenesis of AIP in these patients.

Lindkvist *et al.* (2008) studied the possible involvement of *H. pylori* infection in the increased risk of pancreatic cancer after previous studies had reported conflicting results. Patients with pancreatic cancer ($n = 87$) were compared with 263 age- and sex-matched controls selected from a total cohort of 33,346 subjects. The authors found no association between *H. pylori* seropositivity and risk of pancreatic cancer in the total cohort; however, adjustment for variables such as smoking and alcohol consumption suggested that patients with healthier lifestyles had an increased risk of pancreatic cancer if they were *H. pylori*-positive (Lindkvist *et al.*, 2008).

5.8 Concluding Comment

Despite many thousands of studies investigating the role of *H. pylori* in a diverse range of extragastric diseases, in most cases the epidemiological data remain inconsistent. Plausible biological explanations for these putative associations are commonly not well formed, and evidence that eradication of *H. pylori* leads to clinical improvement is missing. Unless well-designed prospective studies that control for the many factors that can confound such studies are undertaken, the putative association between *H. pylori* infection and these extragastric manifestations is likely to remain unresolved.

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6 *Helicobacter pylori*-induced Acquired Immunity and Immunoregulation

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6.1 Introduction

Helicobacter pylori infections are commonly acquired during early childhood (Goodman and Correa, 1995) and colonization persists lifelong unless antibiotic treatment is administered, as natural clearance of the infection is rare (Xia and Talley, 1997). How the bacterium persists in the gastric mucosa of its host for decades remains incompletely understood. There is evidence to suggest that *H. pylori* has co-evolved with man and has adapted to manipulate and evade the immune response, aiding its survival, but also contributing to pathogenesis (Blaser and Atherton, 2004). Following uptake by antigen-presenting cells, probably in intestinal Peyer's patches (Gewirtz and Sitaraman, 2007), the bacteria stimulate an acquired immune response consisting of high antibody titres and specific T cells which are detectable in both the gastric mucosa and peripheral blood (O'Keeffe and Moran, 2008). The components thought to be necessary for clearance of many bacterial infections, such as antibody, complement components and phagocytic cells (Mueller-Ortiz *et al.*, 2004), can be detected in the gastric mucosa (Berstad *et al.*, 1997), yet colonization persists regardless. The possibility that an ineffective or inappropriate type of immune response is provoked by the

bacteria has been of recent research interest, especially since a marked regulatory T-cell response has been demonstrated (Lundgren *et al.*, 2005b; Rad *et al.*, 2006; Robinson *et al.*, 2008). These suppressive cells are thought to reduce the inflammatory response and inhibit protective immune mechanisms (reviewed by Joosten and Ottenhoff, 2008). A recent hypothesis is that *H. pylori*-induced immune responses may also play a general regulatory role in preventing other immunological or inflammatory conditions, since inverse epidemiological associations have been noted between these and *H. pylori* prevalence (Blaser *et al.*, 2008). This chapter reviews recent findings on the acquired immune response to *H. pylori*, its role in modulating gastroduodenal pathology and disease, and also discusses possible implications for the immune system in general and for other diseases.

6.2 B-cell and Antibody Responses

H. pylori stimulates the production of mucosal and systemic immunoglobulin (Ig) A and IgG antibodies, and this forms the basis for some commercial diagnostic tests (Kindermann *et al.*, 2001). Traditionally, bacterial vaccines have depended upon the stimulation of a high-titre humoral response for protective efficacy, but

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the effect of antibody upon *H. pylori* colonization is unclear. *H. pylori* is susceptible to complement-mediated killing in the presence of serum *in vitro* (Gonzalez-Valencia *et al.*, 1996; Berstad *et al.*, 2001), IgG is detectable in the gastric juice of infected people (Meining *et al.*, 2002), and *H. pylori* bacteria in the human gastric mucosa are coated in IgG, IgA and IgM (Wyatt *et al.*, 1986), but the successful immunization of B-cell-deficient mice (Ermak *et al.*, 1998; Sutton *et al.*, 2000) indicates that antibodies are not essential for protection.

Human studies have found that the presence of anti-*H. pylori* IgA in maternal milk is associated with delayed colonization in infants, suggesting that IgA can block infection (Thomas *et al.*, 2004). Others have tested the effects of orally administered bovine colostrum containing high levels of *Helicobacter*-specific IgA, showing protective effects in mouse models (Casswall *et al.*, 2002; Marnila *et al.*, 2003), but results from human volunteer studies were disappointing (Casswall *et al.*, 1998). Intragastric administration of specific monoclonal IgA antibodies to mice provided protection against *Helicobacter felis* infection (Czinn *et al.*, 1993), but others have shown that specific IgA and IgG may potentially promote bacterial colonization and inhibit protective immune mechanisms in mice (Akhiani *et al.*, 2004b). Human studies have also shown that the presence of an elevated IgA response to infection is associated with a significantly increased risk of gastric cancer (Kosunen *et al.*, 2005; Knekt *et al.*, 2006). The humoral response to *H. pylori* may even play a role in pathogenesis by triggering autoimmune processes (D'Elios *et al.*, 2004), where antibodies cross-react with host antigens such as Lewis blood-group antigens on gastric epithelial cells (Appelmek *et al.*, 1996) and the parietal cell H⁺,K⁺-ATPase (Amedei *et al.*, 2003). Sera from approximately half of infected individuals contain parietal cell-reactive antibodies (Negrini *et al.*, 1991), which could potentially contribute to local inflammation and tissue damage.

While B cells are usually involved in the positive induction of immune responses, a suppressive subset of CD1d^{high}CD5⁺ regulatory B cells (Bregs) has recently been discovered (Yanaba *et al.*, 2008). These form part of

the early defence against infection via production of polyreactive IgM, but also suppress the responses of T cells and natural killer T (NKT) cells by secreting interleukin (IL)-10 and may promote the expansion of regulatory T cells (Tregs) (Bouaziz *et al.*, 2008; Mauri and Ehrenstein, 2008). Bregs appear to be important in some bacterial infections (Majlessi *et al.*, 2008); however, studies on *H. pylori* infection in B-cell knockout mice reported no significant increase in gastric pathology (Ermak *et al.*, 1998; Garhart *et al.*, 2003b), thus any role of Bregs in *H. pylori* infection remains speculative.

6.3 CD4⁺ T-cell Responses and Cross-talk with Innate Immunity

In vitro work has shown that *H. pylori* can utilize many different mechanisms for down-regulating T-cell activity. Lymphocyte cell cycle arrest can be induced by several virulence factors including cytotoxin-associated gene A protein (CagA) (Paziak-Domanska *et al.*, 2000), proliferation-inhibiting protein (Knipp *et al.*, 1996) and γ -glutamyl transpeptidase (Gerhard *et al.*, 2005; Schmees *et al.*, 2007). Vacuolating toxin (VacA) inhibits T-cell responses indirectly by blocking major histocompatibility complex class II-mediated antigen presentation (Molinari *et al.*, 1998) and by direct suppression of T-cell activation (Boncristiano *et al.*, 2003; Gebert *et al.*, 2003; Torres *et al.*, 2007). Furthermore, expression of the co-stimulatory molecule B7-H1 by gastric epithelial cells is elevated during *H. pylori* infection *in vivo*, and this molecule can suppress T-cell activity (Das *et al.*, 2006).

Aside from inhibiting T-cell activity in general, the response to infection is greatly influenced by the type of T-cell response elicited. CD4⁺ T cells orchestrate the immune response in mammals: they direct the class and subclass of antibodies, dictate properties of professional phagocytes, and can up- or down-regulate the level of inflammation (Romagnani, 2006). Certain T-helper (Th) subsets are important in immunity to different types of infection and these are summarized in Fig. 6.1. Th1 cells help to eliminate

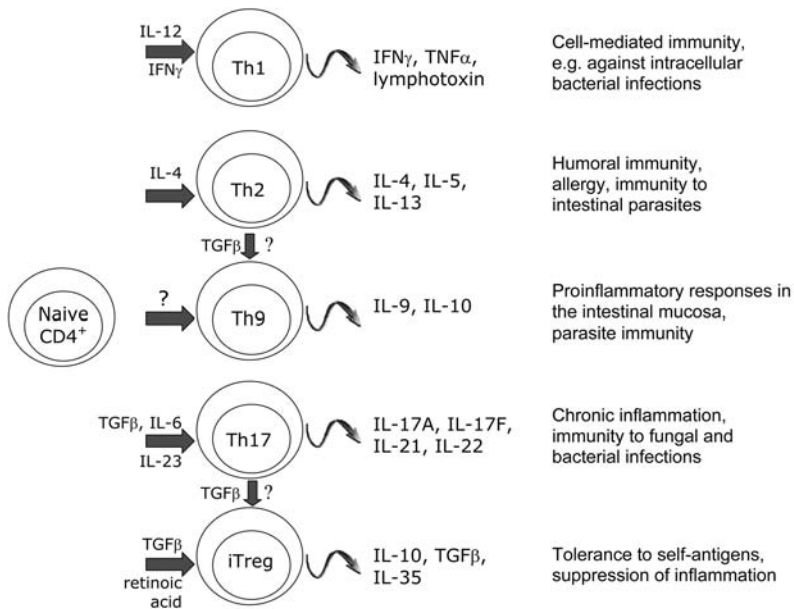


Fig. 6.1. Subsets of CD4⁺ T-helper (Th) cells, showing the innate factors that influence their differentiation from naive T cells, the cytokines they secrete and their main functional roles. Abbreviations: IFN γ , interferon- γ ; IL, interleukin; iTreg, inducible regulatory T cell; TGF β , transforming growth factor- β ; TNF α , tumour necrosis factor- α .

intracellular bacteria and viruses, whereas Th2 cells are protective against intestinal helminth parasites. Th2 cells may develop into Th9 cells, which may also play a role in parasite immunity (Veldhoen *et al.*, 2008). Th17 cells may be important in infections with fungi and extracellular bacteria (MacDonald and Maizels, 2008; Curtis and Way, 2009). Tregs comprise a suppressive subset, which prevent immune attack against self-antigens or innocuous environmental antigens (Belkaid, 2008; Costantino *et al.*, 2008b) and also regulate inflammation. These T-cell subsets are discussed in more detail below.

During the development of Th cells from naive precursors, the cells are exposed to an array of signals, which directs them down particular differentiation pathways (Romagnani, 2006; Kaiko *et al.*, 2008). Until recently it was thought that mature Th cells were terminally differentiated and subsets were fixed. In fact there is a degree of plasticity in Th cells as they may respond to signals

such as innate cytokines and acquire the properties of a different subset (Rowell and Wilson, 2009; Wei *et al.*, 2009). For example, Th2 cells may be converted into interferon- γ (IFN γ)-secreting Th1 cells in the presence of Th1-associated cytokines (Krawczyk *et al.*, 2007). *H. pylori* has recently been shown to induce IL-12p40 and IL-18 expression by gastric epithelial cells and monocytes (Shimada *et al.*, 2008; Yamauchi *et al.*, 2008; Takeshima *et al.*, 2009), thus it is possible that these cytokines could contribute to this conversion. Under the direction of transforming growth factor- β (TGF β) and IFN γ , CD4⁺CD25⁻ effector T cells can be induced to acquire the suppressive attributes of Tregs (Wang *et al.*, 2006). High levels of these cytokines are present in the *H. pylori*-infected gastric mucosa. The balance between Tregs and Th17 cells is known to be influenced by the presence of TGF β , IL-6 and retinoic acid; reduced levels of retinoic acid (a factor favouring Treg differentiation) are associated with increased pathology in the *H. pylori*-infected

gastric mucosa (Matsumoto *et al.*, 2005a). Similarly, Th17 cells can shift towards a Th1 phenotype in the presence of reduced TGF β and increased IL-12 and IL-23 (Lee *et al.*, 2009), or be induced by IL-6 and TGF β to express IL-10 (McGeachy *et al.*, 2007).

Th1, Th2, Th17 and Treg cells have all been reported in the *H. pylori*-infected gastric mucosa (Bamford *et al.*, 1998; D’Elios *et al.*, 2003; Caruso *et al.*, 2008; Robinson *et al.*, 2008). Given new findings on Th subset plasticity, we should view these data with caution, especially where cells have been isolated from their natural innate cytokine environment and cultured for extended periods prior to phenotyping. In addition to the conversion of one subset into another, one must also consider that hybrid cell types exist, e.g. Th1 cells may secrete IL-10 (O’Garra and Vieira, 2007) and IL-17-producing FOXP3⁺ Tregs have recently been described in humans (Voo *et al.*, 2009). Previously reported gastric IFN γ ⁺ Th1 cells might also secrete IL-17 and may actually be Th17 cells (Annunziato *et al.*, 2007). Also IL-10, thought to indicate a Treg response, could be derived from Th1 cells. In summary, the field of T-helper subset responses has recently

become more complex, making it important that panels of markers for the different cell types are used in such studies.

6.3.1 T-helper subset responses

T-helper 1

In humans, a predominant IFN γ -producing CD4⁺ T-cell response is found in the *H. pylori*-infected gastric mucosa (Fan *et al.*, 1994; D’Elios *et al.*, 1997; Bamford *et al.*, 1998), and is associated with the expression of proinflammatory cytokines such as tumour necrosis factor- α (TNF α), IL-12 and IL-18. When macrophages are activated in a Th1 cytokine environment they become ‘angry’. ‘Angry’ macrophages secrete proinflammatory factors and have enhanced bactericidal activity as compared with Th2-activated macrophages (Ma *et al.*, 2003). The number of IFN γ -secreting cells in the infected human gastric mucosa correlates with both the severity of gastritis (Lehmann *et al.*, 2002) and the presence of peptic ulcer disease (Robinson *et al.*, 2008) (Fig. 6.2). *H. pylori* infection influences the levels of gastric hormones, reducing somatostatin release and

T-helper subset responses			
Th1	Th17	Th2	Treg
Inflammation, pathology and disease			
Required for vaccine-induced protection in mice (Akhiani <i>et al.</i> , 2002; Garhart <i>et al.</i> , 2003a)	Required for vaccine-induced protection in mice (DeLyria <i>et al.</i> , 2009; Velin <i>et al.</i> , 2009)	Not required for successful vaccination in mice (Garhart <i>et al.</i> , 2003b)	Inhibits vaccine-induced protection in mice (Watanabe <i>et al.</i> , 2002)
Associated with reduced colonization densities in mice and humans (Lucas <i>et al.</i> , 2001; Holck <i>et al.</i> , 2003)	Associated with reduced colonization densities in mice (Shiomi <i>et al.</i> , 2008; Velin <i>et al.</i> , 2009)	Associated with increased colonization densities in mice (Fox <i>et al.</i> , 2000)	Associated with increased colonization densities in mice and humans (Raghavan <i>et al.</i> , 2003; Robinson <i>et al.</i> , 2008)

Fig. 6.2. Role of T-helper (Th) subsets in *Helicobacter pylori* infection, pathology, disease and vaccination. Abbreviation: Treg, regulatory T cell.

stimulating gastrin production. This inhibits Th2 responses and increases Th1 activity (Zavros and Merchant, 2005). C57BL/6 mice, which mount a strong Th1 response to *H. pylori*, develop severe gastritis when infected (Smythies *et al.*, 2000; Garhart *et al.*, 2002). A Th1 response is associated with reduced bacterial colonization density both in humans (Holck *et al.*, 2003) and in mouse models (Lucas *et al.*, 2001; Stoicov *et al.*, 2004). An expression profiling study of the *H. pylori*-infected murine stomach found that genes associated with an IFN γ -dependent Th1 response were up-regulated (Vivas *et al.*, 2008), and several groups have shown that a Th1 response is required for successful immunization (Eaton *et al.*, 2001; Akhiani *et al.*, 2002; Garhart *et al.*, 2003b; Taylor *et al.*, 2008).

A number of *H. pylori* virulence factors have been reported to promote Th1 responses. For example, administration of *H. pylori* neutrophil-activating protein (HP-NAP) to mice suppresses Th2 responses to intestinal parasites (Amedei *et al.*, 2006; Del Prete *et al.*, 2008) and down-regulates Th2-mediated allergy in a model for asthma (Codolo *et al.*, 2008). The *cag* pathogenicity island is associated with increased Th1, IL-12 and IL-18 responses (Wang *et al.*, 2007; Yamauchi *et al.*, 2008; Takeshima *et al.*, 2009), outer inflammatory protein A (OipA) stimulates IL-18 (Yamauchi *et al.*, 2008) and VacA induces T cells to express IL-12p40 (Takeshima *et al.*, 2009). The Th1 response is also influenced by the plasticity region locus *jhp0947-jhp0949* (de Jonge *et al.*, 2004), genomic DNA recombination (Robinson *et al.*, 2005) and phase-variable expression of Lewis blood-group antigens via interaction with the cellular innate immune receptor, dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) (Bergman *et al.*, 2004). The fact that so many features of *H. pylori* strongly stimulate host Th1 immunity indicates that this type of response is important for successful chronic colonization of the gastric mucosa by these bacteria.

T-helper 2

Although a predominant Th1 response has been reported in the infected human gastric

mucosa, Th2 cells have also been detected (Serrano *et al.*, 2007; Robinson *et al.*, 2008). Initial vaccine studies in mice found that a Th2 response correlated with reduced bacterial loads (Mohammadi *et al.*, 1997), although subsequent gene knockout studies found that *IL4* is unnecessary for induction of protective immunity (Garhart *et al.*, 2003b), whereas Th1-associated genes such as *IL12* and *IL18* are required (Garhart *et al.*, 2003a; Akhiani *et al.*, 2004a) (Fig. 6.2). Gastritis in mice is Th1 mediated and is more severe in IL-4-deficient mice, implying that a Th2 response suppresses inflammation (Smythies *et al.*, 2000). Moreover, the co-administration of an intestinal parasite in order to skew the Th1/Th2 balance towards Th2 in mice resulted in reduced gastric pathology, but *Helicobacter* colonization densities were increased (Fox *et al.*, 2000). Regarding human infections, several groups have shown that there is a shift from Th1 immunity in active gastritis, towards IL-4⁺ or IL-13⁺ Th2 responses in patients with gastric cancer or precancerous changes (Ren *et al.*, 2001; Marotti *et al.*, 2008). The significance of this is unclear.

A new T-helper subset (Th9), which secretes IL-9 and IL-10, has recently been discovered in the mouse (Tato and Cua, 2008). These cells are derived from Th2 cells under the influence of TGF β 1 and perform specialized functions in the gut, mediating protection from parasites (Veldhoen *et al.*, 2008) and contributing to inflammation in a colitis model (Dardalhon *et al.*, 2008). There are no data regarding Th9 cells in *H. pylori* infection, but elevated IL-9 and IL-10 expression in the human gastric mucosa is associated with both *H. pylori*-positive status and incidence of gastric adenocarcinoma (Ellmark *et al.*, 2006).

T-helper 17

The presence of IL-17 in the *Helicobacter*-infected gastric mucosa was reported before the discovery of Th17 cells (Luzza *et al.*, 2000). IL-17 is produced mainly by CD4⁺ (Th17) and CD8⁺ (Tc17) cells, and is also reportedly expressed by some $\gamma\delta$ T cells and invariant NKT cells. Th17 cells express IL-17A and IL-17F, which have similar functions (Dong,

2008). The role of IL-17A and IL-17F in immunity to extracellular infections involves activation of fibroblasts, endothelial and epithelial cells, and macrophages to secrete cytokines and chemokines, leading to the neutrophil accumulation. They also express antibacterial peptides and matrix metalloproteinases (Matsuzaki and Umemura, 2007; Iwakura *et al.*, 2008). As neutrophilic gastritis is a prominent feature of human *H. pylori* infection, IL-17 and Th17 cells have become of particular interest.

Greatly reduced levels of neutrophil infiltration were reported in the gastric mucosa of *H. pylori*-infected IL-17-deficient mice (Shiomi *et al.*, 2008). Neutrophil numbers in the infected human gastric mucosa correlate with IL-17 expression, with the highest concentrations being found at ulcer sites (Mizuno *et al.*, 2005). Human gastric epithelial cells and lamina propria mononuclear cells express receptors for, and are capable of responding to, IL-17. Stimulation of MKN28 gastric adenocarcinoma cells with IL-17 induced activation of extracellular signal-regulated protein kinase 1/2 (ERK 1/2), resulting in activator protein-1 (AP-1) and nuclear factor (NF)- κ B activation and IL-8 expression (Sebkova *et al.*, 2004). Neutralization of IL-17 in mucosal mononuclear cell cultures markedly reduced the IL-8 response to *H. pylori* (Luzza *et al.*, 2000), implying that the Th17 response is key in inflammation. One study investigated associations of *IL17A* and *IL17F* genetic polymorphisms and the incidence of gastric disease, showing increased inflammation in *H. pylori*-infected individuals with the *IL17F* 7488T allele (Arisawa *et al.*, 2007). A recent paper reported that IL-17A has anti-inflammatory effects on *H. pylori*-induced gastritis (Otani *et al.*, 2009), where the administration of neutralizing anti-IL-17A antibody increased Th1 cytokine expression and gastritis severity in infected mice. Other groups have also reported anti-inflammatory properties of IL-17A, and that Th17 cells negatively regulate Th1 responses (Nakae *et al.*, 2007; Ke *et al.*, 2009). The Th17 response may therefore also limit gastric inflammation and pathology by suppressing Th1 cell differentiation.

IL-23 stimulates differentiation and expansion of Th17 cells, and is involved in

several intestinal inflammatory conditions and cancer development (Tan *et al.*, 2009). IL-23 is over-expressed in the *H. pylori*-infected human gastric mucosa, and both Th17 and Tc17 cells are present (Caruso *et al.*, 2008). Th17 cells are present at higher levels than normal in the stomach and peripheral blood of patients with gastric cancer (Zhang, B. *et al.*, 2008). Similar observations have been made with ovarian, renal and pancreatic malignancy (Kryczek *et al.*, 2007) and increasing frequencies of Th17 cells correlated with advancing tumour progression. IL-17 (as with IL-23) is associated with reduced anti-tumour immunity while promoting angiogenesis and tumour growth (Numasaki *et al.*, 2003; Langowski *et al.*, 2006).

In addition to participating in the inflammatory response to *H. pylori*, two recent studies report a requirement for IL-17 in vaccine-induced protective immunity. The first publication demonstrated that intranasal immunization using a lysate of the *H. pylori* SS1 strain with cholera toxin adjuvant induced a CD4⁺ T-cell response characterized by IL-17 and IFN γ secretion upon their antigenic re-stimulation *in vitro* (DeLyria *et al.*, 2009). When animals were subsequently infected, there was a more substantial IL-17 response, increased gastritis and reduced colonization density compared with unvaccinated mice. Antibody-mediated depletion of neutrophils inhibited clearance of the infection in vaccinated animals, indicating that neutrophils play a role in protective immunity. This result contrasts with a previous study, however, which showed that neutrophil depletion had no effect on vaccine-induced reductions of colonization (Velin *et al.*, 2005). A second publication on the contribution of Th17 cells to vaccination-mediated bacterial clearance examined the effect of neutralizing IL-17 antibody treatment on *H. felis* infection in mice (Velin *et al.*, 2009). This group also found a more dramatic influx of CD4⁺IL-17⁺ cells in the infected gastric mucosa, but found that anti-IL-17 antibody treatment (administered at the same time as immunization) inhibited clearance of the infection and reduced gastric inflammation. Both papers show that Th17 cells contribute greatly to protective immunity.

6.4 CD8⁺ Cytotoxic T Cells

Although most studies on cellular immunity to *H. pylori* have focused on CD4⁺ cells, increased numbers of CD8⁺ cytotoxic T cells (Tc) are also found in the gastric mucosa and blood of infected human subjects and the stomachs of infected mice (Stromberg *et al.*, 2003; Rossi *et al.*, 2004; Nurgalieva *et al.*, 2005; Munoz *et al.*, 2007). Increased numbers of activated CD8⁺ cells were found in the peripheral blood of children with duodenal ulcers (Figueiredo Soares *et al.*, 2007). More severe gastritis, mediated by CD8⁺ T cells, is also observed in *H. pylori*-infected CD4⁺ cell-deficient mice (Fukui *et al.*, 2007; Tan *et al.*, 2008). Most human gastric mucosal Tc cells are memory cells, which could readily be activated by culture with *H. pylori*-exposed B cells (Azem *et al.*, 2006). These cells may express IL-17 (Caruso *et al.*, 2008), and CD8⁺ T cells in inflamed lesions of the infected human stomach have been shown to contain cytolytic granules and express the proinflammatory chemokine RANTES (Ohtani *et al.*, 2004). Together these data indicate a potential role for CD8⁺ T cells in *H. pylori* inflammation and disease.

6.5 Regulatory T Cells

The term 'regulatory T cell' (Treg) refers to a cell type that actively controls or suppresses the function of other cells. They are usually CD4⁺; however, some CD8⁺ cells and even NKT cells may have regulatory activity (Coleman *et al.*, 2007). Suppressive T cells were first described several decades ago (Gershon and Kondo, 1971), but the field fell into disrepute because of inconsistent findings and a lack of cellular markers to conduct rigorous studies (Germain, 2008). These cells, renamed 'regulatory' rather than 'suppressor' T cells, are now recognized as being extremely important as they can prevent inflammatory and immune-mediated disease conditions (e.g. autoimmunity, allergy, inflammatory bowel disease) (Larche, 2007; Costantino *et al.*, 2008a; Izcue *et al.*, 2009). Because of suppressive activity on antitumour immunity, Tregs also play an important role in cancer

progression (Ghiringhelli *et al.*, 2006; Kono *et al.*, 2006; Gallimore and Godkin, 2008). Treg responses are even exploited by some infections (probably including *H. pylori*) to prevent their clearance (Belkaid, 2007; Coleman *et al.*, 2007).

While several types of Treg have been described that express different profiles of markers (listed in Table 6.1), based on the location of their differentiation the two main categories are natural (nTregs) and inducible (iTregs). nTregs are produced in the thymus and due to their specificity for self antigens are believed to play a primary role in preventing autoimmune reactions. In contrast, iTregs are generated in the periphery from naive T cells, similar to Th cells, and are generally thought to be specific for non-self antigens. Since *H. pylori* expresses a number of antigens with similarity to host molecules, both of these Treg types could be present in the infected gastric mucosa. Identification is difficult because of the diversity of Tregs and the lack of cellular markers that can distinguish these subsets. CD4⁺ Tregs commonly express high levels of CD25 (IL-2R α) and the transcription factor FOXP3 (which represses effector genes and induces expression of suppressive mediators) (Wing *et al.*, 2005; Coleman *et al.*, 2007). Tregs act via a variety of mechanisms (reviewed by Vignali, 2008); for example, by secreting suppressive cytokines such as IL-10 and TGF β (which may have bystander activity on antigenically unrelated effector cells), or in a contact-mediated fashion where a negative signal is delivered to another cell to suppress its activity.

H. pylori infection of mice stimulates a Treg response that regulates gastric inflammation and is thought to blunt protective immune mechanisms to maintain a chronic infection. Initial studies showed that Tregs suppress inflammatory and Th1 responses to *H. pylori* infection. Adoptive transfer of CD4⁺CD25⁺-depleted lymph node cells into athymic *nu/nu* C57BL/6 mice, followed by infection with *H. pylori*, induced earlier and more severe gastritis compared with the recipients of lymph node cells including Tregs (Raghavan *et al.*, 2003, 2004). Following on from this, other groups showed that *H. pylori* induces a high-level gastric CD25⁺FOXP3⁺

Table 6.1. Main markers and suppressive factors expressed by regulatory T cells.

Molecule	Description
<i>Main identifying markers</i>	
FOXP3	Forkhead transcription factor; strong marker for murine CD4 ⁺ CD25 ⁺ Tregs but not expressed by all human Tregs; required for murine Treg function; represses effector genes and regulates expression of Treg-associated genes
CD25 ^{high}	IL-2R α chain; high Treg expression levels induce cytokine deprivation-mediated apoptosis in Teffs
GITR	Constitutively expressed by Tregs; protection from glucocorticoid-induced apoptosis; mediates survival during thymic generation of natural Tregs; interaction with its ligand abrogates Treg suppressive activity
CD127 ⁻ CD45RO	IL-7R; absence correlates with expression of FOXP3 by Tregs Tregs are antigen-specific memory T cells
<i>Cytokines and secreted factors</i>	
IL-10	Suppressive cytokine; inhibits NF- κ B activation; inhibits activity of Teffs and differentiation of naive T cells
TGF β 1	Suppressive cytokine; inhibits inflammation, activity of Teffs and differentiation of naive T cells
IL-35	Suppressive cytokine; inhibits inflammation and activity of Teffs; suppression of Th17 development
Granzymes A and B, perforin Adenosine	Cytolytic molecules; kill target cells Binds type 1 purinergic adenosine A2A receptor on Teffs and inhibits activity by metabolic disruption
IDO	Induces conversion of tryptophan into pro-apoptotic metabolites
<i>Cell-associated molecules</i>	
CTLA-4 (CD152)	Constitutively expressed on CD4 ⁺ CD25 ⁺ Tregs; interacts with CD80/86 on DCs and delivers a negative signal; induces IDO
LAG3 (CD223)	Binds MHC class II on immature DCs to suppress maturation and capacity to stimulate an immune response
Neuropilin-1 (Npl-1)	Constitutively expressed on CD4 ⁺ CD25 ⁺ Tregs; expression linked with suppressive function
CD39 and CD73	Generate pericellular adenosine production

Abbreviations: CTLA-4, cytotoxic T lymphocyte-associated antigen 4; DC, dendritic cell; GITR, glucocorticoid-induced TNF receptor-related protein; IDO, indoleamine 2,3-dioxygenase; IL, interleukin; LAG3, lymphocyte activation gene 3; MHC, major histocompatibility complex; NF, nuclear factor; Teff, effector T cell; TGF, transforming growth factor; TNF, tumour necrosis factor; Treg, regulatory T cell.

Treg response in mice (Kaparakis *et al.*, 2006; Rad *et al.*, 2006). *In vivo* depletion of Tregs by administration of an anti-CD25 monoclonal antibody resulted in more severe gastritis in infected C57BL/6 mice (Rad *et al.*, 2006), a markedly diminished Th1 cytokine response in infected BALB/c mice (Kaparakis *et al.*, 2006), and also enhanced clearance of infection following immunization with *H. pylori*-pulsed dendritic cells (Zhang, M. *et al.*, 2008). In addition, CD25⁺ Tregs were shown to induce anergy in effector T cells during *H. pylori* infection (Stuller *et al.*, 2008).

Human studies also found increased numbers of Tregs in *H. pylori*-infected gastric mucosal tissues (Lundgren *et al.*, 2005a,b; Rad *et al.*, 2006; Harris *et al.*, 2008; Robinson *et al.*, 2008). These cells suppressed memory T-cell responses to *H. pylori* (Lundgren *et al.*, 2003), and an elevated Treg response is reported to be associated with higher colonization densities, lower-level expression of inflammatory cytokines and lower frequencies of Th1 and Th2 cells in gastric tissue (Robinson *et al.*, 2008). Two studies have also shown that high-level Treg responses are found when peptic ulcer

disease is absent, indicating that Tregs may protect against the development of disease (Harris *et al.*, 2008; Robinson *et al.*, 2008).

The Treg response to *H. pylori* is described in more detail below.

6.5.1 Interleukin-10-secreting regulatory T cells

The importance of IL-10 secretion as a mechanism of action for *H. pylori*-induced Tregs is clear from mouse models. Chronic infections cannot be established in IL-10-deficient mice, probably due to the development of a severe unregulated inflammatory response in these animals (Chen *et al.*, 2001; Matsumoto *et al.*, 2005b). Adoptively transferred Tregs from IL-10-deficient mice, unlike cells from wild-type animals, are unable to inhibit *H. pylori*-induced gastritis (Lee *et al.*, 2007). IL-10 is also highly expressed in the gastric mucosa and peripheral blood of *H. pylori*-infected human subjects (Goll *et al.*, 2007; Lundin *et al.*, 2007; Serrano *et al.*, 2007; Kandulski *et al.*, 2008). Particularly high IL-10 responses were found in those colonized by *cagA*⁺ strains (Wang *et al.*, 2007; Robinson *et al.*, 2008). Studies on *IL10* gene polymorphisms have yielded contradictory results (see Sutton *et al.*, Chapter 7, this volume), but low IL-10 producer genotypes have been associated with increased gastritis and premalignant pathology (Zamboni *et al.*, 2005; Achyut *et al.*, 2008). A large proportion of the CD4⁺CD25^{high} Tregs in human gastric tissues express IL-10, and peptic ulcer disease more commonly occurs when there are low numbers of these cells (Robinson *et al.*, 2008). *IL10* mRNA levels also correlate inversely with IL-8 response and colonization densities (Robinson *et al.*, 2008). Therefore, as in mouse models, the IL-10⁺ Treg response influences persistence of *H. pylori* and the level of inflammation induced.

6.5.2 TGF β -secreting regulatory T cells

TGF β 1 is another Treg-associated cytokine with an important role in *H. pylori* infection,

since loss of TGF β signalling in infected mice leads to increased gastric inflammation and incidence of adenocarcinoma (Hahm *et al.*, 2002). Moreover, infected human gastric mucosa also has elevated TGF β 1 expression (Lindholm *et al.*, 1998; Li and Li, 2006; Goll *et al.*, 2007; Harris *et al.*, 2008; Robinson *et al.*, 2008). *In vitro*, *H. pylori* induces TGF β release from gastric epithelial cells and leucocytes (Wu *et al.*, 2007), and gastric *FOXP3* mRNA expression is positively correlated with the TGF β response. Colonization density is also positively correlated with TGF β expression (Kandulski *et al.*, 2008). TGF β 1⁺ cells in gastric tissues are predominantly CD25⁺ and FOXP3⁺, i.e. Tregs. Increased numbers coincide with reduced levels of gastritis in children (Harris *et al.*, 2008). Our own studies found that, in contrast to IL-10, low TGF β 1 expression levels were not associated with the incidence of peptic ulceration (Robinson *et al.*, 2008). This implies that, although TGF β 1 inhibits inflammation, it is not sufficient to prevent *H. pylori*-mediated ulceration in humans.

6.5.3 Other known regulatory T-cell mechanisms in *H. pylori* infection

One of the best characterized contact-mediated mechanisms for Treg suppression involves the co-stimulatory molecule cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) where, following binding of CD80/86, a negative signal is delivered directly into dendritic cells to inhibit their maturation and function (Vignali, 2008). After transduction of this signal, dendritic-cell cytokine production is reduced, thereby inhibiting inflammation. The ability of dendritic cells to present antigens would also be impaired leading to a reduced capacity to stimulate T-cell responses. Increased CTLA-4⁺ cells have been detected in the *H. pylori*-infected human gastric mucosa (Stromberg *et al.*, 2003; Lundgren *et al.*, 2005b), and blockade of CTLA-4 in *H. pylori*-infected mice reverses T-cell anergy, resulting in increased inflammation and reduced bacterial loads (Watanabe *et al.*, 2004; Anderson *et al.*, 2006). Hyporesponsive T cells induced by intraperitoneal injection of bacteria into mice, which inhibited the

development of *H. pylori*-induced gastritis, were also found to express higher levels of CTLA-4 (Watanabe *et al.*, 2002). Human *CTLA4* gene polymorphisms are associated with susceptibility to mucosa-associated lymphoid tissue (MALT) lymphoma (Cheng *et al.*, 2006) and differential regulation of *H. pylori*-induced indoleamine 2,3-dioxygenase (IDO) activity. Tregs induce IDO expression by dendritic cells in a CTLA-4/CD80/86-dependent manner, resulting in the catabolism of tryptophan into pro-apoptotic compounds and the inhibition of effector T-cell function (Mellor and Munn, 2004). *TGFB1* polymorphisms also affect IDO (Raitala *et al.*, 2007).

In addition, it has been shown that interaction of naive T cells with the programmed cell death ligand 1 (B7-H1) on gastric epithelial cells favoured their development into CD25⁺FOXP3⁺ Tregs. *H. pylori* stimulates increased expression of B7-H1 (Beswick *et al.*, 2007), possibly contributing to increased Tregs in the gastric mucosa.

Many other functional mechanisms for the inhibition or killing of effector cells have been attributed to Tregs (see Table 6.1), including metabolic disruption, perforin- and granzyme-mediated killing, IL-2 sequestration and secretion of IL-35 (reviewed by Vignali, 2008). However, as yet, there is no data on their involvement in *H. pylori* immune responses.

6.5.4 Regulatory T cells and associations with *H. pylori* disease

We have previously reported that low numbers of Tregs in the human gastric mucosa are associated with increased Th1 responses, inflammation and the occurrence of peptic ulcer disease in adults (Robinson *et al.*, 2008), indicating that Tregs may protect against ulcer development. In contrast, another group showed that elevated Tregs in the duodenum are linked with the presence of duodenal ulcers (Stromberg *et al.*, 2003). There are several differences between the two studies. In Robinson *et al.* (2008), data from patients with gastric or duodenal ulceration were combined together into one group and compared with samples from patients without ulcers. Treg responses during gastric and

duodenal ulceration were not significantly different, but the group sizes were small. In addition, gastric CD4⁺CD25^{high} cells were quantified by flow cytometry, where levels of CD25 staining were higher than in activated CD4⁺CD25⁺ cells. In contrast, Stromberg *et al.* (2003) quantified CD4⁺CD25⁺ cells, which would have included activated effector T cells with intermediate CD25 expression as well as Tregs. In this study, higher levels of CTLA-4⁺ cells were also detected, which is indicative of a Treg response. In children, where *H. pylori*-induced pathology is much less severe, Harris *et al.* (2008) showed that the Treg response is of a higher level than that observed in adults. This may confirm that Tregs are protective against disease; however, firm conclusions are difficult from these sorts of association studies. There may be differences in the populations studied and also in sampling and analysis methods. Animal models do not tend to result in ulceration, which means that it is difficult to show whether Tregs prevent peptic ulcer disease or not.

However, mouse infection studies have firmly demonstrated that Tregs can reduce gastric inflammatory responses and this has been confirmed by co-culturing human gastric Tregs with gastric epithelial cells *in vitro* (Stromberg *et al.*, 2005). Because the development of gastric adenocarcinoma is strongly linked with a chronic inflammatory response, it would seem likely that Tregs are protective against this malignancy. Moreover, Tregs have been shown to prevent the induction of colonic adenocarcinoma in a mouse model by suppressing the *Helicobacter hepaticus*-induced inflammatory response (Erdman *et al.*, 2003). One suggestion for the wide variation in the worldwide incidence rates of gastric cancer is variability in the cellular immune response mounted to *H. pylori* infection. This could be affected by immune responses generated against other commonly occurring infections. For example, in mice, a concurrent intestinal helminth infection modulated the Th1 response to *H. felis* and reduced gastric premalignant pathology (Fox *et al.*, 2000). Many intestinal helminths induce strong Treg responses and are now being tested as anti-inflammatory therapeutics (Maizels and Yazdanbakhsh, 2008).

Increased levels of Tregs have been detected in the blood and tumour tissues of patients with different types of cancer (including gastric), and these responses are associated with advancing cancer progression and a poor prognosis (Sasada *et al.*, 2003; Kono *et al.*, 2006; Miller *et al.*, 2006). Some tumours secrete factors that expand Treg numbers and thus inhibit antitumour immunity (Ghiringhelli *et al.*, 2006). Increased numbers of FOXP3⁺ Tregs have been found in gastric adenocarcinoma tissue compared with surrounding normal tissue (Enarsson *et al.*, 2006). Also, systemic and mucosal Tregs from patients with gastric adenocarcinoma produced high levels of IL-10, which suppresses both the antitumour cytotoxic T-cell response in the stomach (Lundin *et al.*, 2007) and the transendothelial migration of T cells in a cell contact-mediated manner (Enarsson *et al.*, 2007).

We hypothesize that a strong Treg response is initially beneficial in inhibiting inflammation and carcinogenesis. However, if a tumour subsequently develops, then protective immune mechanisms are suppressed leading to more rapid cancer progression. This poses a problem if Tregs were to be targeted in an immunomodulatory strategy to prevent *H. pylori* disease.

6.6 *H. pylori*-mediated Immunomodulation of Other Diseases

6.6.1 Allergy

A number of epidemiological studies have found a protective association between *H. pylori* infection and the incidence of atopy, allergic rhinitis, dermatitis and asthma (Kosunen *et al.*, 2002; Pessi *et al.*, 2005; Chen and Blaser, 2007, 2008; Seiskari *et al.*, 2007; see Mitchell *et al.*, Chapter 5, this volume). A potential role for *H. pylori* has been proposed, whereby childhood exposure to certain micro-organisms (including *H. pylori*) is needed for healthy development of the immune system (Rook and Brunet, 2005). The typical age of acquisition of this infection (during early childhood) coincides with the common age

for asthma onset. Indeed, stronger protective associations against childhood rather than adult asthma have been reported (Chen and Blaser, 2007). Higher IL-10 responses have been found in younger children with *H. pylori*, which may perhaps protect against allergy (Oderda *et al.*, 2007). Higher levels of *IL10* mRNA and a stronger IL-10⁺ Treg response have been found in CagA⁺ compared with CagA⁻ human gastric tissue samples (Hida *et al.*, 1999; Robinson *et al.*, 2008), perhaps explaining the stronger protective effects associated with CagA⁺ infections (Chen and Blaser, 2007). As increased numbers of Tregs are present in the blood of *H. pylori*-infected patients (Kenefick *et al.*, 2007; Wang *et al.*, 2007), these cells could play a general immunosuppressive role.

As *H. pylori* infection induces a strong Th1 response (Bamford *et al.*, 1998), any protective effects of these bacteria against asthma could potentially be mediated by skewing the Th subset balance away from Th2. Infection with *cagA*⁺ strains is reported to elicit increased human Th1 (Hida *et al.*, 1999) and reduced Th2 responses (Orsini *et al.*, 2003). One recent study found that the presence of *H. pylori* was associated with reduced Th2 responses and reduced risk of atopy (Cam *et al.*, 2009). Significantly, administration of purified HP-NAP, a Th1-promoting *H. pylori* virulence factor, has been shown to inhibit allergy in a mouse model (Codolo *et al.*, 2008).

6.6.2 Autoimmunity

Several groups have shown a significantly lower prevalence of *H. pylori*-specific antibodies among people with autoimmune diseases such as multiple sclerosis (Wender, 2003; Li *et al.*, 2007) and systemic lupus erythematosus (Sawalha *et al.*, 2004). This suggests that *H. pylori* infection may also protect against, or attenuate, autoimmune diseases. Indeed, it has even been suggested that *H. pylori*-containing nanoparticles could be used as a treatment for multiple sclerosis (Pezeshki *et al.*, 2008). As yet there is no information regarding the mechanisms behind these protective associations, but Tregs are

likely to be good candidates because they are key suppressors of autoimmune reactions.

6.7 Conclusion

Gaining a better understanding of the immune response to *H. pylori* is important. The recent discovery of new types of T cells and the phenomenon of T-helper subset plasticity means that many long-established dogmas now need revision. Detailed knowledge of the immune mechanisms required for clearance of the infection is vital to underpin effective vaccine development. The need for vaccines is growing, with increasing prevalence of antibiotic-resistant strains, especially in countries where gastric cancer is common (Wong *et al.*, 2003).

Investigating the balance between the Treg response and proinflammatory Th1 or Th17 subsets may also reveal new indicators for who is most at risk of peptic ulceration or gastric cancer as a consequence of their infection. Only 10–15% of infected individuals have symptomatic disease, and infection may mediate protection against other immune and inflammatory diseases. Humans have co-evolved with *H. pylori* and are normally meant to have these bacteria in the stomach. It is possible that a *Helicobacter*-free person is missing various immunological stimuli needed for good health. Thus, a targeted approach to *H. pylori* management, focusing on people at greatest risk of developing *H. pylori*-associated disease, would appear sensible.

Many key questions remain. There have been no interventional human or animal studies to prove whether *H. pylori* protects against allergy and autoimmunity. If present, it is also unknown whether such effects would be diminished following *H. pylori* eradication. Further into the 21st century, it will be interesting to see whether *H. pylori* preparations can be used to treat or prevent such conditions. *H. pylori*-induced Treg responses may also potentially impact upon the disease sequelae of other infections, or even the effectiveness of some vaccines. It has been reported that a concurrent *H. pylori* infection can suppress virus-specific CD8⁺ T cells and

prevent clearance of a viral infection in mice (Shirai *et al.*, 1998), most likely via the stimulation of Tregs. We know that Tregs inhibit the efficacy of *H. pylori* vaccination (Zhang, M. *et al.*, 2008); perhaps there are also deleterious effects on other immunizations that have not been identified. Indeed, a recent study in Bangladesh showed that intestinal parasite infections are responsible for some cholera vaccine failures (Harris *et al.*, 2009). These issues, coupled with the fact that Tregs can increase the rate of cancer progression, indicate that immunity and the immunoregulatory responses to *H. pylori* should now be investigated in more detail. This will not only provide information on how to manage *H. pylori*-associated conditions, but could also have an important impact on extragastric diseases.

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7 Host Genetic Factors in Susceptibility and Resistance to *Helicobacter pylori* Pathogenesis

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7.1 Introduction

In the years following the discovery of *Helicobacter pylori* as a common infection of the human stomach, there was great controversy regarding whether this infection was responsible for a range of disease states, including peptic (duodenal and gastric) ulcers and stomach cancer. A major reason for this controversy was the diverse pathologies that appeared to be associated with this infection. It was hard to understand why many individuals infected with *H. pylori* would be asymptomatic, while others would develop ulcers and yet others cancer. The fact that the majority of infected persons do not develop disease remains a key argument for those sceptical of a role for *H. pylori* in the aetiology of these diseases.

Over the last 20 years, however, the reason for the diversity of disease outcomes resulting from *H. pylori* infection has started to become clearer. Our understanding now indicates that the development of symptomatic disease in response to *H. pylori* is a result of the chronic inflammation that is typically present for decades following infection during childhood (see Malaty, Chapter 1, this volume). The site of inflammation is particularly important. Persons who develop *Helicobacter*-associated gastritis in the acid-secreting corpus region of the stomach are

susceptible to developing gastric ulcers, as well as the most serious consequence of *H. pylori* infection, namely gastric adenocarcinoma (see Correa and Piazuelo, Chapter 3, this volume). The most important feature in development of this cancer is believed to be the loss of acid-secreting cells due to tissue changes resulting from chronic inflammation of the gastric corpus. In contrast, individuals who develop inflammation of the antrum and/or duodenum are prone to duodenal ulcers and, as the inflammation does not damage the acid-secreting regions of the stomach, appear protected against developing gastric adenocarcinoma.

Another key feature that appears to play an important role in determining which *H. pylori*-positive individuals will develop disease is the severity of the inflammation. The greater the severity of the inflammation resulting from *H. pylori* infection, the more chance there is for an associated disease to occur. It is logical that more severe inflammation increases the risk of tissue ulceration; similarly more severe inflammation in the corpus means more chance of destructive loss of acid secretion, thus increasing susceptibility to cancer progression.

A key question is what dictates the severity of *Helicobacter*-associated inflammation? Why is there a high prevalence of *H. pylori* infection but a low incidence of gastric cancer

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in India and Thailand, while Japan and some areas of China suffer much greater incidences of this malignancy (Singh and Ghoshal, 2006)? This is also not a simple matter, as it is clearly a result of a complex interaction of three broad areas (Fig. 7.1). Environmental factors, such as smoking, a high-salt diet and a low intake of fresh fruit and vegetables, clearly play a role. Bacterial genetic factors are certainly extremely important in pathogenesis and there is a quite remarkable degree of diversity in important *H. pylori* virulence factors (see Backert *et al.*, Chapter 11, this volume). The third area is that of host genetic factors, i.e. variability in genes of the infected host that make an individual more likely to develop *Helicobacter*-associated disease. That is the subject of this chapter.

Information regarding the importance of host genetic factors has been obtained predominantly from two sources. A large number of studies have examined the association between human gene polymorphisms (i.e. differences in gene sequence that alter the functional activity of encoded proteins when expressed) and susceptibility to *H. pylori* infection and/or associated diseases, in particular gastric cancer. These findings are supported by observing the severity of inflammation and disease progression following *Helicobacter* infection of genetically modified

mice that are deficient for key host genetic factors.

7.2 Human Polymorphism Studies Indicate that Host Variability in Immune-related Genes can Impact upon Susceptibility to *H. pylori*-associated Pathologies

Strong associations have been made between a number of human gene polymorphisms and susceptibility to *Helicobacter*-associated disease. However, this can be extremely complicated as there is also ethnic variation, with genes that appear to be of considerable significance in Caucasians being possibly irrelevant in an Asian population, and vice versa. The majority of the important host factors identified involve gene products that regulate or impact upon the host immune response to infection, in particular cytokines, as well as receptors of the host immune system that detect the presence of pathogens (Toll-like and nucleotide-binding oligomerization domain-like receptors). Other factors, such as mucins that limit access of the bacteria to the stomach surface, may also play an important role. A summary of known associations between human gene polymorphisms and *H. pylori* infection and disease state is presented in Table 7.1 and Fig. 7.2. The potentially most important findings are discussed further in the following text. It should be remembered that the statistical power of studies involving small numbers of patients is limited, and in these cases false negative associations between particular gene polymorphisms and disease are possible.

7.2.1 Cytokines produced by T-helper lymphocytes

The T-helper lymphocyte (Th cell) is the conductor of the host acquired (specific and memory) immune response to infection. This is achieved by the secretion of a range of cytokines that impact heavily upon and control the activities of other immune cells, in particular those involved in the inflammatory response to infection. Based on the cytokine

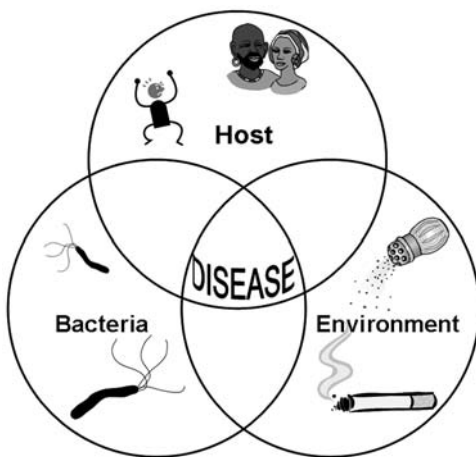


Fig. 7.1. Multiple factors influence whether an individual infected with *Helicobacter pylori* will develop an associated disease.

Table 7.1. Some human polymorphisms associated with *Helicobacter pylori* pathogenesis.

Gene (polymorphism)	Association with <i>H. pylori</i> pathogenesis	Key references
<i>IFNGR</i> (<i>IFNGR1</i> -56)	Increased risk of gastric cancer in Portuguese	Canedo <i>et al.</i> (2008b)
<i>HLA</i> (<i>DQA</i> *1012)	Reduced risk of pathology in Japanese	Azuma <i>et al.</i> (1998); Yoshitake <i>et al.</i> (1999)
<i>IL1B</i> (-31, -511, <i>RN</i> *2)	Significant risk of gastric ulcers and gastric cancer; effects more obvious in Caucasian rather than Asian populations	El-Omar <i>et al.</i> (2000, 2003); Xuan <i>et al.</i> (2005)
<i>TNFA</i> (-308, -857)	Increased risk of gastric ulcers and gastric cancer in Caucasian and some Asian populations	El-Omar <i>et al.</i> (2003); Machado <i>et al.</i> (2003); Sugimoto <i>et al.</i> (2007)
<i>IL6</i> (-174)	Increased risk of gastric cancer in Brazilians, but not in Korean or Japanese populations	Hwang <i>et al.</i> (2003); Gatti <i>et al.</i> (2007)
<i>IL8</i> (-251)	Increased risk of intestinal metaplasia and gastric cancer in Asian, but not Caucasian populations	Taguchi <i>et al.</i> (2005); Canedo <i>et al.</i> (2008a); Kim <i>et al.</i> (2008)
<i>IL10</i> (-1082, -819, -592)	Unclear; possible increased risk of gastric cancer due to hyperinflammatory response	El-Omar <i>et al.</i> (2003); Garcia-Gonzalez <i>et al.</i> (2007)
<i>TGFB</i> (+509, +869, +905)	Reduced risk of duodenal ulcers in Caucasians, but no association with gastric cancer	Garcia-Gonzalez <i>et al.</i> (2006)
<i>TLR4</i> (+299)	Unclear; possible reduced risk of MALT lymphoma in Caucasians	Hellmig <i>et al.</i> (2005a); Hofner <i>et al.</i> (2007); Trejo-De La <i>et al.</i> (2008)
<i>CD14</i> (-260)	Increased risk of gastric cancer and intestinal metaplasia	Zhao <i>et al.</i> (2007)
<i>TLR2</i> (-196)	Unclear; possible increased risk of gastric cancer in Japanese	Tahara <i>et al.</i> (2007b); Moura <i>et al.</i> (2008)
<i>NOD1</i> (G796A)	Increased risk of duodenal ulcer in Caucasians; unknown in other populations	Hofner <i>et al.</i> (2007)
<i>MUC1</i> (short allele)	Increased risk of gastric adenocarcinoma and gastritis in Caucasians; unknown in others	Carvalho <i>et al.</i> (1997); Vinall <i>et al.</i> (2002)
<i>COX1</i> (-1676)	Associated with gastric ulcers and functional dyspepsia in Japanese	Arisawa <i>et al.</i> (2007a, 2008a)
<i>COX2</i> (-1195, -765)	Gastric cancer in Chinese (-1995) and gastric cancer and peptic ulcers in Indians (-765)	Liu <i>et al.</i> (2006); Saxena <i>et al.</i> (2008)
<i>MDR1</i> (+3435)	Contradictory reports on <i>MDR1</i> polymorphisms and gastric cancer in Japanese	Tahara <i>et al.</i> (2007a); Sugimoto <i>et al.</i> (2008)
<i>MBL</i> (+54)	Increased risk of gastric cancer in Japanese but no association with gastritis in Polish	Bak-Romaniszyn <i>et al.</i> (2006); Wang <i>et al.</i> (2008)
<i>MIF</i> (-173, -794)	Increased risk of mucosal atrophy in Japanese	Arisawa <i>et al.</i> (2008b)
<i>IL2</i> (-330)	Increased risk of atrophic gastritis in Japanese but not Koreans	Togawa <i>et al.</i> (2005); Shin <i>et al.</i> (2008)
<i>IL17</i> (+7488, -197A)	Increased inflammation during <i>H. pylori</i> infection, and increased risk of gastric cancer in Japanese	Arisawa <i>et al.</i> (2007b); Shibata <i>et al.</i> (2009)
<i>IL18</i> (-137, -607)	Increased severity of gastritis in Japanese with possible effects on <i>H. pylori</i> eradication	Leung <i>et al.</i> (2006); Sakai <i>et al.</i> (2008)

MALT, mucosa-associated lymphoid tissue.

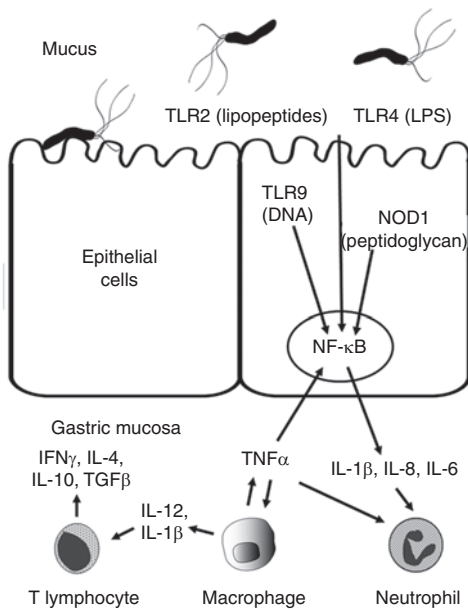


Fig. 7.2. Components of the immune response to *Helicobacter pylori* infection where host variability may influence disease outcome. Bacterial components (shown in parentheses) potentially trigger a range of host receptors to drive an inflammatory response. Variability in host immune receptors or cytokines can influence the severity of the resulting inflammation and disease sequelae. Abbreviations: IFN γ , interferon- γ ; IL, interleukin; LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; NOD, nucleotide-binding oligomerization domain; TGF β , transforming growth factor- β ; TNF α , tumour necrosis factor- α ; TLR, Toll-like receptor.

profiles produced, Th cells are separated into distinct subpopulations. For example, Th1 cells produce proinflammatory cytokines including interferon- γ (IFN γ) and interleukin (IL)-12, while Th2 cells produce cytokines including IL-4 that suppress inflammation. More recently, Th17 cells (producing IL-17) have risen in prominence as important mediators of *H. pylori*-driven inflammation (Shiomi *et al.*, 2008; DeLyria *et al.*, 2009). The importance of Th cells in *Helicobacter* pathogenesis is reviewed in detail elsewhere (see Robinson and Atherton, Chapter 6, this volume). Here, we discuss how variability in host genes for T-helper cytokines may contribute to selecting individuals who progress to *Helicobacter*-associated pathologies.

Interferon- γ

IFN γ is an important cytokine, produced primarily by Th1 cells, that is a key mediator of the inflammatory Th1 immune response. It has been shown to be crucial for *H. pylori*-mediated immunopathology, which is dominated by IFN γ -producing Th1 cells in the gastric mucosa (Bamford *et al.*, 1998). Studies using knockout mice have shown that, in the absence of IFN γ , *Helicobacter* gastric colonization is significantly increased but pathology scores are significantly decreased compared with wild-type mice (Eaton *et al.*, 2001; Akhiani *et al.*, 2002). While polymorphisms in the human *IFNG* gene (i.e. +874 (A/T)) have been found to increase cytokine production, studies investigating the significance of *IFNG* polymorphisms in German, Italian and Japanese populations have found no association with *H. pylori*-induced disease, including gastric cancer (Rad *et al.*, 2004; Zamboni *et al.*, 2005; Seno *et al.*, 2007).

The strongest associations with regard to *Helicobacter* pathogenesis have been made with the receptors of this cytokine (IFNGR1 and IFNGR2). Whole-genome scanning of Senegalese patients found the polymorphisms in *IFNGR1* were strongly associated with high levels of anti-*H. pylori* serum antibodies (Thye *et al.*, 2003). While a -56 (C/T) polymorphism potentially led to loss of receptor function, -1004 (A/C) and -1400 (T/C) polymorphisms both resulted in amino acid substitutions with the potential to affect IFNGR1 signalling (Thye *et al.*, 2003; Canedo *et al.*, 2008b). Further, a study in Portuguese patients found that the *IFNGR1* -56 polymorphism was significantly associated with increased risk of gastric cancer. A polymorphism in *IFNGR2* has been associated with increased risk of gastric cancer in Polish subjects, with the highest risk present in those carrying both the *IFNGR2* and *TNFA* -308 polymorphisms (Hou *et al.*, 2007).

Interleukin-4

IL-4, the key mediator of the Th2 immune response, is anti-inflammatory due to its inhibition of IFN γ and IL-2 production by

Th1 cells (Tanaka *et al.*, 1993). During *Helicobacter* infection, IL-4 levels decrease in the human gastric mucosa (Orsini *et al.*, 2003). While most studies have found no association between *IL4* polymorphisms and *H. pylori*-induced disease in Caucasian (El-Omar *et al.*, 2003; Garcia-Gonzalez *et al.*, 2007; Zambon *et al.*, 2008) and Japanese (Togawa *et al.*, 2005) patients, one study did find that Japanese carriers of both *IL4* -984/-2983 AA/GA were significantly protected against gastric cancer (Seno *et al.*, 2007), although the mechanism of protection is unknown.

Major histocompatibility complex antigens

Major histocompatibility complex (MHC) antigens are critical components of the acquired immune response to infection and MHC loci are among the most genetically variable in humans, with >1500 MHC class I allele and >5000 MHC class II allele combinations (Marsh *et al.*, 2005).

As MHC class II antigens (HLA-DR, HLA-DP and HLA-DQ) are used by antigen-presenting cells (such as macrophages and dendritic cells) to present antigen to and activate Th cells, variability in these alleles can significantly influence the host immune response to infection. Therefore, MHC class II alleles were among the first components of the host response to *Helicobacter* infection to be examined. These studies revealed an ethnic bias in the importance of human leucocyte antigen (HLA) type on the host response to *Helicobacter*, with the most pronounced associations being observed in the high-risk Japanese population.

A number of Japanese studies have found that *HLA-DQA*0102*, *HLA-DQB1*0601* and *HLA-DRB1*1502* are associated with a significantly reduced risk of *H. pylori*-induced atrophic gastritis, duodenal ulcer, gastric ulcer and gastric adenocarcinoma (Azuma *et al.*, 1998; Yoshitake *et al.*, 1999; Hirata *et al.*, 2007), although a similar study found that *HLA-DQB*0601*, along with *HLA-DQA*0103*, conferred a significantly increased risk of *H. pylori*-associated mucosa-associated lymphoid tissue (MALT) lymphoma (Kawahara *et al.*, 2005), suggesting that the *HLA-DQB*0601* allele can affect different

pathological outcomes. Other *HLA* alleles have been associated with an increased risk of *H. pylori* disease outcomes in the Japanese. *HLA-DQA*0301* was significantly lower in uninfected controls compared with duodenal ulcer patients (Azuma *et al.*, 1995) and *HLA-DQB1*0401* was significantly higher in atrophic gastritis patients (Sakai *et al.*, 1999), both indicating an increased risk of disease. *HLA-DRB1*04051* has also been linked with increased risk of gastric cancer in the Japanese, although this was not associated with *H. pylori* infection (Ohtani *et al.*, 2003).

In contrast, however, while associations have been made between *HLA-DQB1*0301* and gastric cancer in American Caucasians (Lee *et al.* 1996), *HLA-DQA1*0503* and gastric cancer in Mexicans (Garza-Gonzalez *et al.*, 2004), and *HLA-DQB*0602* and gastric cancer in Spanish Caucasians (Quintero *et al.*, 2005), many studies on Caucasian patients have found no association between *HLA* molecules and gastric pathologies (Karhukorpi *et al.*, 1999; Magnusson *et al.*, 2001; Kunstmann *et al.*, 2002; Perri *et al.*, 2002). This has reinforced the belief that *HLA* molecules have a greater impact on host response to *Helicobacter* in the Japanese population compared with other ethnic groups.

7.2.2 Inflammation-modulating cytokines produced by cells of the innate immune system

Interleukin-1 β

The best studied and potentially most important host gene is that encoding the cytokine IL-1 β . IL-1 β is a proinflammatory cytokine, produced by macrophages and epithelial cells, that is up-regulated by *H. pylori* infection (Noach *et al.*, 1994; Jung *et al.*, 1997). It induces T- and B-lymphocyte activation, as well as the production of other proinflammatory cytokines (Dinarello and Savage, 1989). However, perhaps the most important feature of IL-1 β with regard to *Helicobacter* pathogenesis is its potent inhibition of gastric acid secretion, with reportedly 100-fold greater activity than proton pump inhibitors (Wolfe and Nompleggi, 1992; Beales and Calam, 1998). Low gastric acid production (hypo-

chlorhydria) is strongly associated with the development of gastric ulcers and gastric cancer. Suppression of gastric acid secretion by increased IL-1 β production has significant effects on cancerous changes in the stomach. With lower stomach acid levels, bacterial toxins and by-products of inflammation that would normally be destroyed are accumulated (some of which are known mutagens), and bacteria that normally find the stomach inhospitable are able to colonize, leading to increased production of carcinogenic bacterial by-products.

IL-1 β and its endogenous receptor antagonist IL-1R α are encoded by the related genes *IL1B* and *IL1RN*. Three polymorphisms in the human *IL1B* gene have been reported, all representing C–T base transitions at positions –511, –31 and +3954 base pairs from the transcriptional start site (El-Omar *et al.*, 2000). In addition, individuals who carry the less common allele 2 of the *IL1RN* gene (*IL1RN**2) have a prolonged response to inflammatory stimuli, and this polymorphism has been associated with a number of conditions including ulcerative colitis, Crohn's disease, alopecia, psoriasis and systemic lupus erythematosus (Witkin *et al.*, 2002). The association of *IL1B* polymorphisms and gastric cancer has been studied in a number of different human populations, with some variable results.

Numerous studies have found associations between *IL1B* polymorphisms and gastric cancer in populations of European and African origin: *IL1B* –31, *IL1B* –511 and *IL1RN**2 were independently associated with hypochlorhydria and increased frequency of atrophic gastritis, intestinal metaplasia and gastric cancer in *Helicobacter*-infected Scottish, Polish and German patients (El-Omar *et al.*, 2000; Rad *et al.*, 2003). While an increased risk of *H. pylori*-associated gastric cancer has been noted in American Caucasians with the *IL1B* –511 or *IL1RN**2 polymorphism (El-Omar *et al.*, 2003), a study of African American and Caucasian patients in the USA found that the *IL1B* +3954 polymorphism, but not *IL1B* –31, *IL1B* –511 and *IL1RN**2 polymorphisms, was associated with increased risk of *H. pylori*-dependent multi-atrophic gastritis (Zabaleta *et al.*, 2006). Mexicans with the *IL1B* –31 poly-

morphism alone or in combination with *IL1RN**2 appear to have an increased risk of *Helicobacter*-associated gastric cancer (Sicinschi *et al.*, 2006), while no association between *IL1B* polymorphisms and gastric cancer was found in Spanish Caucasian patients (Garcia-Gonzalez *et al.*, 2007). Two studies investigated *IL1B* polymorphisms in the Portuguese, who have a high incidence of *H. pylori* infection and gastric cancer. One found that *IL1B* –511 and *IL1RN**2 polymorphisms were independently associated with an increased risk of gastric cancer, with a substantial increase in gastric cancer risk in individuals carrying both polymorphisms (Machado *et al.*, 2001). A second found an increased risk of gastric cancer in patients with *IL1B* –511 and *IL1RN**2 polymorphisms, but not *IL1RN**2 alone (Figueiredo *et al.*, 2002). While studies in central Italy, Costa Rica and Oman (areas of high gastric cancer prevalence) found no association between *IL1B* –31 and *IL1B* –511 polymorphisms and gastric cancer, carriers of the *IL1RN**2 allele had an increased risk of gastric cancer (Alpizar-Alpizar *et al.*, 2005; Palli *et al.*, 2005; Al-Moundhri *et al.*, 2006).

The story in Asia appears more complicated. Increasing evidence suggests that *IL1B* polymorphisms are less important to gastric cancer development in Japanese populations, with no correlation between *IL1B* polymorphisms and expression of the IL-1 β cytokine in the stomach, the severity of *H. pylori*-induced inflammation, or atrophy (Kato *et al.*, 2001; Xuan *et al.*, 2005; Seno *et al.*, 2007; Sugimoto *et al.*, 2007). It has even been suggested that the *IL1B* –511 polymorphism may indicate less risk for gastric cancer in the Japanese (Ito *et al.*, 2007), although a different study found *H. pylori*-infected Japanese patients with the *IL1B* –511 polymorphism had higher gastric pH, associated with more widespread infection and more severe inflammation (Furuta *et al.*, 2002). Similarly in Korea (another country with a high prevalence of gastric cancer), studies have found no association between *IL1B* polymorphisms and *H. pylori*-induced pathologies, including gastric cancer (Chang *et al.*, 2008; Kim *et al.*, 2008; Shin *et al.*, 2008), with the exception of a possible link with *IL1RN**2 (Kim *et al.*, 2006). In contrast, however, many studies in China

reflect the Caucasian findings, with *IL1B* -511 and *IL1RN**2 polymorphisms associated with increased risk of *H. pylori*-induced pathologies, including gastric cancer (Zeng *et al.*, 2003; Chen *et al.*, 2004; Leung *et al.*, 2006; Li *et al.*, 2007; Feng *et al.*, 2008). One study from China did find that *IL1B* -511 polymorphisms did not influence gastric acid secretion in *H. pylori*-infected young healthy individuals, suggesting the association of this polymorphism with gastric cancer may be via an acid-independent mechanism (Hu *et al.*, 2005).

Overall, these observations indicate a strong ethnic effect on the relative importance of IL-1 β and *Helicobacter* pathogenesis, with associations between *IL1B* polymorphisms and gastric cancer depending upon the country and/or ethnic origin of the infected population. While the effect of IL-1 β on acid secretion is clearly important in some populations, this variability likely reflects the interaction of a range of factors in this disease process, including bacterial virulence factors (see Backert *et al.*, Chapter 11, this volume), lifestyle and environmental factors, as well as other host genes.

Tumour necrosis factor- α

Another proinflammatory cytokine up-regulated by *H. pylori* infection in humans is tumour necrosis factor- α (TNF α) (Crabtree *et al.*, 1991). Produced predominantly by macrophages in response to bacterial products, TNF α activates nuclear factor- κ B (NF- κ B) (a transcription factor that triggers production of a range of inflammatory mediators) in a range of immune and non-immune cells, as well as attracting other immune cells to sites of inflammation. TNF α also inhibits gastric acid production (Beales and Calam, 1998) and TNF α -deficient mice develop significantly less severe gastritis than wild-type controls after *H. pylori* infection (Yamamoto *et al.*, 2004), suggesting an important role for TNF α in *Helicobacter*-driven inflammation. Polymorphisms of the *TNFA* gene have been associated with increased susceptibility or severity of a number of conditions including malaria (McGuire *et al.*, 1994), Crohn's disease (Negoro *et al.*, 1999) and lymphoma (Fitzgibbon *et al.*, 1999).

The *TNFA* -308 polymorphism is associated with enhanced TNF α expression (Wilson *et al.*, 1997; Gonzalez *et al.*, 2003) and this polymorphism has been linked with an increased risk of peptic ulcers in German and Spanish Caucasians (Kunstmann *et al.*, 1999; Lanas *et al.*, 2001) and gastric cancer in Caucasian patients from Portugal, Poland and the USA (El-Omar *et al.*, 2003; Machado *et al.*, 2003; Hou *et al.*, 2007). However, other reports found no association between *TNFA* -308 polymorphism and increased cytokine production or increased *H. pylori*-induced pathology (Rad *et al.*, 2004; Garcia-Gonzalez *et al.*, 2007). Similarly, studies in Japanese and Korean patients found no association between *TNFA* -308 polymorphism and *H. pylori*-induced gastroduodenal disease (Kim *et al.*, 2006; Sugimoto *et al.*, 2007). These differences are supported by a meta-analysis of 24 independent studies, which found a statistically significant association between *TNFA* -308 polymorphism and gastric cancer only in Western populations (Gorouhi *et al.* 2008).

While the *TNFA* -857 polymorphism has been associated with peptic ulcers and B-cell lymphoma in Italian and German patients (Hellmig *et al.*, 2005b; Zamboni *et al.*, 2005), it was negatively associated with MALT lymphoma in Chinese patients (Wu *et al.*, 2004). *TNFA* -863 and *TNFA* -1031 polymorphisms have been associated with higher TNF α levels and increased risk of peptic ulcers in Chinese patients (Lu, C.C. *et al.*, 2005), and a study of *H. pylori*-infected Japanese found that these polymorphisms, along with *TNFA* -857, are independently associated with increased risk of gastric ulcers as well as gastric cancer, while simultaneous carriage of all three polymorphisms greatly increases risk of both pathologies (Sugimoto *et al.*, 2007).

Interleukin-12

IL-12 is a proinflammatory cytokine produced by dendritic cells and macrophages that induces IFN γ production and leads to the development of Th1 cells, the primary immune cell involved in *Helicobacter*-mediated gastritis (Delios *et al.*, 1997). While IL-12 is up-regulated in *H. pylori* infection (Hida *et al.*, 1999), IL-12 deficiency in mice has no effect

on *H. pylori* colonization or resulting pathology (Akhiani *et al.*, 2002).

IL-12 is a heterodimer, comprised of two subunits (IL-12p35 and IL-12p40) encoded by the genes *IL12A* and *IL12B*. Again, the importance of *IL12* polymorphisms is unclear, as one study in Italians identified *IL12* polymorphisms (*IL12A* -504 and *IL12B* VNTR 9/11) that were significantly associated with gastric cancer (Navaglia *et al.*, 2005), while studies in other European Caucasian (Garcia-Gonzalez *et al.*, 2007; Hou *et al.*, 2007) as well as Japanese (Seno *et al.*, 2007) populations found no association between *IL12* polymorphisms and risk of *H. pylori*-associated gastric cancer.

Interleukin-6

IL-6 is a multifunctional cytokine produced by many cells including T cells, macrophages and intestinal epithelial cells. It can act either to promote inflammation by inducing the production of acute-phase proteins such as C-reactive protein and serum amyloid A (Gabay, 2006), or as an anti-inflammatory cytokine by controlling the level of proinflammatory cytokines including TNF α , IFN γ and macrophage inflammatory protein 2 (MIP-2) (Xing *et al.*, 1998). A number of polymorphisms in the *IL6* gene have been identified; one notable finding was that the *IL6* -174 polymorphism can lead to increased IL-6 production, which has been linked with chronic arthritis (Fishman *et al.*, 1998).

IL-6 expression is up-regulated in the gastric mucosa of *Helicobacter*-infected patients (Crabtree *et al.*, 1991) and is also increased in early gastric cancer tissue (Yamaoka *et al.*, 2001). However the relevance of *IL6* polymorphisms to *Helicobacter* disease outcome is unclear. While one study in Brazil found an association between *IL6* -174 and gastric cancer (Gatti *et al.*, 2007), other studies found no association between this polymorphism and *Helicobacter* disease outcome in Caucasian (El-Omar *et al.*, 2003), Korean and Japanese (Hwang *et al.*, 2003) populations.

Interleukin-8

IL-8 is a chemokine produced primarily by macrophages, but also by other cells (includ-

ing bacterial-activated epithelial cells), that plays an important role in attracting neutrophils to sites of inflammation/infection, and activating them to release by degranulation a range of antibacterial and proinflammatory mediators (Ogra, 1999). IL-8 is up-regulated after *H. pylori* infection (Crabtree *et al.*, 1994) and is potentially the most important cytokine produced by the host in response to *H. pylori* infection. The infiltration of neutrophils into the stomach mucosa in response to *H. pylori* infection (termed 'active' gastritis) is associated with more severe disease outcomes.

A single polymorphism in the *IL8* gene, at position -251, is associated with increased IL-8 production (Taguchi *et al.*, 2005). A number of studies have suggested that this *IL8* polymorphism plays an important role in *Helicobacter* pathogenesis in Asian populations. The clearest association occurs in Japanese populations, with *IL8* -251 being associated with increased risk of atrophic gastritis, gastric cancer and ulcers (Ohyauchi *et al.*, 2005; Taguchi *et al.*, 2005). In Korean and Chinese populations, the *IL8* polymorphism has also been associated with an increased risk of gastric cancer, but not intestinal metaplasia or atrophic gastritis (Lee *et al.*, 2005; Lu, W. *et al.*, 2005; Leung *et al.*, 2006; Kim *et al.*, 2008; Kang *et al.*, 2009; Ye *et al.*, 2009).

The effect of this polymorphism on *Helicobacter* infection in non-Asians is unclear. While studies found an association between *IL8* -251 and increased risk of duodenal ulcers and gastritis in Hungarian patients (Gyulai *et al.*, 2004; Hofner *et al.*, 2007) and gastric cancer in Mexicans (Garza-Gonzalez *et al.*, 2007), no association was found with gastric cancer risk in Finnish (Kamangar *et al.*, 2006) or Portuguese (Canedo *et al.*, 2008a) subjects. This suggests the effect of the *IL8* polymorphism may be ethnic specific.

Interleukin-10

Anti-inflammatory IL-10 is produced by a wide range of cells including monocytes, macrophages, mast cells, T and B lymphocytes, regulatory T cells and dendritic cells (Mosser and Zhang, 2008). IL-10 is a potent inhibitor of antigen presentation (limiting the acquired

immune response) as well as dendritic cell activation and maturation, thereby suppressing production of a range of important inflammatory cytokines including IL-1, IL-6, IL-12 and TNF α (Mosser and Zhang, 2008). IL-10 is up-regulated by *Helicobacter* infection in man (Karttunen *et al.*, 1997) and given its wide-ranging effects on mediators of inflammation, it is perhaps not surprising that *Helicobacter* infection of IL-10-deficient mice results in increased severity of gastritis (Matsumoto *et al.*, 2005).

Three main polymorphisms in the *IL10* promoter have been identified (-1082 (G/A), -819 (C/T) and -592 (C/A)), which combine to form three main haplotypes: GCC (associated with increased IL-10 production), ACC and ATA (associated with reduced IL-10 production) (Eskdale *et al.*, 1999; Rad *et al.*, 2004). Once again, there are conflicting reports regarding *IL10* polymorphisms and *Helicobacter* infection. One study found that American Caucasians with the *IL10* ATA haplotype had an increased risk of *H. pylori*-induced gastric cancer (El-Omar *et al.*, 2003). It is hypothesized that people carrying this low IL-10-producing haplotype are at increased risk of gastric cancer due to the increased inflammatory response resulting from reduced levels of this protective cytokine. *IL10* polymorphisms have also been associated with increased risk of gastric cancer and intestinal metaplasia in Mexican and Korean patients (Sicinschi *et al.*, 2006; Kim *et al.*, 2008), gastritis in Indian patients (Achut *et al.*, 2008) and gastric cancer in a Chinese population (Lu, W. *et al.*, 2005).

However, an equal number of studies have failed to find any association between *IL10* polymorphisms and increased risk of *H. pylori*-induced pathology, including atrophic gastritis and gastric cancer in European (Rad *et al.*, 2004; Hellmig *et al.*, 2005c; Garcia-Gonzalez *et al.*, 2007; Forte *et al.*, 2008), Chinese (Leung *et al.*, 2006) and Japanese (Saijo *et al.*, 2007; Seno *et al.*, 2007) patients.

Transforming growth factor- β

Transforming growth factor (TGF β) is produced in three isoforms by many different cell types, including monocytes/macrophages,

dendritic cells and lymphocytes. TGF β is a pleiotropic cytokine with potent immunoregulatory properties, inhibiting both antigen presentation (Takeuchi *et al.*, 1998) and lymphocyte proliferation (Letterio and Roberts, 1998; Gorelik and Flavell, 2000). It is chemotactic for monocytes, from which it can induce cytokine secretion. The role of TGF β in regulatory T-cell development and function is complex, with TGF β having been shown to be required for the induction, development and enhancement of regulatory T-cell function (Fantini *et al.*, 2004). Enhanced expression of TGF β has been observed in patients with gastric cancer and gastric ulcers (Ebert *et al.*, 2000; Milani and Calabro, 2001) and administration of TGF β can accelerate the healing of experimental gastric ulcers in animals (Ernst *et al.*, 1996), suggesting TGF β may play an important role in *H. pylori* pathogenesis.

Three main polymorphisms in the *TGFB1* gene have been identified at +509, +869 and +915, the latter two leading to amino acid substitutions. A study on Spanish Caucasians found that the +869 polymorphism was associated with reduced risk of duodenal ulcer (Garcia-Gonzalez *et al.*, 2006), and a second study on Russian Caucasians supported this by showing that carriage of all three *TGFB1* polymorphisms was significantly reduced in those with duodenal ulcer (Polonikov *et al.*, 2007). This cytokine appears to be most important in ulcer development, as studies on Caucasian and Japanese populations have found no association between *TGFB1* polymorphisms and gastric cancer (Garcia-Gonzalez *et al.*, 2007; Seno *et al.*, 2007). However, one Chinese study found that polymorphisms in *TGFB1* +509 and the TGF β receptor *TGFBRII* were associated with a decreased risk of gastric cancer (Jin *et al.*, 2007).

7.2.3 Impact of host variability in pathogen recognition receptors on *H. pylori* pathogenesis

The mammalian immune system possesses a range of pathogen detection receptors, including Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD)

family members (see Kaparakis *et al.*, Chapter 8, this volume). When activated, these receptors trigger an immune response to combat these infections, typically via the production of proinflammatory cytokines.

Toll-like receptor 4 and CD14

TLR4, expressed on the surface of many cells including monocytes/macrophages, dendritic cells and gastrointestinal epithelial cells, associates with CD14 (mainly on macrophages) and MD2 to form a receptor that detects lipopolysaccharide from Gram-negative bacteria. While *H. pylori* infection can up-regulate TLR4 expression (Asahi *et al.*, 2007), the role of TLR4 in *Helicobacter* pathogenesis is controversial. While some *in vitro* studies found *H. pylori* can activate TLR4 on gastric cell lines as well as primary human cells (Su *et al.*, 2003; Alvarez-Arellano *et al.*, 2007), others found no TLR4 expression or activation in similar studies (Backhed *et al.*, 2003; Smith *et al.*, 2003). Results from *in vitro* experiments using mouse cells are similarly unclear (Mandell *et al.*, 2004; Obonyo *et al.*, 2007), although *Helicobacter felis* infection of mice with non-functional TLR4 did not induce gastritis (Sakagami *et al.*, 1997), suggesting TLR4 may be important for *Helicobacter*-induced inflammation.

Several polymorphisms of human *TLR4* have been identified, including an Asp/Gly replacement at +299 that has been shown to attenuate receptor signalling and diminish inflammation in certain disease models (Lorenz *et al.*, 2002; Schmitt *et al.*, 2002) and another at +399. Both of these polymorphisms have been associated with altered production of a range of inflammation-modulating cytokines in response to *Helicobacter* infection, including IL-1 β , TNF α , IL-10 and IL-8 (Trejo-De La *et al.*, 2008). However, while a study of German and Austrian Caucasian patients found the *TLR4* +299 polymorphism was significantly associated with a reduced risk of MALT lymphoma (Hellmig *et al.*, 2005a), no similar association was found in Hungarian (Hofner *et al.*, 2007), Venezuelan (Kato *et al.*, 2007), Mexican (Garza-Gonzalez *et al.*, 2007) or Indian (Achyut *et al.*, 2007) patients. Also, while a polymorphism at *TLR4*

+399 was associated with atrophy and metaplasia in an Indian population (Achyut *et al.*, 2007), it was not associated with disease outcome in Hungarian, Mexican or American populations (Hofner *et al.*, 2007; Trejo-De La *et al.*, 2008; Murphy *et al.*, 2009).

Two polymorphisms in the *CD14* gene have been identified (positions -159 and -260). In Taiwanese, Japanese and Caucasian patients, *CD14* -159 was not associated with gastric malignancy or dyspepsia (Wu *et al.*, 2006; Tahara *et al.*, 2008a; Hold *et al.*, 2009) but was associated with a marginally increased risk of MALT lymphoma in Turkish patients (Türe-Ozdemir *et al.*, 2008). However, the *CD14* -260 polymorphism has been associated with an elevated risk of gastric carcinoma in Chinese (Zhao *et al.*, 2007) and intestinal metaplasia in Venezuelan (Kato *et al.*, 2007) patients.

Toll-like receptor 2

TLR2 is a cell surface receptor that recognizes a range of ligands, including bacterial glycolipids, lipopeptides and lipoproteins. Expression of TLR2 is widespread on immune cells, including monocytes/macrophages, dendritic cells, B cells and subsets of T cells, but is not believed to be expressed on the normal gastric epithelium. *In vitro* studies have shown that *Helicobacter* can activate TLR2 on cell lines and primary immune cells from mice and human subjects (Smith *et al.*, 2003; Mandell *et al.*, 2004; Alvarez-Arellano *et al.*, 2007). A Japanese study found that a 22 bp deletion (-196 to -174) in the *TLR2* gene that can affect promoter activity was present at a significantly higher frequency in gastric cancer patients (Tahara *et al.*, 2007b), and was significantly associated with intestinal metaplasia in females and those over 60 years of age. However, no association was found between *TLR2* polymorphisms and peptic ulcers in Japanese and Brazilian subjects (Moura *et al.*, 2008; Tahara *et al.*, 2008b).

Nucleotide-binding oligomerization domain-1/2

Intracellular NOD receptors sense intracellular bacteria and bacterial products. NOD1 is

expressed in gastric epithelial cells, as well as macrophages and dendritic cells (Hofner *et al.*, 2007) and recognizes a mucopeptide found in peptidoglycan from Gram-negative bacteria (Girardin *et al.*, 2003a). NOD2 is expressed in intestinal epithelial cells including Paneth cells and monocytes and recognizes a different mucopeptide (muramyl dipeptide) found in all bacteria (Girardin *et al.*, 2003b). *Helicobacter* peptidoglycan introduced into gastric epithelial cells by a type IV secretion system (*cag* pathogenicity island) is recognized by NOD1, inducing a signal cascade ultimately resulting in activation of the important transcription factor NF- κ B and the production of key proinflammatory cytokines (Viala *et al.*, 2004). Both NOD1 and NOD2 are up-regulated in the gastric epithelium by *H. pylori* infection (Rosenstiel *et al.*, 2006).

Few studies have evaluated NOD polymorphisms and *Helicobacter*-associated disease. Two studies in Caucasian patients found no association between NOD1 polymorphism and gastritis and gastric ulcers, but one did find a significant association between NOD1 G796A and duodenal ulcers (Rosenstiel *et al.*, 2006; Hofner *et al.*, 2007). The NOD2 polymorphism R702W (previously associated with increased risk of Crohn's disease; Cuthbert *et al.*, 2002) has been associated with significantly increased risk of gastric lymphoma (Rosenstiel *et al.*, 2006). No studies have been published from other high infection prevalence regions, so any ethnic effects of NOD1/NOD2 polymorphisms remain to be discovered.

7.2.4 The gastric epithelial mucin, MUC1

Mucins are a family of large complex glycoproteins that play an important role in protecting the mucosal surface against infection (Lindén *et al.*, 2008). While some form gels and are key constituents of mucus, others are expressed by epithelial cells. MUC1, the main mucin expressed on the gastric epithelial surface, is an extremely long molecule, largely due to the presence of a heavily glycosylated, variable number of tandem repeat (VNTR) extracellular region comprising up to 120 repeat units of 60 nucleotides. Owing to its

length, MUC1 extends above other cell surface molecules and is therefore most likely the first structure encountered by *H. pylori* approaching the epithelial surface.

Examination of human MUC1 gene sequences revealed a significant association between the possession of a short MUC1 allele and gastric cancer in Portuguese (Carvalho *et al.*, 1997), *H. pylori*-induced gastritis in British (Vinall *et al.*, 2002) and *H. pylori*-induced gastritis and intestinal metaplasia in Colombian (Silva *et al.*, 2003) patients. Moreover, the *in vitro* binding of *H. pylori* is significantly decreased to human gastric cell lines with shorter MUC1 alleles (Costa *et al.*, 2008), indicating that MUC1 plays an important role in limiting the attachment of *H. pylori* to the epithelial surface. MUC1 can limit attachment of *H. pylori* to the gastric epithelium both by acting as a releasable decoy and by steric hindrance (Lindén *et al.*, 2009). The potential importance of MUC1 in *Helicobacter* pathogenesis was further demonstrated when infection of Muc1-deficient mice with *H. pylori* resulted in increased levels of colonization and more severe pathology compared with wild-type controls (McGuckin *et al.*, 2007).

A short allele for MUC1 would translate into a short MUC1 molecule at the epithelial surface, and such a change could readily explain the increased association with *H. pylori*-induced gastritis and gastric cancer (Fig. 7.3).

7.2.5 The effect of gender on *Helicobacter pylori* infection and its pathological consequences

It is becoming increasingly recognized that simply being male is a risk factor for *H. pylori* infection and associated sequelae. In general, males are significantly more likely to develop *H. pylori*-associated pathologies, including gastric adenocarcinoma (Parkin *et al.*, 2005) and peptic ulcer disease (World Health Organization, 2008). Globally, the burden due to peptic ulcer disease, resulting in loss of life and loss of productivity, is 3.3 million disability-adjusted life years (DALYs) in males, twice that of females at 1.67 million DALYs (World Health Organization, 2008).

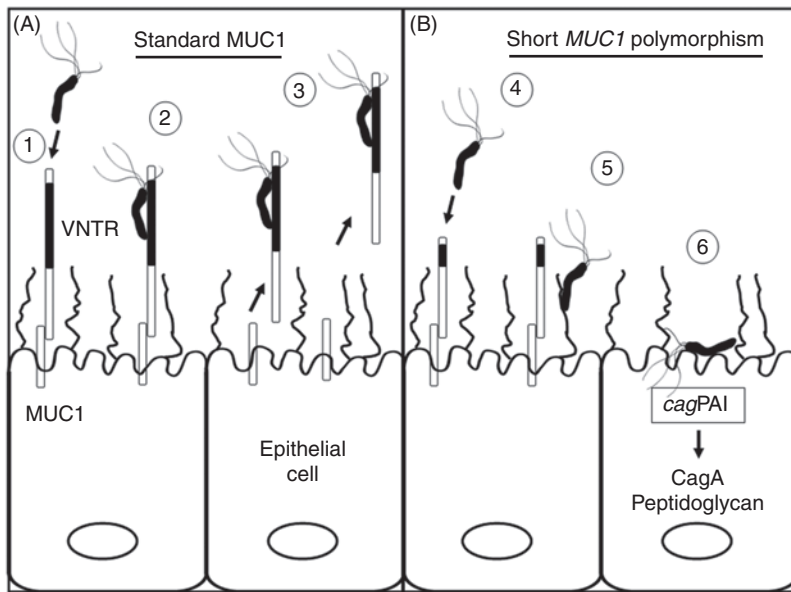


Fig. 7.3. Hypothesis: Short *MUC1* allele polymorphisms are associated with susceptibility to *Helicobacter pylori*-induced gastritis and gastric cancer. (A) Standard length *MUC1*: (1) propelled by flagella, and moving in response to chemotactic signals and pH gradients, *H. pylori* migrate to the gastric epithelial surface. Owing to its extreme length, *MUC1* mucin towers above the other cell surface molecules and is therefore the first structure encountered by approaching bacteria. (2) Using specific adhesins (i.e. BabA and SabA; see Lindén *et al.*, Chapter 12, this volume), *H. pylori* attach to the heavily glycosylated, variable number of tandem repeats (VNTR) region of *MUC1*. (3) The extracellular domain of *MUC1* is only loosely associated with its transmembrane region. Upon bacterial binding, *MUC1* undergoes a conformational change resulting in shedding of the extracellular domain, complete with adherent *H. pylori*, thereby limiting bacterial attachment to the epithelial cell surface. (B) Short *MUC1* polymorphism: individuals with short *MUC1* alleles have variable number of tandem repeat (VNTR) regions that are greatly reduced in length. (4) These shortened *MUC1* molecules allow *H. pylori* to get closer to the epithelial surface where (5) they can attach to other glycosylated cell surface molecules that cannot be shed by the host cell. (6) Once attached, *H. pylori* strains with the *cag* pathogenicity island (*cagPAI*) inject peptidoglycan (NOD1 activator) and cytotoxin-associated gene A protein (CagA) (see Backer *et al.*, Chapter 11, this volume), with proinflammatory consequences.

Similarly, in 2004, it was estimated that 500 million males died from stomach cancer, compared with 300 million females (World Health Organization, 2008). This difference probably involves the influence of gender biology on *H. pylori* infection and associated pathologies. However, a highly complex interaction of social, economic and environmental gender effects (i.e. differences in smoking, diet, occupations and social interactions) is also likely to play a significant role. There is a surprising dearth of literature investigating the role of gender in susceptibility to *H. pylori*-associated pathologies, with

the factors causing this disparate outcome in males versus females remaining poorly understood.

Sexual dimorphism in H. pylori infection and pathology in human subjects

A meta-analysis published in 2006 recognized that several factors hamper our understanding of gender, *H. pylori* infection and disease outcomes (De Martel and Parsonnet, 2006). These include: (i) study populations not being representative of the general population;

(ii) failure to adjust for confounding factors such as socio-economic status; and (iii) failure to present gender data in the absence of statistically significant correlations. This meta-analysis of large population-based studies revealed that, on average, after adjusting for age and socio-economic status, men were infected with *H. pylori* 16% more often than women (De Martel and Parsonnet, 2006). As this meta-analysis included only one Chinese study, which found no sexual dimorphism in infection rates, it is difficult to reach a definitive conclusion of whether this is a global phenomenon. Interestingly, a concurrent, but limited analysis of paediatric studies indicated that the male predominance of infection appears to be confined to the adult population (De Martel and Parsonnet, 2006). This latter observation suggests that sex hormones, synthesized in great quantities from the onset of puberty, may contribute to this gender bias.

It is difficult to delineate the contribution of sex-specific factors on *H. pylori* colonization and subsequent development of pathology. Is the increased incidence of peptic ulcer disease and gastric cancer in males due to increased susceptibility to infection, or due to a qualitatively and quantitatively different response to infection? Indeed, sex-specific factors could potentially influence both susceptibility to infection and the response to infection.

While epidemiological studies recognize that gastric cancer is more prevalent in males than females, it is less clear whether gender influences the incidence of peptic ulcers. An investigation of gender, *H. pylori* infection and peptic ulcer disease in 55,000 dyspeptic Chinese patients found that male sex increased the risk of developing gastric and duodenal ulcers independently of *H. pylori* infection (Wu *et al.*, 2008). The authors postulated that this reflects a protective effect of female sex hormones, as the sex difference dropped markedly in groups above 50 years of age, and was absent in the group aged 70 years or more. Broader epidemiological studies may reveal whether this is a global phenomenon. Such studies are, in part, confounded by sociological and environmental factors that contribute to gender effects. For example, the aforementioned Chinese study (Wu *et al.*, 2008) does not

adjust for smoking habits, known to also increase the risk of developing gastric cancer; in China, males are 67 times more likely to smoke than females (Pan and Hu, 2008).

Gender effects observed from animal models

Examination of gender effects on *H. pylori* infection in controlled animal studies provides an ideal means to investigate the influence of genetic and physiological sex-specific factors independently of socio-economic and environmental factors. While very few studies have used both male and female animals (mainly due to a tendency to only use female mice), experimental infections suggest that sex-specific factors do indeed influence colonization levels and development of pathology, although little is known about why sexual dimorphism exists.

Two studies have identified a gender difference in *H. pylori* colonization levels in mice. Colonization levels have been reported to be higher in males than females in C57BL/6 mice (Raghavan *et al.*, 2002), as well as in BALB/c mice lacking the IL-4R α cytokine receptor (Aebischer *et al.*, 2001), highlighting a potential interaction between sex-specific factors and the immune response to infection. In contrast, *H. pylori* colonization of B6129SvEv mice was equivalent in males and females (Rogers *et al.*, 2005).

Similarly, few reports have investigated gender and the development of *Helicobacter*-induced gastritis. Infected male mice that over-express gastrin developed more severe inflammation of the gastric corpus (with atrophy) than female mice after 5 months of infection (Fox *et al.*, 2003). In contrast, infection of female C57BL/6 mice with *H. felis* (a relative of *H. pylori* that induces more severe gastritis in mice) induced more severe inflammation, with a marginal increase in colonization, than infection of male mice, but only 1 year post-infection (Court *et al.*, 2003).

When infected with *H. pylori*, Mongolian gerbils can develop gastritis and gastric adenocarcinoma in a fashion akin to *H. pylori*-infected human subjects. Crabtree *et al.* (2004) found that female Mongolian gerbils develop more severe gastritis after 36 weeks of *H. pylori* infection, accompanied by increased

expression of IFN γ and IL-12p40, again highlighting a potential interaction of sex-specific factors with the immune response to *H. pylori*. While both male and female gerbils can develop adenocarcinoma (Zheng *et al.*, 2004), it remains unclear whether there is a gender bias in the development of gastric cancer, and further studies are required.

Care must be taken extrapolating gender observations made in animal models to the human situation. With the exception of the hypergastrinaemic mice, more severe pathology is generally observed in female rodents, the reverse of that observed in human subjects. Despite this, animal studies may still represent the best means of investigating the influence of sex-specific factors on *H. pylori* colonization and subsequent development of disease, as they allow for the control of socioeconomic and environmental factors.

How does gender contribute to H. pylori infection and associated disease?

It is well recognized that women in many parts of the world experience lower socioeconomic status (a significant risk factor in *Helicobacter* infection and pathogenesis), often with reduced access to health care, than their male counterparts. It is intriguing, therefore, that males are more prone to infection, peptic ulcer disease and gastric cancer than females (Green, 1992; Fish, 2008), suggesting this gender influence is potentially mediated by physiological factors.

Environmental factors, many of which may exhibit a gender bias, can also impact the outcome of *H. pylori* infection. Smoking, for example, is globally four times more prevalent in males than females (Corrao *et al.*, 2000). One study in Japanese men found that the risk of developing gastric cancer increased if they also smoked, with 50% of gastric cancer cases attributed to a combination of *H. pylori* infection and smoking (Shikata *et al.*, 2008). This increased the risk of developing gastric cancer 11-fold over either *H. pylori* infection or smoking alone. Another Japanese study found that *H. pylori* infection in men increased the risk of developing gastric cancer independently of smoking habits (Yamagata *et al.*, 2000). These studies suggest that, while

sex differences in smoking levels may contribute to increased progression to gastric cancer in Japanese males, it is not the only gender factor to play a role.

Evidence is accumulating that gender biology (including physiology and anatomy) has a strong influence on *H. pylori* infection and pathogenesis. The most obvious physiological difference between males and females is the sex hormones. The predominant male hormone, testosterone, and the predominant female hormones, oestrogen and progesterone, can all influence immunity. It has long been recognized that inflammatory responses in men and women are disparate. Broadly speaking, males tend to develop more severe sequelae from infectious diseases, whereas females are more prone to autoimmune diseases (Whitacre, 2001). It is generally thought that females generate more robust cellular and humoral immune responses and thus are more resistant to certain infections than males. Consequently, however, they are more prone to autoimmune disease. The reasons for this are not well understood, but sex hormone receptors are expressed by various immune cells, including CD4⁺ and CD8⁺ T cells, B cells, natural killer cells and macrophages (Lang, 2004). However, the actions of oestrogen on immune cells are complex and could result from direct interaction with the receptor or indirectly as a result of interaction with other hormones such as androgens, progesterone, prolactin and gonadotrophin-releasing hormone, all of which are thought to possess immunomodulatory functions (Lang, 2004). Thus it is easily conceivable that sex hormones – directly and indirectly – can modify the response to *H. pylori* infection (Sipponen and Correa, 2002). Indeed, considering the widespread expression of sex hormone receptors, sex hormones could alter the function of many cells and tissues that may change resistance to *H. pylori* infection and could influence development of disease.

Another obvious gender difference is that females possess two X chromosomes, while males possess one X and one Y chromosome. Many Y chromosome genes, mostly involved in development of testes, are specific to males, but curiously little is known about them. Apart from the effects of sex hormones on the

immune response and other biological processes, little is known about the specific influence of the sex chromosomes on disease development. One elegant study used transgenic mice that were XY but lacking the testis-determining *Sry* gene, or XX with the *Sry* gene inserted on an autosome (Smith-Bouvier *et al.*, 2008). Effectively, this allowed comparison of XX and XY mice on either a female or male hormonal background. They concluded that XX chromosome genes conferred greater susceptibility to experimental autoimmune disease, via a mechanism that was hormone independent. Several genes encoding immunomodulatory factors are present on the X chromosome, including those for CD40 ligand, Foxp3 and TLR-7, all important modulators of the inflammatory response. While dogma dictates that one X chromosome is inactivated, recent evidence suggests that X-inactivation can be incomplete (Carrel and Willard, 2005). Thus, a gene dosage effect of some of these genes could contribute to differential regulation in males and females of the inflammatory response to infections such as *H. pylori*.

Hence, while epidemiological data clearly show that gender plays an important role in host susceptibility to *H. pylori*-associated pathologies, our general ignorance of how sex-related factors interact with the host immune system and its response to infectious disease means no study has truly investigated the mechanism(s) behind this sexual dimorphism. Socio-economic and environmental factors certainly contribute to disease progression, but the mechanism(s) whereby biological factors play a role are less clear. Much can still be learned about the poorly understood impact of gender on *Helicobacter* pathogenesis. Controlled animal studies could shed light on genetic, physiological, hormonal or immunological factors that contribute to susceptibility to *H. pylori* infection and disease progression.

7.3 Conclusion

In summary, host genetics clearly play an extremely important role in deciding which *H. pylori*-infected individuals will progress to develop associated pathologies. Variations in a range of host genes, particularly those that

modify the scale of the inflammatory response to *Helicobacter* infection, can influence disease susceptibility. The overriding message emerging from the numerous studies investigating these genes, however, is that their relative importance can vary greatly between human populations. For example, polymorphisms in *IL1B* seem important for *H. pylori* pathogenesis in Caucasian populations, *IL8* polymorphisms may be more relevant in Asians, while *TNFA* may be significant for both.

This variation may be due to a number of factors. First, a relevant gene polymorphism within a particular ethnic group may interact with other gene variants within that population, producing a combination that leads to either increased or decreased susceptibility. Additionally, it is well recognized that virulence factor expression by *H. pylori* is affected by geography, with some countries such as Japan having a high frequency of more virulent strains of these bacteria. Hence the importance of a host gene polymorphism may be affected by the type of *H. pylori* strain with which an individual is infected. Most likely it is a combination of both of these, with further contribution from social and environmental factors.

Future studies may help further dissect the complex interactions of host genes in *Helicobacter* pathogenesis, perhaps by examining ethnic groups that have grown up in different countries. An investigation of polymorphisms in, say, persons of Chinese origin born in Western countries with different social and environmental exposures may be informative. However, it is also recognized that *H. pylori* infections often pass down from mother to child, meaning infected Chinese in the USA are likely to still carry *H. pylori* strains of Chinese origin. Hence it is not straightforward to delineate the role of host genetics from bacterial and environmental factors. Despite these problems, such efforts are worthwhile. Currently, we are incapable of predicting the prognosis of *H. pylori* infection in an individual. Understanding the role of host genes, within the context of other variables, would be invaluable for explaining the pathogenic process that occurs with *H. pylori* infection and would potentially provide indicators of disease susceptibility.

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8 Innate Immune Initiators and Effectors in *Helicobacter pylori* Infection

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8.1 Introduction

Bacterial pathogens employ different strategies to induce responses in host cells. These strategies typically involve cell invasion, adhesion and/or secretion of bacterial products. During *Helicobacter pylori* infection, most of the bacteria occupy a predominantly extracellular niche within the gastric mucus layer of the human stomach (Chan *et al.*, 1992; Dubois and Boren, 2007) and only 1% of *H. pylori* appear to be interacting directly with epithelial cells (Peterson, 1991; Ko *et al.*, 1999). Despite the location of *H. pylori* within the gastric mucosa, the host develops a robust inflammatory response and a variety of diseases varying in severity (reviewed in Montecucco and Rappuoli, 2001; Peek and Blaser, 2002).

Due to the location of *H. pylori* *in vivo*, it is likely that interactions of these bacteria with epithelial cells represent a key step in the induction of host immune responses. Epithelial cells are not, however, of lymphoid origin, so how does *H. pylori* initiate an inflammatory response in these cells? Furthermore, what are the host and *H. pylori* signals mediating these responses and how do these products interact with one another to facilitate the development of a chronic inflammatory condition?

In this chapter we discuss the innate immune responses generated by the host in an attempt to control *H. pylori* infection. Specifically, we address the roles of host pathogen-recognition molecules (PRMs) in the identification of *H. pylori* bacteria within the gastric mucosa and the downstream signalling events that these molecules trigger. We also identify some of the key *H. pylori* products that facilitate the initiation of inflammation *in vivo*. Finally, we discuss how these processes result in the production of pro-inflammatory cytokines, ultimately leading to the development of gastritis within the host.

8.2 Innate Immune Initiators in Host Immune Responses to *H. pylori*

The last decade has seen a renaissance in the perceived importance of the innate immune system in host defence against microbial infection. Indeed, it is now recognized that the innate immune system is not only important for the initiation of broad host defence responses to microbial pathogens, but also in the development of adaptive immune responses to these microorganisms (Napolitani *et al.*, 2005; Pasare and Medzhitov, 2005; Fritz *et al.*, 2007). The initiation of innate immune responses to microbial pathogens is

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dependent on the recognition of conserved microbial structures or products by host PRMs, which are evolutionarily conserved across the plant and animal kingdoms (Akira *et al.*, 2006). The key PRM signalling pathways for bacteria, in most cases, converge on the transcription factor nuclear factor- κ B (NF- κ B), a master regulator of genes encoding a broad range of inflammatory and immune products. Indeed, *H. pylori* is known to induce NF- κ B activation both *in vitro* (Philpott *et al.*, 2002; Viala *et al.*, 2004; Brandt *et al.*, 2005; Hirata *et al.*, 2006a,b; Ferrero *et al.*, 2008) and *in vivo* (Ferrero *et al.*, 2008; Yanai *et al.*, 2008). Discussed below are the various PRMs that have been implicated in innate immune recognition of *H. pylori*, leading to NF- κ B activation and the generation of proinflammatory responses to this pathogen.

8.2.1 The Toll-like receptor family

The first mammalian PRMs to be identified as playing a role in host defence against bacterial pathogens belonged to the Toll-like receptor (TLR) protein family (Akira *et al.*, 2006). One of these proteins, TLR4, was shown to share structural similarities to the fungal defence protein, TOLL, in *Drosophila* (Medzhitov *et al.*, 1997). Since the identification of TLR4 as the first known TLR, a total of 13 TLRs have been described, of which ten are expressed in humans (reviewed in Takeda and Akira, 2007; O'Neill, 2008). TLRs are expressed by multiple cells of the innate immune system, such as dendritic cells (DCs), macrophages and non-lymphoid cells such as epithelial cells (Takeda *et al.*, 2003).

TLRs and the interleukin (IL)-1 receptors belong to a superfamily of receptors that shares conserved Toll/IL-1 receptor (TIR) domains that are vital to the transduction of signals in the cell (Medzhitov *et al.*, 1997; O'Neill, 2008). TLR molecules are comprised of three regions corresponding to extracellular, transmembrane and TIR intracellular signalling domains. A defining characteristic of the TLRs and PRMs is the extracellular leucine-rich repeat (LRR) domain, which is responsible for the recognition of molecular

structures present within microbes (Takeda *et al.*, 2003). The structures recognized by TLRs are referred to as pathogen-associated molecular patterns (PAMPs). This term, however, is a misnomer because most PAMPs are actually also present in commensal and non-pathogenic microorganisms.

As depicted in the representative diagram of TLR signalling (Fig. 8.1), microbial products mediate the dimerization of TLR molecules that, in turn, trigger the recruitment of adaptor proteins to the intracellular TIR domains (Rakoff-Nahoum and Medzhitov, 2009). These interactions result in a signalling cascade that facilitates the recruitment of one or more of the following TIR-containing adaptor proteins: myeloid differentiation primary response gene 88 (MyD88); MyD88 adapter-like protein (MAL) (also known as TIR domain-containing adaptor protein, TIRAP); TIR domain-containing adapter-inducing interferon- β (TRIF); or TRIF-related adaptor molecule (TRAM) (Fitzgerald *et al.*, 2001; Kenny and O'Neill, 2008). The type of adaptor and co-adaptor molecules involved will vary depending on the specific TLR pathway that has been activated. For the present purposes, however, we discuss MyD88 and its interacting partners.

The recruitment of MyD88 to the TLR complex leads to the formation of a signalling scaffold involving the adaptor molecules IL-1 receptor-associated kinase-1 (IRAK1) and IRAK4, which interact with MyD88 via homophilic interactions involving death domains. These co-adaptor molecules are subsequently phosphorylated by IRAK4, leading to the activation and autophosphorylation of IRAK1. Both IRAK1 and IRAK4 then dissociate from the MyD88 complex (Brikos *et al.*, 2007) and interact with tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6). This results in TRAF6-mediated activation of the I κ B kinase (IKK) complex, as well as phosphorylation of the mitogen-activated protein kinases (MAPKs): extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. In turn, the IKK complex becomes phosphorylated, leading to the phosphorylation and degradation of the inhibitor of κ B (I κ B) proteins, thereby liberating transactivating

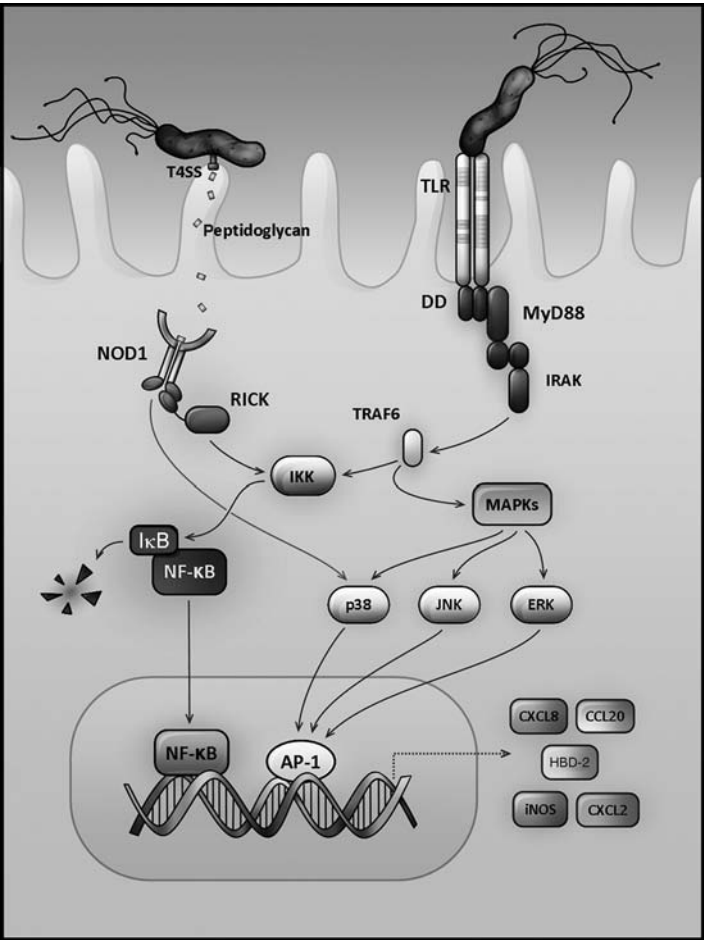


Fig. 8.1. *Helicobacter pylori* induces PRM signalling and innate immune responses. Pathogen-recognition molecules (PRMs) recognize specific ligands, which are broadly classified as pathogen-associated molecular patterns (PAMPs). Once PRMs detect their specific PAMP, they dimerize and recruit adaptor proteins to their signalling domains. This interaction results in the initiation of a signalling cascade, which ultimately activates the IκB kinase (IKK) complex and the degradation of inhibitor of κB (IκB) proteins, and/or the phosphorylation of mitogen-activated protein kinases (MAPKs). Degradation of IκB and phosphorylation of the MAPKs initiates the translocation of the transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) into the nucleus, resulting in the production of proinflammatory cytokines and antimicrobial peptides. Abbreviations: CXCL2, CXCL8 and CCL20, cytokines; DD, death domains; ERK, extracellular signal-regulated kinase; HBD-2, human β-defensin-2; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; MyD88, myeloid differentiation primary response gene 88; NOD1, nucleotide-binding oligomerization domain-1; RICK, RIP-like interacting CLARP kinase; T4SS, type IV secretion system; TLR, Toll-like receptor; TRAF6, TNF receptor-associated factor 6.

NF-κB complexes to translocate into the nucleus. These translocated NF-κB molecules then bind to conserved binding sites within the promoter regions of inflammatory or

immune genes, resulting in the up-regulated expression of various proinflammatory mediators, such as the chemokine CXCL8 (or IL-8) (Rakoff-Nahoum and Medzhitov, 2009).

Toll-like receptor expression in epithelial cells within H. pylori-infected tissues

Although there are ten known human TLRs, only TLR2, -4, -5 and -9 are expressed by epithelial cells in the human stomach (Schmausser *et al.*, 2004) and thus may be important for *H. pylori* recognition *in vivo*. Within healthy human gastric mucosa, TLR5 and TLR9 are present on the apical and basolateral surfaces; however, within inflamed gastric mucosa they are located only on the basolateral surfaces (Schmausser *et al.*, 2004). Moreover, gastric levels of TLR4 and its co-receptor MD2 are increased in *H. pylori*-infected tissues (Ishihara *et al.*, 2004; Schmausser *et al.*, 2004).

Many early studies implicated TLR2, -4 and -5 in the recognition of *H. pylori*. Given the importance of epithelial cells in the host response to *H. pylori* infection, this discussion primarily focuses on PRM recognition in this cell type. It is important to note, however, that many of the studies in this area have been performed using epithelial cell lines transfected to over-express specific TLR molecules. The findings generated from such studies have been either confirmed or contested by subsequent investigations using established cell lines, primary cells and/or by the genetic 'knockout' or 'knockdown' of specific PRMs in different cell types.

TLR2 AND TLR4. TLR2 detects and responds to stimulation with a wide variety of microbial products, including lipoproteins from Gram-negative bacteria, lipoteichoic acid, Gram-positive peptidoglycan and fungal cell wall components (Takeuchi *et al.*, 1999; Akira *et al.*, 2006). In contrast, TLR4 has a very narrow specificity for Gram-negative lipopolysaccharide (LPS) (Poltorak *et al.*, 1998). Nevertheless, some forms of Gram-negative LPS have been reported to signal preferentially via TLR2 (Akira *et al.*, 2006). Indeed, from over-expression studies in TLR2/TLR4-deficient cells and/or gastric epithelial cells constitutively expressing TLR, it was reported that TLR2, and not TLR4, was involved in the recognition of *H. pylori* LPS (Smith *et al.*, 2003; Lepper *et al.*, 2005; Chaouche-Drider *et al.*, 2009). It was even suggested that *H. pylori*

LPS may act as an antagonist for TLR4 signalling (Lepper *et al.*, 2005). The findings in favour of a role for TLR2 recognition of *H. pylori* LPS were indirectly supported by the work of Backhed and colleagues. These workers showed that *H. pylori* could induce CXCL8 responses in primary gastric epithelial cells that do not express TLR4, as well as in several epithelial cell lines that, for the most part, lack the CD14 co-receptor needed for TLR4 signalling (Backhed *et al.*, 2003). It is noteworthy that *H. pylori* LPS has a 100-fold lower biological activity compared with LPS molecules of other Gram-negative bacteria, such as *Escherichia coli* or *Salmonella enterica* (Muotiala *et al.*, 1992; Birkholz *et al.*, 1993). Moreover, it was reported that *H. pylori* LPS, in contrast to that of *E. coli*, is poorly bound by LPS-binding protein (Cunningham *et al.*, 1996). This would be expected to have an effect on the transfer of *H. pylori* LPS to CD14, and thus may explain the low TLR4 agonist activity of *H. pylori* LPS.

Some authors have nevertheless identified TLR4 as being important for the induction of proinflammatory responses by *H. pylori* LPS, both in guinea pig gastric pit cells and in transfected epithelial cells (Kawahara *et al.*, 2001; Mandell *et al.*, 2004). For example, human HEK293 epithelial cells, which do not express endogenous TLR2 or TLR4, became responsive to *H. pylori* LPS when transfected to express TLR4 (Mandell *et al.*, 2004). The divergent results were attributed to the different amounts of LPS used in the experimental work (i.e. nanograms versus micrograms), and the purity of the *H. pylori* LPS preparations used, which lacked trace amounts of TLR2 agonist (Mandell *et al.*, 2004). Consistent with this suggestion, highly purified preparations of *H. pylori* LPS induced cytokine responses in macrophages from TLR2-knockout animals, whereas TLR4-deficient cells were non-responsive (Mandell *et al.*, 2004). Although these findings could be interpreted as confirming the role of TLR4 in *H. pylori* LPS recognition, it is also possible that the data simply reflect the reported differences in LPS responsiveness of macrophages and epithelial cells (Maeda *et al.*, 2001; Su *et al.*, 2003).

If there is still controversy regarding the identity of the specific TLR involved in the

recognition of *H. pylori* LPS, the situation for TLR recognition of live *H. pylori* bacteria seems slightly clearer with multiple reports describing a role for TLR2. For example, human HEK293 epithelial cells, stably transfected to express functional TLR2, recognized intact *H. pylori* whereas TLR4-transfected cells were non-responsive (Mandell *et al.*, 2004; Torok *et al.*, 2005). These findings highlight the difference in results one can obtain when studying *H. pylori* LPS-induced responses using purified *H. pylori* LPS compared with viable *H. pylori* bacteria. Using TLR neutralizing antibodies, it was also found that TLR2 in concert with TLR9 mediated prostaglandin E2 release and angiogenic responses by gastric epithelial cells in response to *H. pylori* stimulation (Chang *et al.*, 2005). These findings were supported by experiments in which macrophages from TLR2-deficient mice were found to be non-responsive to stimulation with *Helicobacter* (Mandell *et al.*, 2004; Obonyo *et al.*, 2007). In contrast, macrophages from both wild-type and TLR4-deficient mice responded to *H. pylori* by the production of IL-6 and CCL2 (or monocyte chemotactic protein-1, MCP-1) (Mandell *et al.*, 2004). Interestingly, however, another group reported that TLR4-deficient macrophages exhibited reduced responses to stimulation with *H. pylori* (Obonyo *et al.*, 2007). The inter-study differences in the findings may be attributed to the origin of the macrophages used (peritoneal versus bone marrow derived, respectively) as well as the cytokines measured (IL-6 and MCP-1 versus IL-10 and IL-12, respectively) (Mandell *et al.*, 2004; Obonyo *et al.*, 2007).

Further evidence for the proposed role of TLR2 as the dominant TLR required for *H. pylori* recognition arises from mouse vaccination studies, which showed a significant reduction in the bacterial loads of TLR2-deficient mice vaccinated against *H. pylori* when compared with wild-type animals (Panthel *et al.*, 2003). To our knowledge, similar animal studies have yet to be conducted with genetically constructed TLR4-deficient mice.

The bacterial components responsible for the effects of whole live *H. pylori* on TLR2 signalling have yet to be identified. Nevertheless, one potential candidate is

the *H. pylori* neutrophil-activating protein (HP-NAP; see discussion in Section 8.3.1). HP-NAP is capable of stimulating neutrophils and monocytes to enhance their production of IL-12, thereby facilitating the differentiation of naive T-helper (Th) cells into the Th1 phenotype (D'Elios *et al.*, 2007) (Fig. 8.2). Moreover, stimulation of TLR2-transfected HEK293 cells with HP-NAP activated NF- κ B in a dose-dependent manner, whereas NF- κ B activation was absent in HEK293 cells not expressing TLR2 (Amedei *et al.*, 2006).

Despite the conflicting data regarding the role of TLR4 in epithelial and macrophage responses to *H. pylori* stimulation, there is evidence that TLR4 may play a partial role in initiating DCs to produce cytokines in response to *H. pylori* (Rad *et al.*, 2009). Indeed, while *H. pylori* stimulation of bone marrow-derived DCs from wild-type mice resulted in the induction of IL-6, stimulation of DCs from both TLR2- or TLR4-deficient mice had a reduced level of IL-6 production, which was significantly reduced in TLR2-deficient mice (Rad *et al.*, 2009). These findings suggest that TLR2, and to a much lower extent TLR4, are the predominant surface TLRs involved in the response of DCs to *H. pylori* lysates (Rad *et al.*, 2009). Furthermore, two separate studies examining detailed immune responses to *H. pylori*, by murine chromosome mapping or microarray analysis, implicated the involvement of additional PRMs, including TLR9 (Anderson *et al.*, 2007; Rad *et al.*, 2009) and retinoic acid-inducible gene I (RIG-I), which recognize unmethylated CpG DNA and RNA, respectively (Rad *et al.*, 2009).

TLR5. Several reports have identified the ability of bacterial flagellin to function as a major agonist of proinflammatory responses in human epithelial cells (Gewirtz *et al.*, 2001a,b; Zeng *et al.*, 2003). Bacterial flagellin is specifically recognized by TLR5 (Hayashi *et al.*, 2001). Given that *H. pylori* expresses four to six polar, sheathed flagella (Eaton *et al.*, 1996), it seemed reasonable to assume that TLR5 may play an important role in *H. pylori* recognition by epithelial cells. Indeed, two reports described the ability of TLR5 to recognize *H. pylori* flagellin and to initiate signalling in HEK293 cells over-expressing TLR5 (Smith *et al.*, 2003; Torok *et al.*, 2005). Other workers,

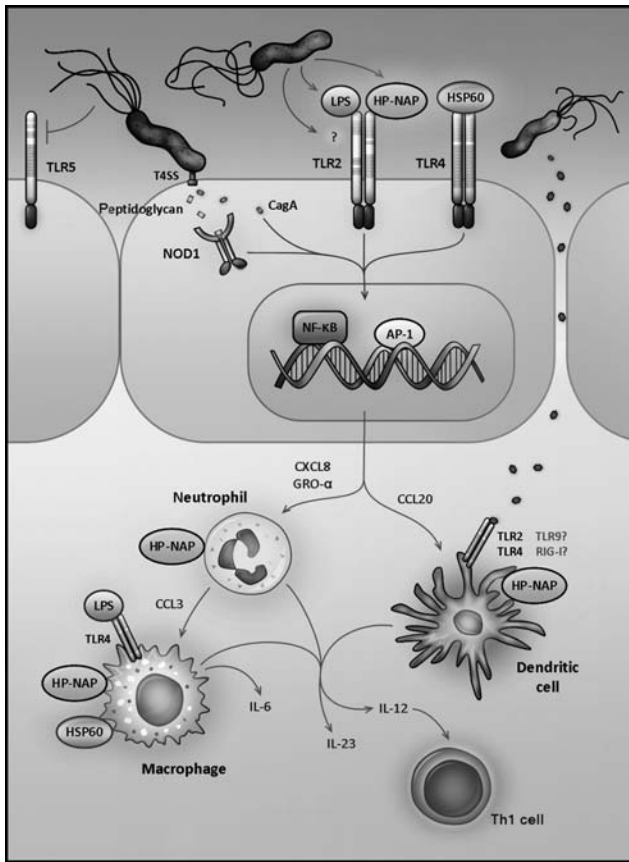


Fig. 8.2. Signalling via PRMs results in the development of local inflammation and the induction of the adaptive immune response. *Helicobacter pylori* pathogen-associated molecular patterns (PAMPs) are detected by pathogen-recognition molecules (PRMs) expressed by epithelial cells, which results in the activation of nuclear factor- κ B (NF- κ B) and/or activator protein-1 (AP-1) and the production of proinflammatory cytokines. These proinflammatory cytokines function to recruit macrophages, neutrophils and dendritic cells to the site of infection, which can be further activated by PAMPs that pass through the epithelial cell barrier. These activated innate immune cells subsequently produce cytokines that facilitate the recruitment of inflammatory cells and T-helper (Th) cells, resulting in the progression of inflammation and the development of an adaptive immune response. Abbreviations: CagA, cytotoxin-associated gene A protein; CXCL8 and CCL20, cytokines; GRO- α , growth-regulated oncogene- α ; HP-NAP, *H. pylori* neutrophil-activating protein; HSP60, heat-shock protein 60; IL, interleukin; LPS, lipopolysaccharide; NOD1, nucleotide-binding oligomerization domain-1; RIG-I, retinoic acid-inducible gene I; T4SS, type IV secretion system; TLR, Toll-like receptor.

however, subsequently demonstrated that flagellin-responsive epithelial cell lines did not detect *H. pylori* flagellin, suggesting that this component of the bacterium has very low immunostimulatory ability and may thus be able to evade TLR5 recognition (Lee *et al.*, 2003; Gewirtz *et al.*, 2004). Furthermore, non-flagellated *H. pylori* mutant bacteria retained

most, if not all, of their ability to induce CXCL8 production in flagellin-responsive cell lines (Lee *et al.*, 2003; Gewirtz *et al.*, 2004). It is now established that *H. pylori* flagellin molecules cannot be sensed by TLR5, because these molecules lack the conserved amino acids required for full TLR5 agonist activity (Andersen-Nissen *et al.*, 2005). Interestingly,

enteric *Helicobacter* species (Chaouche-Drider *et al.*, 2009), as well as species within the closely related *Campylobacter* genus (Andersen-Nissen *et al.*, 2005), also seem to have evolved flagellins that are able to escape TLR5 detection.

8.2.2 The nucleotide-binding oligomerization domain-like receptor family

In addition to TLRs, host cells may also express one or more types of cytosolic PRMs. One of these protein families, the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family, has been shown to contain members that play very important roles in host defence against microbial pathogens, as well as in the recognition of 'danger signals' from within eukaryotic cells (reviewed in Ferrero, 2005; Fritz *et al.*, 2006; Kaparakis *et al.*, 2007).

Nucleotide-binding oligomerization domain proteins

The archetypal NLR, NOD1, is specifically involved in host defence against Gram-negative bacterial infection (Viala *et al.*, 2004). NOD1 is comprised of three domains: a central nucleotide-binding oligomerization domain (NOD), a C-terminal LRR domain and a caspase-activated recruitment domain (CARD). The LRR domain in NOD1 is required for the sensing of Gram-negative peptidoglycan, whereas NOD2 is a sensor of all forms of bacterial peptidoglycan (reviewed in Ferrero, 2005; Fritz *et al.*, 2006; Kaparakis *et al.*, 2007). The NOD component of these proteins is important for protein self-oligomerization (Inohara and Nunez, 2003; Fritz *et al.*, 2006), whereas the CARD facilitates the interaction of NOD1 with downstream signalling molecules through homophilic and heterophilic protein interactions (Inohara and Nunez, 2003). NOD1 has one CARD, whereas NOD2 has two of these regions in its C terminus. NOD1 is predominantly contained within epithelial cells and, to a lesser extent, within lamina propria monocytes, whereas NOD2 seems to

be more abundant in cells of the monocyte/macrophage lineage (Bertin *et al.*, 1999; Ogura *et al.*, 2001; Hisamatsu *et al.*, 2003).

Human NOD1 senses a specific structure that is most commonly found in Gram-negative bacterial peptidoglycan (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003). This mucopeptide structure is composed of a disaccharide moiety, *N*-acetyl glucosamine-*N*-acetyl muramic acid (GlcNAc-MurNAc), linked to a tripeptide of which the terminal amino acid is *meso*-diaminopimelate (mDAP) (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003). Interestingly, human NOD1 was shown to specifically recognize this so-called GM-TriDAP structure, whereas murine NOD1 is most responsive to a tetrapeptide mucopeptide containing *L*-alanine-*D*-glutamate-mDAP-*D*-alanine (GM-TetraDAP) (Magalhaes *et al.*, 2005).

Although *H. pylori* is primarily an extracellular pathogen, it can activate NOD1 signalling via its type IV secretion system (T4SS), encoded by the cytotoxin-associated gene (*cag*) pathogenicity island (*cagPAI*) (Viala *et al.*, 2004) (see Backert *et al.*, Chapter 11, this volume). Specifically, *H. pylori* activates NOD1 by transfer of its peptidoglycan into the cytoplasm of non-phagocytic epithelial cells via a *cagPAI*-dependent mechanism (Viala *et al.*, 2004). As in other models, *H. pylori*-mediated NOD1 signalling results in the induction of NF- κ B activation and CXCL8 secretion *in vitro*. Interestingly, *cagPAI*-positive *H. pylori* strains are associated with higher levels of CXCL8 expression *in vivo* than *cagPAI*-negative strains (Crabtree *et al.*, 1995; Peek *et al.*, 1995), suggesting that NOD1 may play an important role in the development of the more severe immunopathology associated with *cagPAI*-positive strains (see the more detailed discussion below). Indeed, Western blot analyses of inflamed gastric tissues obtained from *H. pylori*-positive patients revealed that NOD1 synthesis was up-regulated *in vivo* during inflammatory conditions (Rosenstiel *et al.*, 2006). Similarly, NOD2 protein levels were also increased in these tissues; moreover, NOD2 was reported to mediate responses to *cagPAI*-positive *H. pylori* in NOD2-transfected epithelial cells (Rosenstiel *et al.*, 2006). The importance of NOD2 for *H. pylori* infection, however, awaits further investigation. In

contrast, animal studies showed that *Nod1*-deficient mice were more susceptible than wild-type animals to infection by *H. pylori* strains harbouring a functional T4SS, but not to isogenic T4SS mutant bacteria (Viala *et al.*, 2004). This suggests a role for NOD1 in host defence to infection by *cagPAI*-positive *H. pylori* bacteria.

NOD1 sensing of peptidoglycan initiates a signalling cascade that ultimately results in the up-regulation of NF- κ B and the production of proinflammatory cytokines, including CXCL8 and its murine homologue, CXCL2 (or macrophage inflammatory protein-2, MIP-2) (Girardin *et al.*, 2003; Viala *et al.*, 2004). NOD1 activation was demonstrated to mediate the recruitment and oligomerization of the RIP-like interacting CLARP kinase (RICK) (Inohara *et al.*, 2000; Girardin *et al.*, 2001; Hasegawa *et al.*, 2008), a member of the receptor-interacting protein kinase family (Meylan and Tschopp, 2005). RICK is also known as receptor-interacting protein (RIP)-2 or CARD-containing ICE-associated kinase (CARDIAK) (Thome *et al.*, 1998). RICK was shown to interact with NOD1 (or NOD2) via its CARD region, resulting in the formation of a transient complex comprising NOD1 (or NOD2), RICK and the IKK complex (Inohara *et al.*, 2000; Girardin *et al.*, 2001; Hasegawa *et al.*, 2008) (Fig. 8.1). Ultimately, this complex initiates a signalling cascade that results in the phosphorylation and degradation of the IKK complex. As for TLR signalling, the activation of the IKK complex promotes the degradation of the I κ B proteins, which enables the disassociated NF- κ B complex to translocate into the nucleus and bind to consensus binding sites within the promoter regions of proinflammatory genes.

Although the downstream responses to *H. pylori*-mediated NOD1 signalling have yet to be established, recent data from our laboratory suggest that RICK does play an important function in this pathway (Allison *et al.*, 2009). Additionally, Hirata and co-workers demonstrated that *H. pylori cagPAI*-dependent activation of IKK subunit α , as well as I κ B α phosphorylation, are critical for CXCL8 responses in epithelial cells (Hirata *et al.*,

2006a,b). Interestingly, a TRAF6/MyD88-dependent pathway for CXCL8 responses to *H. pylori* was reported, although IRAK1 and -2 were not required (Hirata *et al.*, 2006b). It should be noted that these authors observed no effect for NOD1 siRNA on epithelial cell responses to *H. pylori*; however, they did not present data confirming the effect of this siRNA on NOD1 signalling per se.

As predicted by the model of NOD signalling presented above, *H. pylori*-mediated activation of this pathway results in the translocation to the nucleus of the transactivating NF- κ B complex, consisting of p65 and p50 subunits. Moreover, the downstream proinflammatory responses of this signalling pathway, as measured by CXCL8 production, were found to be enhanced by a concomitant up-regulation of AP-1 (Allison *et al.*, 2009). We propose that the additional binding of this transcription factor to the promoter region of the *CXCL8* gene enhances chemokine responses in cells. It is also noteworthy that *H. pylori*-mediated NOD1 signalling involves the activation of p38 MAPK, and to a lesser extent ERK, rather than JNK, as was reported for *Shigella flexneri* (Allison *et al.*, 2009). These data highlight the fact that although different infectious agents may activate the same PRM signalling cascades, the mechanisms by which they do so are likely to vary, as are the downstream effector responses regulated by these cascades. Despite these differences, many of the PRM signalling pathways merge to NF- κ B. This transcription factor typically targets the genes encoding proinflammatory cytokines and chemoattractants (e.g. CXCL8, CXCL5 and CCL20), which function to recruit neutrophils and DCs (Kim *et al.*, 2001; Tomimori *et al.*, 2007) (Fig. 8.2).

In addition, PRMs may regulate the expression of various antimicrobial peptides. Indeed, NOD1 was shown to be essential for the up-regulated expression in epithelial cells of the gene (*DEFB4*) encoding the antimicrobial peptide, human β -defensin-2 (HBD-2), by *cagPAI*-positive *H. pylori* strains (Boughan *et al.*, 2006; Grubman *et al.*, 2010). In contrast, a NOD1/*cagPAI*-independent

mechanism was involved in *H. pylori* up-regulated expression of *DEFB3*, which encodes another major member of the HBD family, HBD-3 (Boughan *et al.*, 2006; Grubman *et al.*, 2010). A more detailed discussion of these and other antimicrobial factors is provided in Section 8.4.2. Significantly, culture supernatants from *cagPAI*-positive *H. pylori*-stimulated AGS control cells, but not those in which NOD1 was stably 'knocked down' by siRNA, significantly reduced *H. pylori* numbers in a salt- and cathepsin L-dependent manner, suggestive of a role for HBD-2 in bacterial killing (Grubman *et al.*, 2010). This finding was confirmed by the pre-treatment of *H. pylori*-stimulated cells with siRNA directed against the *DEFB4* gene (Grubman *et al.*, 2010). Taken together, it appears that NOD1 may have a dual role in host defences against bacterial pathogens: first by inducing chemokine production that results in the recruitment of proinflammatory cells to the site of infection; and second by initiating the synthesis of antimicrobial peptides with direct activity against these micro-organisms.

Although *H. pylori* is a potent activator of NOD1 signalling, it is also able to avoid detection by this PRM, and thus probably also NOD1-mediated clearance by the host. *H. pylori* can evade NOD1 detection through the actions of an amidase, AmiA, which mediates modifications of cell wall peptidoglycan; these modifications are characterized by a decrease in the quantities of GM-TriDAP and the concomitant accumulation of an *N*-acetyl-D-glucosaminyl- β (1,4)-*N*-acetylmuramyl-L-Ala-D-Glu structure (Chaput *et al.*, 2006). As *H. pylori* AmiA is also essential for the morphological transition of *H. pylori* from the rod to coccoid form, the coccoid forms are no longer detected by human NOD1 and are incapable of inducing NF- κ B activation or CXCL8 secretion by gastric epithelial cells (Chaput *et al.*, 2006). These findings are consistent with work in other infection models describing the ability of different bacterial pathogens to avoid detection by NOD1 or NOD2 through changes in their peptidoglycan structures (Lenz *et al.*, 2003; Boneca *et al.*, 2007; Wolfert *et al.*, 2007).

8.2.3 NACHT-LRR-PYD-containing proteins

The NACHT-LRR-PYD-containing proteins (NALPs) represent another important group of proteins belonging to the NLR family. The name of these proteins derives from their conserved three-domain structure comprising: a central domain NACHT (an acronym for NAIIP (neuronal apoptosis inhibitor protein), C2TA (MHC class 2 transcription activator), HET-E (incompatibility locus protein from *Podospora anserine*) and TP1 (telomerase-associated protein)); a C-terminal LRR region; and an N-terminal pyrin domain (PYD) (Martinon *et al.*, 2002). NALPs are activated by microbial products including toxins (Gurcel *et al.*, 2006) and a variety of danger-associated molecular patterns (Martinon *et al.*, 2002; Fritz *et al.*, 2006). Proteins of the NLR family regulate the activation of caspase-1 (Martinon and Tschopp, 2004), which is responsible for the maturation of proinflammatory cytokines. Both IL-1 β and IL-18, which play key roles in host defence against infection, are synthesized as inactive cytoplasmic precursors and are proteolytically cleaved by caspase-1 to produce the biologically active mature forms (Thornberry *et al.*, 1992).

Upon activation by their ligand, NALPs associate via their PYD domains with apoptosis-associated speck-like protein (ASC). ASC subsequently interacts with the CARD of procaspase-1, leading to the formation of a complex called the 'inflammasome' and the cleaving of IL-1 β and IL-18 (Martinon *et al.*, 2002, 2004). Although the role of NALPs in innate immune responses to *H. pylori* have yet to be explored in detail, it was found that ASC-deficient mice challenged with *H. pylori* exhibited higher bacterial loads and significantly lower levels of gastritis when compared with wild-type animals (Benoit *et al.*, 2009). Moreover, ASC-deficient mice were incapable of producing any IL-1 β or IL-18, and produced substantially less interferon- γ (IFN γ) in response to *H. pylori* infection compared with wild-type animals (Benoit *et al.*, 2009). It is therefore possible that ASC functions to up-regulate IL-1 β and IL-18 production, thereby enhancing the development of IFN γ .

dependent inflammatory responses and the reduction of gastric *H. pylori* loads in the host. These findings suggest that the inflammatory may thus play a role in host immune responses to *H. pylori*. Further studies are, however, required to confirm this possibility.

8.2.4 The impact of polymorphisms in genes encoding pathogen-recognition molecules on *H. pylori* disease

Given the importance of PRMs in host recognition and responses to pathogens, several studies have investigated whether PRM gene polymorphisms may contribute to the varying disease outcomes associated with *H. pylori* infection. Although the findings of such studies have thus far been largely inconclusive, there does seem to be a general trend between gene polymorphisms in certain TLR signalling molecules and the development of more severe gastritis.

A -159C/T promoter polymorphism in the gene encoding CD14 was associated with an increased risk of precancerous lesions in *H. pylori*-infected Caucasians (Hold *et al.*, 2009). A similar association with precancerous outcomes was found for a -1237T/C polymorphism in *TLR9* (Hold *et al.*, 2009). However, no such associations were found between either of these gene polymorphisms and gastric adenocarcinoma risk in Caucasians (Hold *et al.*, 2009). Similarly, no association could be found between the -159C/T *CD14* gene polymorphism and the risk for gastric carcinoma, or mucosa-associated lymphoid tissue (MALT) lymphoma, in a Taiwanese Chinese population (Wu *et al.*, 2006). Interestingly, however, a -196 to -174 deletion in the *TLR2* promoter region was more frequently found in Japanese patients with gastric cancer (Tahara *et al.*, 2007, 2008). Moreover, the *TLR2* -196 to -174 deletion allele also correlated with an increased risk of intestinal metaplasia, a precursor of gastric adenocarcinoma, but only in individuals over 60 years of age (Tahara *et al.*, 2008). On the other hand, no association was found between this polymorphism and the severity of neutrophil or mononuclear cell infiltrates (Tahara *et al.*, 2008). In contrast, Achyut and

colleagues (2007) were not only able to show an association between a *TLR4* gene polymorphism (T399I) and an increased risk for precancerous lesions among *H. pylori*-infected individuals from an Indian population, but also found a correlation with plasma cell infiltration, atrophy and intestinal metaplasia. Neither the T399I *TLR4* polymorphism nor another common polymorphism (A299G) was found to increase the risk of duodenal ulcer (Hofner *et al.*, 2007). Similarly, no association was found between several *NOD1* or *NOD2* gene polymorphisms in *H. pylori*-related gastric ulcer, although a polymorphism in the *NOD2* gene was associated with an increased risk of developing MALT lymphoma (Rosenstiel *et al.*, 2006). Taken together, the data suggest that several PRM gene polymorphisms may favour the development of precancerous lesions in *H. pylori*-infected subjects. Nevertheless, it is unlikely that a single polymorphism will be linked to gastric carcinogenesis risk (see Sutton *et al.*, Chapter 7, this volume). This probably reflects the multifactorial determinants (i.e. host, bacterial and environmental) that influence *H. pylori* disease severity (Peek and Blaser, 2002).

8.2.5 The role of triggering receptor expressed on myeloid cells in innate host responses to *H. pylori*

Receptor proteins belonging to the new family of molecules known as triggering receptor expressed on myeloid cells (TREM) have been reported to have diverse functions, including a role in innate immunity and host defence against bacterial infection (Sharif and Knapp, 2008). One of these proteins, TREM-1, was shown to be expressed on the surface of neutrophils, monocytes and macrophages (Sharif and Knapp, 2008). TREM-1 initiates a signal upon association with DAP12, an immunoreceptor tyrosine-based activation motif (ITAM)-containing transmembrane adaptor protein (Bouchon *et al.*, 2000; Bleharski *et al.*, 2003). To date, the natural ligand that activates TREM-1 is unknown; however, stimulation of TREM-1 in neutrophils and monocytes leads to the secretion of CXCL8 and

TNF α (Bouchon *et al.*, 2000). TREM-1 is expressed on gastric epithelium *in vitro* and *in vivo* in response to *H. pylori* stimulation (Schmausser *et al.*, 2008). Interestingly, *in vitro* stimulation of gastric epithelial cells with CXCL8 or LPS alone could not up-regulate TREM-1; however, TREM-1 could be up-regulated by CagA-positive *H. pylori* (Schmausser *et al.*, 2008). Furthermore, stimulation of gastric epithelial cells with an anti-TREM-1 antibody resulted in the up-regulation of CXCL8, suggesting that TREM-1 may amplify inflammation by up-regulating CXCL8 (Schmausser *et al.*, 2008). Further investigations are required to identify the *H. pylori* factor that is responsible for TREM-1-induced chemokine production, as well as the role that TREM-1 plays in the innate immune response to *H. pylori* infection.

8.3 Bacterial Danger Signals that Initiate Innate Immune Responses in Host Cells

8.3.1 *H. pylori* neutrophil-activating protein

The influx of neutrophils into the gastric mucosa is characteristic of an acute *H. pylori* infection (Craig *et al.*, 1992; Crabtree, 1996). During infection, *H. pylori* produces the 150 kDa protein HP-NAP, which can adhere to surrounding *H. pylori* facilitating their adherence to host epithelial cells (Namavar *et al.*, 1998). However, HP-NAP also has a pro-inflammatory function as it can rapidly cross the endothelia and promote the adhesion of neutrophils to endothelial cells, facilitating the transendothelial migration of these cells into infected tissues (Polenghi *et al.*, 2007). In addition, HP-NAP is able to further enhance the generation of gastritis by up-regulating the expression of β 2-integrin on the surface of endothelial cells, thereby promoting neutrophil migration (Montecucco and de Bernard, 2003), and by activating neutrophils to produce reactive oxygen radicals and chemokines such as CXCL8, CCL3 (or macrophage inflammatory protein-1 α , MIP-1 α) and CCL4 (or MIP-1 β) (Wang *et al.*, 2006; Polenghi

et al., 2007). These factors are likely to contribute to the further recruitment of neutrophils, monocytes, DCs and lymphocytes to the gastric mucosa. The production of HP-NAP also appears to facilitate the development of a consistent maturation of DCs, as indicated by the elevated surface expression of the major histocompatibility complex (MHC) class II and the co-stimulatory molecules CD80 and CD86 (Amedei *et al.*, 2006). Finally, HP-NAP enhances the development of inflammation by inducing the production of IL-12 and IL-23 by human monocytes, DCs and neutrophils, therefore promoting the development of the Th1 proinflammatory immune response (Amedei *et al.*, 2006) that is typical of an *H. pylori* infection (Fig. 8.2).

8.3.2 Heat-shock protein 60

H. pylori heat-shock protein 60 (HSP60), a homologue of the *E. coli* GroEL chaperone, is an immunodominant antigen of *H. pylori* (Macchia *et al.*, 1993; Suerbaum *et al.*, 1994; Ferrero *et al.*, 1995). This protein is secreted by *H. pylori* *in vivo* (Dunn *et al.*, 1997) and is able to induce IL-6 production in macrophages (Gobert *et al.*, 2004). HSP60 was identified by the screening of soluble *H. pylori* fractions, which had been isolated by ion exchange and size exclusion chromatography, for their ability to induce IL-6 secretion in RAW 264.7 macrophages via a TLR2- and MyD88-independent mechanism (Gobert *et al.*, 2004). In contrast, TLR2/4 and MyD88 were reported to be important in CXCL8 responses to HSP60 in Kato III cells, a gastric epithelial cell line (Takenaka *et al.*, 2004).

8.3.3 Cytotoxin-associated gene A protein

Cytotoxin-associated gene A protein (CagA), which is encoded by the *cagPAI*, is an important virulence determinant of *H. pylori* (Peek and Blaser, 2002). The ability of CagA to induce cytoskeletal changes and to cause damage to the gastric epithelial cell barrier is discussed elsewhere (see Backert *et al.*,

Chapter 11, this volume). Nevertheless, it should be noted that CagA also has a role in inducing CXCL8 production by epithelial cells (Brandt *et al.*, 2005; Kim *et al.*, 2006). Specifically, it was reported that the T4SS-dependent delivery of CagA into epithelial cells, or via the process of transfection, induced MAPK signalling and the secretion of CXCL8 (Brandt *et al.*, 2005; Kim *et al.*, 2006; Kwok *et al.*, 2007). These responses occur after prolonged exposure of cells to CagA (i.e. at 36–48 h post-exposure) and appear to be induced via a MAPK-dependent but NOD1-independent mechanism (Brandt *et al.*, 2005).

8.4 Innate Immune Effector Responses to *H. pylori* Infection

A variety of antimicrobial factors are produced in a rapid manner by host epithelial cells in response to a bacterial stimulus, thus functioning as a first line of defence against microbial pathogens. The various factors that are specifically produced by host epithelial cells in response to *H. pylori* infection are discussed below.

8.4.1 Lactoferrin

One of the earliest reported antimicrobials identified as being induced in response to *H. pylori* infection was lactoferrin. This 80 kDa iron-binding glycoprotein can be detected in most biological fluids of mammals (Lonnerdal and Iyer, 1995). Lactoferrin is synthesized by the cardiac and pyloric glandular epithelial cells of the stomach (Luqmani *et al.*, 1991) and by innate immune cells such as neutrophils (Lonnerdal and Iyer, 1995), which secrete lactoferrin from secondary granules (Baggiolini *et al.*, 1970; Leffell and Spitznagel, 1972). Although lactoferrin is constitutively produced, its concentration can be elevated within secretions at sites of infection and inflammation *in vivo*. Therefore, lactoferrins are considered to be an important component of the host defence mechanism due to their function as anti-inflammatory and immunomodulatory molecules (Arnold *et al.*, 1977; Ellison *et al.*, 1988; Yamauchi *et al.*, 1993).

In addition, lactoferrin can inhibit the growth of several human bacterial pathogens (Arnold *et al.*, 1977), including *H. pylori* (Miehlke *et al.*, 1996). There are two mechanisms whereby lactoferrin functions as an antimicrobial agent *in vivo*. First, due to its high iron-binding capacity, lactoferrin can deplete the bacterial environment of free iron, hence hindering growth of the organism (Weinberg, 1984). Second, the N terminus of lactoferrin has a domain, distinct from its iron-binding site, that functions as an antimicrobial against Gram-positive and Gram-negative bacteria (Bellamy *et al.*, 1992). At physiological concentrations, lactoferrin directly damages the outer membrane permeability of Gram-negative bacteria by releasing LPS (Ellison *et al.*, 1988). However, due to its iron-binding abilities, it had been suggested that lactoferrin may serve as a mechanism utilized by *H. pylori* to acquire iron from the environment and to stimulate bacterial growth (Husson *et al.*, 1993). Research has identified that *H. pylori* growth in an iron-restricted environment induced the production of lactoferrin-binding proteins, which were able to bind human lactoferrin (Dhaenens *et al.*, 1997). Hence, *H. pylori* may be increasing its chance of survival within a challenging environment by taking advantage of host innate immune peptides so as to enhance its growth *in vivo*. Nevertheless, given the antimicrobial activity of lactoferrin, it has been suggested that this protein may represent a host defence strategy against *H. pylori* infection (Miehlke *et al.*, 1996; Dial *et al.*, 1998). Indeed, examination of gastric biopsies revealed that the gastric concentrations of lactoferrin were significantly higher in individuals with *H. pylori* infection and were elevated in a gastritis-dependent manner (Nakao *et al.*, 1997a,b).

The ability of lactoferrin to function as an antimicrobial against *H. pylori* has resulted in numerous efforts by researchers to utilize lactoferrins as a treatment for *H. pylori*-infected individuals, although the findings from such trials have thus far been inconclusive (Dial *et al.*, 2000; Di Mario *et al.*, 2003; Huynh *et al.*, 2009). As many of the studies on gastric lactoferrin responses preceded the discovery of PRMs, it is not clear which, if

any, of these innate immune molecules regulate the production of this antimicrobial factor during *H. pylori* infection.

8.4.2 Defensins

More recently, a second group of antimicrobial peptides, termed defensins, have been studied in relation to *H. pylori* infection. Defensins can be divided into two main classes, α and β , according to their genetic and structural features (reviewed in Ganz, 2003). Microarray studies of *H. pylori*-infected human subjects and animals have reported *H. pylori*-mediated modulation of defensin gene expression (Walduck *et al.*, 2004; Resnick *et al.*, 2006; Hornsby *et al.*, 2008).

α -Defensins

The α -defensins are produced and released by neutrophils, granulocytes and Paneth cells. These antimicrobial peptides may play a role in the local host response to *H. pylori* infection (Kocsis *et al.*, 2009). The α -defensins include the human neutrophil peptides (HNPs), which are the major components of neutrophil-derived α -defensins. Studies have revealed significantly increased levels of HNP-1, -2 and -3 in the plasma and gastric juice of *H. pylori*-infected patients, which decline after eradication of *H. pylori* (Isomoto *et al.*, 2004; Nishi *et al.*, 2005), indicating that α -defensin production is part of the response to *H. pylori* infection.

Human β -defensins

Human β -defensins (HBDs) are cationic, cysteine-rich and disulfide-linked low-molecular-weight peptides with immunomodulatory properties required for host defence from bacterial pathogens (Ganz, 2003). While these peptides are endogenously produced by epithelial cells, their expression can be up-regulated during infection (Harder *et al.*, 2000; O'Neil *et al.*, 2000; Krisanaprakornkit *et al.*, 2002). To date, six members of the HBD family have been identified (Zhao *et al.*, 1996; Harder *et al.*, 1997; Garcia *et al.*, 2001a,b;

Yamaguchi *et al.*, 2002). As discussed below, the expression levels of genes encoding several of these defensins have been shown to increase during *H. pylori* infection.

HUMAN β -DEFENSIN-1. HBD-1 is constitutively expressed in several tissues and by various epithelial cells during normal homeostasis *in vivo*, including the gastric epithelium (Zhao *et al.*, 1996; Valore *et al.*, 1998). HBD-1 has antimicrobial activity against both Gram-positive and Gram-negative bacteria, and various isoforms of HBD-1 exist, although the individual role of each isoform is unclear (Valore *et al.*, 1998). Unlike the other HBDs, HBD-1 is not significantly up-regulated during *H. pylori* infection *in vitro* or *in vivo* (Bajaj-Elliott *et al.*, 2002; Grubman *et al.*, 2010). Hence, HBD-1 is not thought to play a key role as an antimicrobial during *H. pylori* infection.

HUMAN β -DEFENSIN-2. Basal HBD-2 expression is detected in the skin, lung, trachea, urogenital and gastrointestinal tracts of man and its expression is strongly regulated (Harder *et al.*, 1997). Most significantly, this β -defensin has been shown to have a role in defending the host against Gram-negative bacterial and fungal infections at mucosal surfaces (Schroder and Harder, 1999). Indeed, recombinant HBD-2 is able to potently kill *H. pylori* in a dose- and salt-dependent manner (Hamanaka *et al.*, 2001; Uehara *et al.*, 2003). Several studies investigating the antimicrobial activity of recombinant HBD-2 have shown that complete inhibition of *H. pylori* growth is possible with as little HBD-2 as 70 $\mu\text{g/ml}$ or 10^{-5} M (Hamanaka *et al.*, 2001; George *et al.*, 2003). Moreover, as discussed above, our own work has shown that HBD-2, which was released into the culture supernatants of gastric epithelial cells stimulated with *cagPAI*-positive *H. pylori*, is effective in killing *H. pylori in vitro* (Grubman *et al.*, 2010). Initial studies showed that direct contact of *cagPAI*-positive *H. pylori* bacteria with gastric epithelial cells was essential for HBD-2 activation in response to this pathogen (Wada *et al.*, 1999). However, paired isogenic parental and *cagPAI* mutant strains were not used, thus some form of bystander effect could not be excluded. It

was subsequently shown that expression of the HBD-2 gene, *DEFB4*, by epithelial cells was dependent on both the presence in *H. pylori* bacteria of a functional T4SS as well as the actions of NOD1 (Boughan *et al.*, 2006; Grubman *et al.*, 2010).

DEFB4 expression can be induced by treatment of cells with TNF α , IL-1 or by exposure to microorganisms such as *H. pylori* (Wada *et al.*, 1999; O'Neil *et al.*, 2000; Hamanaka *et al.*, 2001). The HBD-2 promoter contains three binding sites for NF- κ B and one binding site for AP-1 (Wada *et al.*, 2001), indicating that stimulation of the NF- κ B or MAPK pathway should result in the up-regulation of HBD-2. Given that *H. pylori* activates both pathways in a T4SS- and NOD1-dependent manner (Allison *et al.*, 2009), it is therefore logical that this same mechanism should be implicated in the induction of HBD-2 expression by *cagPAI*-positive *H. pylori* bacteria.

HUMAN β -DEFENSIN-3. HBD-3 has been reported to be the β -defensin with the most potent activity against *H. pylori*. The heightened potency of this peptide has been attributed to the fact that it exists as a dimer, whereas all other HBDs exist as monomers (George *et al.*, 2003). In contrast to other HBDs, HBD-3 possesses salt-insensitive antimicrobial activity against a broad spectrum of Gram-positive and Gram-negative bacteria (Harder *et al.*, 2001; Wu *et al.*, 2003). HBD-3 can reduce the number of viable *H. pylori* by 95% at a concentration of 7 μ g/ml (George *et al.*, 2003) and can render *H. pylori* completely non-viable in as little as 1 h (Uehara *et al.*, 2003). Experimental evidence has shown that maximal levels of HBD-3 in response to *H. pylori* infection were generated 12 h post-stimulation. This is different from HBD-2, which reaches maximal levels at 6–8 h post-infection (Bajaj-Elliott *et al.*, 2002). Thus, it is possible that HBD-3 may have a more delayed effect during the acute phase of infection.

Similar to HBD-2, the expression levels of the gene encoding HBD-3, *DEFB3*, increased in response to *H. pylori* infection in gastric mucosal tissues and in gastric cancer cell lines (George *et al.*, 2003; Kawauchi *et al.*, 2006).

This increase in *DEFB3* expression may be linked to the HBD-3 promoter containing AP-1 and IFN γ binding sites (Jia *et al.*, 2001). Nevertheless, both a functional T4SS and NOD1 seemed to be dispensable for the up-regulation of HBD-3 expression in gastric epithelial cell lines (Boughan *et al.*, 2006; Grubman *et al.*, 2010). *H. pylori*-mediated *DEFB3* expression was found to be dependent on epidermal growth factor receptor (EGFR)-mediated ERK activation (Boughan *et al.*, 2006).

HUMAN β -DEFENSIN-4. HBD-4 has antimicrobial activity against Gram-positive and Gram-negative bacteria (Garcia *et al.*, 2001b). Interestingly, in contrast to other HBDs, the expression of the HBD-4 gene, *DEFB104A*, in gastric epithelial cells is not significantly affected by stimulation with proinflammatory cytokines, such as IL-1 α , IL-6, INF γ or TNF α (Otte *et al.*, 2009). Instead, it appears that p38 MAPK has a role in *DEFB104A* expression (Otte *et al.*, 2009). Similar to other HBD genes, *DEFB104A* is expressed in the gastric mucosa during *H. pylori* infection, and HBD-4 levels are greater in patients infected with CagA-positive *H. pylori* strains compared with those colonized with CagA-negative bacteria (Otte *et al.*, 2009). Nevertheless, the mechanism whereby CagA may regulate HBD-4 expression during *H. pylori* infection, including the PRM involved, has yet to be identified.

Cathelicidin LL-37

The cathelicidins comprise another group of mammalian cationic antibacterial proteins. To date, only a single cathelicidin has been identified in man: the human cationic antibacterial protein (hCAP18), which is an 18 kDa protein whose C-terminal 37-amino-acid peptide is termed LL-37 (Gennaro and Zanetti, 2000; Lehrer and Ganz, 2002). The antimicrobial function of LL-37 is based on its amphipathic α -helical structure, which is thought to integrate within or deform the membrane of bacteria, resulting in cell death (Oren *et al.*, 1999). LL-37 has been shown to have bactericidal activity against Gram-positive and Gram-negative bacteria (Bals *et*

al., 1998; Travis *et al.*, 2000). In concordance with other human antimicrobial peptides, LL-37 has LPS-binding and -neutralizing properties that function as a chemoattractant for monocytes, neutrophils and CD4⁺ T cells (Kirikae *et al.*, 1998).

LL37 mRNA has recently been identified to be constitutively expressed on the surface and upper crypts of the gastric and intestinal epithelium (Hase *et al.*, 2002, 2003). Moreover, using human gastric epithelial cells as a model, it was shown that epithelial LL-37 production was up-regulated in response to *H. pylori* infection via a *cagPAI*-dependent mechanism, but not to proinflammatory cytokines, nor to other activators of NF- κ B. Surprisingly, a synergistic effect was observed for LL-37 and HBD-1 in *H. pylori* killing (Hase *et al.*, 2003).

8.4.3 Inflammatory cells

H. pylori infection and its recognition by various PRMs results in elevated levels of pro-inflammatory chemokines that are produced by epithelial cells at the site of infection. The production of these chemokines facilitates the second stage in the innate immune response, which involves the recruitment of innate immune cells to the site of infection and the development of acute inflammation (Fig. 8.2). Neutrophils are recruited to the stomach, as are macrophages and DCs, by proinflammatory cytokines that are fundamental to the genesis of adaptive immune responses to *H. pylori*. In fact, the recruitment of DCs to the gastric mucosa occurs within 6 h post-infection, whereas after 5 days, these cells have migrated out of the tissue, possibly to draining lymph nodes (Kao *et al.*, 2006). Thus, innate immune cells, such as DCs, can activate Th CD4⁺ cells and initiate the development of the adaptive immune response. For further detail regarding the *H. pylori*-specific adaptive immune response, see Robinson and Atherton, Chapter 6, this volume. Interestingly, it was shown that NOD1 within epithelial cells regulates the development of an adaptive immune response to both inert agonists and infectious agents; of particular note, NOD1 was shown to be required for the

development of the characteristic Th1 responses to *cagPAI*-positive *H. pylori* bacteria in mice (Fritz *et al.*, 2007). In addition, Rad and colleagues (2007) reported that MyD88^{-/-} mice challenged with *H. pylori* displayed a defect in the development of immunoglobulin (Ig)G2c/IgG1 antibody responses, indicative of a Th1 response. The mechanisms whereby PRMs regulate the development of Th responses to *H. pylori* infection have, however, yet to be fully characterized.

Macrophages, in unison with DCs, are responsible for the production of IL-12 and the recruitment of Th cells to the gastric mucosa (Haeberle *et al.*, 1997; Meyer *et al.*, 2000) (Fig. 8.2). Macrophages further amplify the inflammatory response by their production of cytokines such as IL-1, TNF α and IL-6 in response to *H. pylori* antigens (Mai *et al.*, 1991; Harris *et al.*, 1998; Gobert *et al.*, 2004). IL-6 production by macrophages is linked to responses initiated by TLR4, MAPK and NF- κ B (Pathak *et al.*, 2006), and is likely to facilitate the development of IFN γ -producing T cells (Meyer *et al.*, 2000). The requirement for macrophages to initiate acute inflammation has been demonstrated *in vivo* (Karakakis *et al.*, 2008; Yanai *et al.*, 2008). Transient macrophage depletion of *H. pylori*-infected mice or Mongolian gerbils by clodronate treatment significantly reduced the gastric pathology, thus confirming the role of macrophages in the initiation of pathology. In addition, DCs were shown to produce IL-6, CXCL8, IL-10, IL-12, IL-1 β and TNF α when treated with *H. pylori* or their products (Kranzer *et al.*, 2004, 2005; Kao *et al.*, 2006). The presence of a *cagPAI* was not required for the induction of cytokines responses in DCs (Kranzer *et al.*, 2005; Kao *et al.*, 2006). Importantly, MyD88-deficient DCs were found to be affected in their abilities to secrete proinflammatory cytokines and to up-regulate MHC and co-stimulatory molecules, suggesting that TLR recognition of *H. pylori* may be important for DC activation (Rad *et al.*, 2007). Consistent with these *in vitro* data, *in vivo* studies showed that MyD88^{-/-} mice developed lower levels of gastric inflammation and increased bacterial loads in response to *H. pylori* challenge (Rad *et al.*, 2007).

8.5 Conclusions

H. pylori causes chronic inflammation within the host, resulting in disease outcomes of varying severity. This pathogen produces several products or factors that have the potential to be recognized by host PRMs. Consistent with this observation, multiple PRMs have been implicated in innate immune responses to *H. pylori*. We propose that these PRMs are likely to act in a cell type-specific manner with epithelial cells relying primarily on NOD1 and possibly TLR2. It now seems clear, however, that epithelial cells do not use TLR5 to detect *H. pylori*, nor is it likely that TLR4 plays a significant role in epithelial cell responses to the bacterium under physiological conditions.

In contrast to the restricted PRM repertoire involved in epithelial cell responses to *H. pylori*, immune cells utilize multiple PRMs, with macrophages and DCs shown to respond to *H. pylori* via TLR2/4-dependent mechanisms and even TLR-independent pathways (Bleharski *et al.*, 2003; Gobert *et al.*, 2004; Mandell *et al.*, 2004; Rad *et al.*, 2007, 2009). Whether there is any cross-talk or synergism between PRM signalling in different cell types remains to be determined. In any case, PRM 'sensing' of *H. pylori* within gastric tissues is clearly likely to play a significant role in the generation of proinflammatory cytokines by host cells. These cytokines enable inflammatory cells to be recruited to the site of infection, where they mature and progress throughout the periphery and secondary lymphoid tissues, thereby mediating the development of adaptive immune responses. We propose that the innate immune system, via its effects on these responses, is likely to influence the severity of disease outcomes induced by *H. pylori* infection.

Acknowledgements

RLF greatly acknowledges the support of the National Health and Medical Research Council of Australia, the ANZ Charitable Trust and the CASS Foundation, Victoria. Priyangi Alwis is thanked for her design and preparation of the figures used in this chapter.

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9 *Helicobacter pylori* Vaccines

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9.1 Introduction

Helicobacter pylori has been estimated to infect one-half of the world's population. These infections are not readily cleared by host inflammatory and immune responses and may persist for life. While the majority of infected individuals do not exhibit overt signs of severe disease, 10–20% are at high risk of developing peptic ulcers or gastric cancers. Combined antimicrobial and anti-acid therapy can eradicate *H. pylori* infections, but not without significant side-effects or risks for the development of antibiotic-resistant strains (see Mégraud, Chapter 4, this volume). The infection is also so widespread that antimicrobial treatment is an impractical approach for controlling *H. pylori*-associated disease. In addition, animal model studies and anecdotal reports for human infections have shown that antimicrobial 'cure' of *H. pylori* infection does not prevent re-infection. For these reasons, vaccination has been suggested as an alternative approach for the control of *H. pylori* infection and disease. Additionally, most *H. pylori*-infected subjects who develop gastric cancer typically remain asymptomatic until progression of the cancer is well established. Widespread vaccination in populations with higher risks for gastric cancer would prevent

the incidence of gastric cancer without having to identify specific subjects harbouring *H. pylori* infections (Rupnow *et al.*, 2001).

9.2 Candidate Antigens for Immunization Against *H. pylori*

Small animal models, particularly the mouse, have been used extensively to identify both candidate vaccine antigens and adjuvant/delivery systems for an *H. pylori* vaccine (Nedrud, 2001). The initial studies demonstrating the efficacy of a mucosal vaccine against *Helicobacter* infection in mice were performed with whole-cell preparations or crude whole-cell lysates (Chen *et al.*, 1993; Czinn *et al.*, 1993). These preparations have continued to be used by several laboratories for the study of the protective immune response. However, many laboratories soon began investigating specific purified and recombinant proteins, with less potential for undesirable side-effects, as candidates for subunit vaccines in mice. These antigens are involved in virulence, adhesion and normal bacterial activity in the gastric mucosa and some of the proteins tested for efficacy in mice include urease subunits (Lee *et al.*, 1995), cytotoxin-associated gene A protein (CagA)

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and vacuolating cytotoxin (VacA) (Ghiara *et al.*, 1997), catalase (Radcliff *et al.*, 1997), heat-shock protein A (GroES) (Ferrero *et al.*, 1995), *H. pylori* adhesin A (Sutton *et al.*, 2007) and neutrophil-activating protein (NAP) (Satin *et al.*, 2000), as well as others. The rationale for selecting specific proteins includes blocking adhesion, preventing an important activity or neutralizing a virulence mechanism.

There is some debate on the merit of using certain proteins as antigens. Urease, for instance, despite its size and complexity, is not immunodominant in many infected subjects where it has been shown to induce only a weak antibody response (Leal-Herrera *et al.*, 1999). CagA, one of the most immunogenic factors in the infected host, is not present in all strains of *H. pylori*. Three observations seem salient, however, when considering the construction of an effective subunit vaccine.

First, all of these proteins must induce strong immune responses when delivered as vaccine antigens in the context of an appropriate adjuvant, regardless of the immunogenicity displayed during chronic infection.

Secondly, almost every *H. pylori* challenge that has been tested in mice in a challenge model against *H. pylori* or *Helicobacter felis* infection has been, in general, as efficacious as the others. Although biopharmaceutical companies may have utilized the published sequences of the *H. pylori* genome to evaluate vaccine candidates that subsequently proved to have poor efficacy, there does not seem to be a shortage of prospective antigens. The variety of candidate vaccine antigens is particularly instructive when considering antigens such as catalase (Radcliff *et al.*, 1997) that are not present on the cell surface. The role of antibodies in limiting *H. pylori* infection or in contributing to vaccine-induced immunity remains controversial but, as discussed below, mice deficient in antibody production can be protectively immunized against *Helicobacter* infection (Ermak *et al.*, 1998; Blanchard *et al.*, 1999a; Gottwein *et al.*, 2001; Akhiani *et al.*, 2004). These observations, combined with a growing literature that reports bacterial loads inversely proportional to the degree of inflammation in the gastric mucosa (Garhart *et al.*, 2002), suggest that a cell-mediated, proinflammatory response can

be important for bacterial eradication. Thus, using effective adjuvants or delivery systems (see below) that can become re-stimulated upon challenge with an infectious organism to promote a protective gastritis may be one mechanism for protective immunity. As the final effect of such an inflammatory response on the bacterium is non-specific, the location of those antigens on the bacterium becomes less important.

Finally, there is evidence that multiple antigens may be necessary for the development of a truly protective vaccine against *H. pylori*. This concept was first promoted by Ferrero *et al.* (1995) who noted that studies by other investigators with urease were yielding protection in approximately 80% of immunized mice. To address this problem, in the *H. felis* model, they performed oral immunizations with a combination of recombinant *H. pylori* heat-shock protein A (HspA) and urease in mice. The dual antigen immunization induced 100% protection from challenge with *H. felis*, whereas either component given individually induced only 80% protection. It is worth noting that the studies in non-human primates and human subjects described below, in which urease has been used either in its purified or expressed form, have been disappointing in terms of both efficacy and immunogenicity. Murine studies often report 'protection' as a significant reduction in bacterial load. Conversely, some studies in mice in which whole-cell lysates continue to be used as the immunogen do sometimes achieve complete protection as determined by the inability to grow *H. pylori* from gastric biopsies (Garhart *et al.*, 2002). Additionally, a multivalent subunit vaccine that incorporates VacA, CagA and NAP, currently in development for use in humans, was shown to be protective in a therapeutic study using beagles as measured by immunohistochemical detection of *H. pylori* in histological sections (Rossi *et al.*, 2004). Another multivalent vaccine consisting of urease B subunit (UreB), HspA and the surface-localized protein HpaA, delivered either orally with a mutant heat-labile toxin or parenterally with alum, induced greater protection against *H. pylori* in Mongolian gerbils than did mono- or bivalent vaccines (Wu *et al.*, 2008).

9.3 Vaccine Adjuvants/Delivery Systems and Routes of Immunization

Because of the small size, low price and easy manipulation of mice, *H. felis* and then *H. pylori* mouse models became the models of choice for early stage *H. pylori* vaccine studies in the 1990s. Thus the first successful demonstrations of *Helicobacter* vaccine feasibility used these models, plus the germ-free piglet. Formalin-killed *H. pylori* delivered subcutaneously in incomplete Freund's adjuvant to germ-free pigs (Eaton and Krakowka, 1992), live *H. felis* delivered intraperitoneally to mice (Chen *et al.*, 1993) and *H. felis* lysate with cholera toxin (CT) adjuvant delivered by oral gavage (Chen *et al.*, 1993; Czinn *et al.*, 1993) were all able to reduce *Helicobacter* colonization in these first trials. Oral vaccination of mice then dominated subsequent vaccine trials in the 1990s as many investigators moved away from bacterial lysate and killed whole-cell vaccines towards purified and recombinant antigens (detailed above). Although CT and the closely related heat-labile toxin of *Escherichia coli* (LT) were then, and remain today, the 'gold standards' of mucosal adjuvants, it was recognized early on that CT and LT use in human subjects would not be practical because of the watery diarrhoea they induce. Similar to its use as a primary screen for vaccine antigens, the mouse has also been used to screen alternative adjuvants/delivery systems and routes of immunization for *H. pylori* vaccines. Table 9.1 lists published results of mouse immunization experiments using various adjuvants/delivery systems and routes of vaccination where a challenge versus *H. felis* or *H. pylori* was reported. Not included in the table are results where immunogenicity was evaluated in the absence of a bacterial challenge. The balance of this section highlights some of the results from the table. Many of the results obtained in the studies from the pre-clinical mouse models summarized here subsequently made their way into human and non-human primate vaccine trials, which are described in Section 9.6.

9.3.1 CT- and LT-based adjuvants

Because *H. pylori* is a gastrointestinal pathogen, most of the earliest vaccine studies involved oral immunization and the well-established mucosal adjuvants CT and LT. CT and LT are composed of non-toxic pentameric B (binding subunits), non-covalently linked to an enzymatically active A subunit that mediates toxicity (Spangler, 1992). The B subunit binds to ubiquitous gangliosides that are widely expressed on a variety of mammalian cell surfaces. The A subunit has been determined to be essential for intestinal adjuvanticity, as the use of recombinant B subunit was shown to lack oral adjuvant activity (Blanchard *et al.*, 1998), although chemical coupling of an *H. pylori* antigen to an undefined B subunit was reported to yield a protective oral vaccine (Ruiz-Bustos *et al.*, 2000), as was the parenteral delivery (avoids gut toxicity) of an LT or recombinant LT-B subunit-adjuvanted *H. pylori* vaccine (Weltzin *et al.*, 2000). Another approach to reduce the toxicity of CT- or LT-based adjuvants is to mutate the A subunit active site. This approach has been evaluated against several pathogenic microorganisms, including *H. pylori* (Ghiara *et al.*, 1997; Marchetti *et al.*, 1998; Cardenas-Freytag *et al.*, 1999). Yet a third approach to eliminate the toxicity of CT/LT has been to replace the B subunit, which targets many cell types including intestinal epithelium, with another targeting motif. When the CTB subunit was replaced by two copies of the protein A immunoglobulin-binding domain ('D') it yields an adjuvant named CTA1-DD that was determined to be non-toxic and demonstrated to possess murine intranasal adjuvant activity that induced a reduction in bacterial loads when mice were challenged with *H. pylori* (Akhiani *et al.*, 2006). This novel approach retains the enzymatically active portion of CT, which has been shown to be required for optimal adjuvant function, but eliminates toxicity by targeting this activity towards cells of the immune system rather than the epithelium.

Table 9.1. Adjuvant/delivery system and route of vaccination comparison for *Helicobacter pylori* vaccines in rodents.^a

Adjuvant/delivery system ^b	Route ^c	<i>H. pylori</i> antigen ^d	Protection ^e	Reference(s)
CT	IN	Lysate	<i>H. pylori</i> + S	Garhart <i>et al.</i> (2002)
pCTB	Oral	Lysate	<i>H. felis</i> + S	Lee and Chen (1994)
rCTB	Oral	Lysate	<i>H. felis</i> –	Blanchard <i>et al.</i> (1998)
CTB-couple	Oral	HSBP	<i>H. pylori</i> + S	Ruiz-Bustos <i>et al.</i> (2000)
LT	Oral	Urease	<i>H. pylori</i> + S	Weltzin <i>et al.</i> (2000)
LTB	Oral	Urease	<i>H. pylori</i> –	
LT, LTB, No adjuvant	Subcutaneous	Urease	<i>H. pylori</i> + S	
LT	Oral	Urease	<i>H. pylori</i> + S	Kleanthous <i>et al.</i> (2001)
	Rectal	Urease	<i>H. pylori</i> + S	
	Rectal	CagA	<i>H. pylori</i> –	
	Rectal	Urease + CagA	<i>H. pylori</i> + S	
LT	Oral, IN, rectal	Urease	<i>H. pylori</i> + S	Kleanthous <i>et al.</i> (1998)
CT	Oral	Urease, UreA, UreB	<i>H. felis</i> + S	Michetti <i>et al.</i> (1994); Lee <i>et al.</i> (1995)
		HspA, HspB, HspA + UreB	<i>H. felis</i> + S	Ferrero <i>et al.</i> (1995)
LTK63	Oral	CagA, VacA, urease	<i>H. pylori</i> + S	Ghiara <i>et al.</i> (1997); Marchetti <i>et al.</i> (1998)
CT	IN	Urease	<i>H. felis</i> + S	Weltzin <i>et al.</i> (1997)
None	IN	Urease	<i>H. felis</i> –	
CTA1-DD	IN	Lysate	<i>H. pylori</i> + S	Akhiani <i>et al.</i> (2006)
CpG	IN	Lysate	<i>H. pylori</i> + S	Shi <i>et al.</i> (2005)
	Oral	Lysate	<i>H. pylori</i> –	
CpG	Subcutaneous	Lysate	<i>H. pylori</i> + S	Sommer <i>et al.</i> (2004)
CpG+CT	IN	Lysate	<i>H. felis</i> + S	Jiang <i>et al.</i> (2003)
CpG	Oral	No antigen therapeutic	<i>H. pylori</i> + S	Raghavan <i>et al.</i> (2003)
Chitosan	Oral	Lysate	<i>H. pylori</i> + S	Xie <i>et al.</i> (2007)
ISCOM™, ISCOMATRIX™	IN, subcutaneous	HpaA, catalase	<i>H. pylori</i> + S	Skene <i>et al.</i> (2008)
Mannan	Oral, IN, IP	Lysate	<i>H. pylori</i> –	Skene (2009)
No adjuvant	Subcutaneous	Lysate, formalin whole cell	<i>H. pylori</i> + S	Harbour <i>et al.</i> (2008)
No adjuvant	IP	Live <i>H. felis</i>	<i>H. felis</i> + S	Chen <i>et al.</i> (1993)
LT	Oral	Urease	<i>H. pylori</i> + S	Guy <i>et al.</i> (1998)
Saponin, Bay	Subcutaneous	Urease	<i>H. pylori</i> + S	
LT	Intranasal	Urease	<i>H. pylori</i> + S	Ermak <i>et al.</i> (1998)
Alum, Bay	Subcutaneous	Urease	<i>H. pylori</i> + S	
LT–alum (1°–2°)	IN, subcutaneous	Urease	<i>H. pylori</i> + S	
LT–Bay (1°–2°)	IN, subcutaneous	Urease	<i>H. pylori</i> + S	
Alum	IP	Lysate	<i>H. pylori</i> + S	Gottwein <i>et al.</i> (2001); Eisenberg <i>et al.</i> (2003)
IFA	Subcutaneous	Lysate	<i>H. pylori</i> + S	
CFA	IP, subcutaneous	Lysate	<i>H. pylori</i> + S	
Alum, CFA	IP	Lysate	<i>H. felis</i> + S	
M-cell targeting	Oral	Formalin whole cell	<i>H. pylori</i> + S	Chionh <i>et al.</i> (2009)
	Oral	Lysate	<i>H. pylori</i> –	
Bacterial ghost	Oral	Bacterial ghost	<i>H. pylori</i> + S	Pantheil <i>et al.</i> (2003b)
Bacterial ghost+CT	Oral	Bacterial ghost	<i>H. pylori</i> + S	
CT/outer membrane vesicles	Oral	Lpp20 in vesicles	<i>H. pylori</i> + S	Keenan <i>et al.</i> (2000)
Polio replicon	Oral	UreB	<i>H. pylori</i> + S	Smythies <i>et al.</i> (2005)

Table 9.1. *Continued*

Adjuvant/delivery system ^b	Route ^c	<i>H. pylori</i> antigen ^d	Protection ^e	Reference(s)
LT fusion protein in microsphere	Oral	UreB	<i>H. pylori</i> +	Zou <i>et al.</i> (2008)
<i>Salmonella</i>	IN	Urease	<i>H. pylori</i> +	Corthesy-Theulaz <i>et al.</i> (1998)
<i>Salmonella</i>	Oral	Urease	<i>H. pylori</i> +	Gomez-Duarte <i>et al.</i> (1998); DiPetrillo <i>et al.</i> (1999)
<i>Salmonella</i> – 1°	IN 1°	Urease	<i>H. pylori</i> +	Londono-Arcila <i>et al.</i> (2002)
Alum – 2°	Subcutaneous 2°	Urease	<i>H. pylori</i> +	
Lactobacillus	Oral	UreB	<i>H. felis</i> +	Corthesy <i>et al.</i> (2005)
DNA	Intracutaneous	HspA, HspB	<i>H. pylori</i> +	Todoroki <i>et al.</i> (2000)
DNA	Intracutaneous or IN	Catalase	<i>H. pylori</i> +	Miyashita <i>et al.</i> (2002)
DNA	IN	UreB	<i>H. pylori</i> +	Hatzifoti <i>et al.</i> (2006)
CT	Oral	Lysate	<i>H. pylori</i> +	Dzwonek <i>et al.</i> (2004)
DNA	IM	UreB	<i>H. pylori</i> –	
DNA	ID	UreB	<i>H. pylori</i> + S	
DNA	IM or ID	Genomic library	<i>H. pylori</i> + S	
DNA	IN, oral, rectal, IM	UreB	<i>H. pylori</i> –	Zavala-Spinetti <i>et al.</i> (2006)
<i>Salmonella</i> -delivered DNA	Oral	UreB + IL2	<i>H. pylori</i> +	Xu <i>et al.</i> (2007)
CT (gerbils)	Oral	Lysate	<i>H. pylori</i> + S	Jeremy <i>et al.</i> (2006)

^aAll experiments done in mice unless otherwise specified. Only studies where an animal challenge was performed with *Helicobacter felis* or *H. pylori* are included.

^bAbbreviations: CT, cholera toxin; LT, heat-labile toxin of *Escherichia coli*; pCTB, CTB subunit purified from holotoxin; rCTB, recombinant CTB subunit produced in *E. coli*; CTB-couple, CTB subunit (p or r, not specified) covalently coupled to antigen; LTK63, mutated LT with lysine substitution at position 63; Bay, glycolipid-peptide proprietary adjuvant; 1°, primary immunization; 2°, secondary (boosting) immunization; IFA, incomplete Freund's adjuvant; CFA, complete Freund's adjuvant; Polio replicon, polio capsid engineered to express an antigen without any polio RNA.

^cAbbreviations: IN, intranasal; IP, intraperitoneal; IM, intramuscular; ID, intradermal.

^dAbbreviations: HSBP, heparin sulfate binding proteins; CagA, cytotoxin-associated gene A protein; UreA, urease A subunit; UreB, urease B subunit; HspA, heat-shock protein A; HspB, heat-shock protein B; VacA, vacuolating cytotoxin; HpaA, a surface-localized protein; Lpp20, lipoprotein 20; IL, interleukin.

^eProtection: *H. felis* or *H. pylori* colonization significantly less in immunized/challenged group compared with unimmunized/challenged group (+). S indicates at least some animals in the immunized/challenged group had undetectable levels of bacteria (sterilizing immunity). Groups where vaccination did not reduce colonization are designated –.

9.3.2 Other adjuvants

In parallel with advances in the adjuvant field in general, a number of additional adjuvants and combinations of adjuvants have been tested for their capacity to enhance *H. pylori*/*H. felis* immunogenicity and to improve vaccine performance in challenge experiments (Table 9.1). Included are CpG oligonucleotides,

biodegradable microspheres, saponins, chitosan, muramyl dipeptide (MDP), and more established adjuvants such as alum, Freund's complete and incomplete adjuvants, and no adjuvant at all. One popular approach has been the use of immunostimulatory CpG oligonucleotides, Toll-like receptor-9 agonists which have been used both parenterally and mucosally and are adept at stimulation of

T-helper 1 type (Th1) immune responses (Krieg, 2006). Because Th1 immunity appears to be important in *H. pylori* immunity (see Section 9.4 on mechanisms of vaccine-induced *H. pylori* immunity), several laboratories have investigated CpGs for *Helicobacter* vaccines (Table 9.1). In particular, when mice were immunized intranasally with *H. felis* lysate and a combination of CT and immunostimulatory CpGs, sterilizing immunity to *H. felis* was achieved (Jiang *et al.*, 2003). Another recent approach has been the use of ISCOMSTM (immune-stimulating complexes), which are saponin-cholesterol-phospholipid cage-like structures with incorporated antigen (Sjolander *et al.*, 1998). Empty cage-like structures (ISCOMATRIXTM) can be mixed with antigen and also have an adjuvant function. These adjuvants are able to induce both antibody and cell-mediated immune responses (Sjolander *et al.*, 1998). When delivered intranasally or subcutaneously along with recombinant antigens (catalase or HpaA), they induced protective immunity equivalent to CT (Skene *et al.*, 2008). Another interesting approach, which did not involve an adjuvant per se, used a lectin known to bind a glycan expressed on M cells of murine Peyer's patches to target killed whole *H. pylori* to M cells and induce protective immunity (Chionh *et al.*, 2009). Bacterial 'ghosts', which are membrane structures devoid of cytoplasm, and bacterial outer membrane vesicles have also been used successfully (Keenan *et al.*, 2000; Panthel *et al.*, 2003b).

9.3.3 Vector vaccines and DNA vaccines

Another popular approach for vaccination is to use a live attenuated vector or a DNA vaccine. Several laboratories have evaluated attenuated *Salmonella* bacteria as a vector for oral, intranasal or mucosal-prime, parenteral-boost vaccination protocols. While these studies have enjoyed moderately good success in the mouse (Table 9.1), recombinant *Salmonella* vaccines for *H. pylori* have been disappointing in human trials (see Section 9.6). Polio virus replicons that lack viral RNA but can carry heterologous epitopes have also been used successfully in a mouse *H. pylori* vaccine

study (Smythies *et al.*, 2005). Plasmid DNA encoding vaccine antigens can be delivered either as naked DNA or by a vector such as *Salmonella*. Several DNA vaccines for *H. pylori* have been evaluated in mouse models and, in one trial, DNA genomic libraries were used to immunize mice and achieved up to 90% sterilizing immunity (Dzwonek *et al.*, 2004).

9.3.4 Immunization routes

Mucosal immunization

H. pylori infection is recognized as a relatively non-invasive colonizer of the gastric mucosa and, as such, vaccine strategies have largely mimicked those employed for other enteric and mucosal bacterial infections. Immunizations therefore have predominantly been applied to optimize induction of immune responses at mucosal surfaces including oral/gastric, intranasal and rectal administrations, in combination with mucosal adjuvants or delivery vehicles. The earliest studies concentrated on oral immunization using CT or LT adjuvants. However, successful intranasal and rectal immunization has been reported by several groups (Weltzin *et al.*, 1997; Kleanthous *et al.*, 2001; Garhart *et al.*, 2003; Jiang *et al.*, 2003). Garhart and colleagues showed that intranasal immunization was much more effective than oral immunization and in fact could lead to undetectable levels of *H. pylori* in immunized mice (Garhart *et al.*, 2002). This may actually represent 'sterilizing immunity', which has been infrequently reported for *H. pylori* vaccines. A close examination of the literature reveals, however, several other instances where both intranasal and other mucosal vaccinations have yielded sterilizing immunity against *H. pylori*. One such case involved an *H. pylori* strain that colonized mice only at very low levels and rectal immunization with a recombinant antigen plus LT (Kleanthous *et al.*, 2001). An oral sonicate plus CT immunization study in gerbils also reported sterilizing immunity, but the magnitude of infection in unimmunized animals was not reported (Jeremy *et al.*, 2006). A small number of mice immunized orally with *H. pylori* bacterial ghosts also

appeared to achieve sterilizing immunity (Panthel *et al.*, 2003b). Finally, as mentioned above, *H. pylori* DNA genomic library vaccines and intranasal lysate immunization with a combination of CT and CpG adjuvants also achieved sterilizing immunity in mice (Jiang *et al.*, 2003; Dzwonek *et al.*, 2004).

Thus, although many of the published *H. pylori* vaccination trials listed in Table 9.1 have reported 'protection' as a reduction in gastric *H. pylori* bacterial load of only ten- to 100-fold (sometimes even less) when compared with non-immunized challenged animals, it has been possible to achieve sterilizing immunity with several different types of immunization.

Parenteral immunization

Although major efforts have been expended in the development of mucosal *H. pylori* vaccines, there is also considerable evidence that *H. pylori* infection induces inflammation that recruits lymphocytes from the circulation. Thus, immunity to *H. pylori* is not, strictly speaking, limited to effector mechanisms of the mucosal immune system. The induction of protective immunity therefore may not require mucosal immunizations and may be possible through more traditional parenteral vaccination strategies. In retrospect, one of the very first mouse studies in which oral immunization was demonstrated to provide protective immunity to mice against *H. felis* also provided evidence that parenteral immunization might serve as an alternative strategy for *Helicobacter* immunotherapy (Chen *et al.*, 1993). Whereas the authors focused on oral immunization, five weekly doses of live *H. felis* into the peritoneum induced protection in 55% of challenged mice. Several additional studies in mice have confirmed the potential of parenteral immunization (Table 9.1).

For example, Ermak *et al.* (1998) demonstrated that subcutaneous immunization of mice with urease antigen in combination with alum induced significant protection as measured by urease activity in gastric biopsies, although protection was much reduced when determined by bacterial load. Guy *et al.* (1998) also employed subcutaneous immunization using several different experimental adjuvants, including a saponin derivative, a

glyco-lipopeptide, a lipid-based formulation and a phosphopolymer. Each of these formulations induced reductions in bacterial load compared with non-immune controls, although only the saponin derivative and glyco-lipopeptide achieved significant protection. Weltzin *et al.* (2000) tested the mucosal adjuvant LT for its potential to work as an adjuvant when administered with urease subcutaneously, and were able to achieve protection that was equivalent to mice immunized with the previously established protocol for oral immunization. Our own group has also investigated the efficacy of systemic immunization in the mouse model, choosing aluminium hydroxide (alum) as the candidate adjuvant, as this adjuvant is the only one licensed for use in the USA. We demonstrated that a single injection of bacterial lysate plus alum was sufficient to induce protective immunity equivalent to oral vaccination when challenging with *H. pylori* (Gottwein *et al.*, 2001). We then demonstrated that this immunization strategy could induce similar immunity when administered to neonatal mice less than 24 h old, thereby demonstrating the potential use of systemic immunization against *H. pylori* in children (Eisenberg *et al.*, 2003). Parental vaccines for *H. pylori* immunity have been tested in non-human primates and more recently in early human clinical trials (see Section 9.6.2).

9.4 What are the Mechanisms of Vaccine-induced *H. pylori* Immunity?

9.4.1 The role of antibodies

H. pylori is generally assumed to be primarily an extracellular bacterium that should be susceptible to antibody effector mechanisms. This fact, along with observations in early human studies that gastric secretions from *H. pylori*-positive patients contained anti-*H. pylori* antibodies and antibody-coated *H. pylori* (Rathbone *et al.*, 1986; Wyatt *et al.*, 1986), as well as the fact that antibodies could be detected in gastric secretions of orally immunized animals (Czinn and Nedrud, 1991; Lee *et al.*, 1995; Ferrero *et al.*, 1997), led to the

hypothesis that anti-*Helicobacter* antibodies were responsible for vaccination-mediated protective immunity. Further support for antibodies came from passive oral monoclonal immunization studies and a 'backpack tumour' passive monoclonal antibody model, which reduced *H. felis*/*H. pylori* colonization in infected mice (Czinn *et al.*, 1993; Blanchard *et al.*, 1995; Keenan *et al.*, 2000). In a similar manner, therapeutic administration of bovine colostrum preparations from *H. pylori*-immunized cattle, or a monoclonal antibody recognizing an *H. pylori* urease fragment, reduced colonization levels in *H. pylori*-infected adult mice (Casswall *et al.*, 2002; Marnila *et al.*, 2003; Hifumi *et al.*, 2008). There is also considerable evidence that human breast milk can contain anti-*H. pylori* antibodies and that breastfeeding can delay or reduce the incidence of *H. pylori* infections in infants. This was first reported for Gambian subjects (Thomas *et al.*, 1993, 2004) and the topic has recently been extensively reviewed (Chak *et al.*, 2009). Combined, all of these studies are highly suggestive that anti-*H. pylori* antibodies can therapeutically reduce *H. pylori* infections and by inference could be an effector mechanism in prophylactic vaccination as well.

In contrast to the positive evidence for antibody-mediated vaccine protection from *H. pylori* infection, there is considerable evidence that prophylactic immunization does not require antibodies. First, we and others have observed that 'protection' does not always correlate with the magnitude of the anti-*Helicobacter* antibody response. A striking example of this was provided by Weltzin and colleagues in the late 1990s (Weltzin *et al.*, 1997). In that study, mice were intranasally immunized with recombinant *H. pylori* urease 15 times in the absence of adjuvant, or four times in the presence of CT adjuvant, over 3 weeks. Mice immunized in the absence of adjuvant achieved equivalent or even higher levels of serum immunoglobulin (Ig) G and mucosal IgA anti-*H. pylori* urease antibodies than did mice immunized in the presence of adjuvant. When challenged with *H. felis*, however, only one of ten mice immunized in the absence of adjuvant was protected from infection, while nine of ten mice immunized in the presence of CT were protected. In

addition, by 10 weeks post-challenge, non-immunized challenged mice also achieved equivalent levels of antibody yet remained infected (Weltzin *et al.*, 1997). It is of course possible that immunization in the presence of a suitable adjuvant alters the specificity, affinity, capacity to agglutinate bacteria, or another critical characteristic of the induced antibody response. Indeed, we observed alterations in the specificity of the anti-*Helicobacter* antibody response in protectively immunized mice relative to infected but non-immunized mice and in a patient who spontaneously cleared his infection (Blanchard *et al.*, 1999b,c) and recent evidence suggests that agglutinating antibodies may be protective (Skene, 2009).

To more directly address the role of antibodies in vaccine-mediated protection from *Helicobacter* infections, we and others utilized both IgA-deficient and pan-antibody-deficient μ MT mice. In either oral or parenteral prophylactic immunization against both *H. pylori* and *H. felis* (Ermak *et al.*, 1998; Blanchard *et al.*, 1999a; Gottwein *et al.*, 2001; Akhiani *et al.*, 2004) or therapeutic oral immunization against *H. pylori* (Sutton *et al.*, 2000), antibody-deficient mice were uniformly as well protected against *Helicobacter* infections as were wild-type animals. Moreover, protection could not be transferred by passive administration of serum antibodies from successfully immunized mice (Ermak *et al.*, 1998). In addition, although not a vaccine study, human patients with IgA deficiency did not exhibit any apparent increase in *H. pylori* infection, based on seropositivity (Bogstedt *et al.*, 1996). It has even been suggested that the presence of antibodies may facilitate the persistence of *H. pylori* in the stomachs of wild-type or interleukin (IL)-10-deficient mice (Akhiani *et al.*, 2004, 2005). This conclusion was reached after observing that antibody-deficient mice had approximately 300-fold fewer bacteria colonizing their gastric mucosa than did wild-type animals, 8 weeks after infection with *H. pylori*. This reduction in bacterial load was accompanied by an increased gastritis score and an increased number of infiltrating CD4⁺ T cells (Akhiani *et al.*, 2004). In a similar manner, IgA^{-/-} $\times$ IL-10^{-/-} double knockout mice displayed enhanced gastritis, enhanced gastric CD4⁺ T cell levels and reduced bacterial load

(Akhiani *et al.*, 2005) compared with IL-10^{-/-} single knockout mice. Thus not only were antibodies not needed for prophylactic vaccine protection, but their presence may have actually impeded clearance of *H. pylori* in non-immunized mice.

Overall, the evidence suggests that antibodies can influence immunity to *H. pylori* in either a positive or a negative way, but that antibodies are not required for vaccine-mediated immunity to *H. pylori*. These conclusions should be tempered, however, by the fact that they are largely drawn from experiments in mice.

9.4.2 *H. pylori* vaccines and cell-mediated immunity

The absence of a requirement for antibody in vaccine-mediated protective immunity to *H. pylori* implicates some type of cell-mediated immunity. This was first suggested when adoptively transferred spleen cells from orally immunized mice, or an *H. felis*-specific CD4⁺ T-cell line, reduced the bacterial burden in naive recipient mice challenged with *H. felis* (Mohammadi *et al.*, 1997). A role for CD4⁺ T cells in mediating vaccine-mediated immunity was confirmed in experiments in which vaccinated major histocompatibility complex class II knockout mice (CD4⁺ T-cell-deficient) were not protected from *H. pylori* infection (Ermak *et al.*, 1998; Pappo *et al.*, 1999).

Post-immunization gastritis

Enhanced gastritis has also been frequently observed after *H. pylori* challenge of prophylactically immunized mice. This enhanced inflammation, termed 'post-immunization gastritis', was observed as early as 1994 by Michetti and colleagues, who protectively immunized mice with an *H. pylori* lysate or urease plus CT (Michetti *et al.*, 1994). In a year-long kinetic study in mice, we observed that prophylactic immunization of mice did not yield immediate protection from infection, but rather clearance of gastric *H. pylori* began a few days after bacterial challenge and was coincident with the development of post-immunization gastritis (Garhart *et al.*, 2002).

Enhancement of gastritis by vaccination against an organism where inflammation represents a major component of the disease is a significant worry. However, Garhart *et al.* (2002) showed that post-immunization gastritis can resolve over time to background or to levels no higher than observed in infected mice. It is worth noting that the uninfected stomach of both mice and man, unlike the lower gastrointestinal tract, generally lacks organized and diffuse mucosal lymphoid tissue or even white blood cells in general. Thus it may be that for bacterial clearance to occur, some level of white blood cell recruitment (inflammation) must occur and would naturally remain present until the bacteria are eradicated.

Post-immunization gastritis has been observed so frequently in mouse immunization experiments that it has become the accepted norm and experiments without post-immunization gastritis are considered exceptions. Two such exceptions have been reported recently. In the first, Mongolian gerbils were orally immunized three times with bacterial lysate plus CT before challenge with a homologous strain of *H. pylori*. Six weeks later bacterial load and gastric inflammation were measured. No *H. pylori* could be detected in the majority of immunized challenged gerbils and no post-immunization gastritis was observed (Jeremy *et al.*, 2006). This is an exciting result that adds credence to the concept of *H. pylori* vaccination. A kinetic study of bacterial clearance and gastritis would be interesting in this model to see if any parallels with mouse studies exist, or to determine if perhaps some post-immunization gastritis does occur but resolves more quickly than in the mouse. The second exception occurred when BALB/c mice were orally immunized with killed whole bacteria targeted to M cells with a lectin, inducing protection against *H. pylori* challenge, without any gastritis (Chionh *et al.*, 2009). Although BALB/c mice exhibit low levels of gastritis in response to *H. pylori* and *H. felis* infections (Mohammadi *et al.*, 1996; Sakagami *et al.*, 1996), this seems an unlikely reason for the absence of post-immunization gastritis in this experiment, as other laboratories have observed post-immunization gastritis in

BALB/c mice (Michetti *et al.*, 1994; J.G. Nedrud *et al.*, unpublished). Perhaps alternative mechanisms of protective immunity such as the induction of non-inflammatory agglutinating antibodies (Skene, 2009) could explain these results. In spite of these exceptions, it does seem clear that, in many cases of prophylactic immunization, clearance of *H. pylori* from mice is associated with enhanced, but often transient, gastritis.

Th1 immune responses

Humans and animals both develop a Th1, interferon- γ (IFN γ)-producing T-cell predominant response to *H. pylori* infections (Svennerholm and Lundgren, 2007), and it is generally accepted that Th1 responses are most important for *Helicobacter* immunity (Taylor *et al.*, 2008). The role of the signature Th1 cytokine, IFN γ , remains controversial. At least two laboratories have reported that IFN γ is critical for protective immunity in mice (Akhiani *et al.*, 2002; Sayi *et al.*, 2009), while two other laboratories have found it to be less important (Sawai *et al.*, 1999; Garhart *et al.*, 2003). It is not easy to resolve these contradictory results, although numerous laboratory to laboratory differences exist, including the antigens and adjuvants used, the route of immunization, assay time points and the end point used to define protective immunity.

One area on which most laboratories agree is the critical importance of IL-12p40 in anti-*Helicobacter* immunity. IL-12p40-deficient mice failed to achieve protective immune responses after either oral or intranasal immunization with *H. pylori* lysate plus CT adjuvant (Akhiani *et al.*, 2002; Garhart *et al.*, 2003). Because the p40 subunit is shared by IL-12 and IL-23, however, defective protective immunity in these animals could be due to the lack of IL-12, the lack of IL-23, or both. Panthel *et al.* (2003a) tested IL-12p35 null mice and observed mixed results depending on the genetic background of the mouse strain. IL-12-deficient p35^{-/-} mice on the C57BL/6 background were only weakly colonized and exhibited a poor vaccine response, while p35^{-/-} mice on the BALB/c background were colonized at 500% of wild-type BALB/c mice and exhibited a robust vaccine response

(Panthel *et al.*, 2003a). We have also examined p35^{-/-} C57BL/6 mice and, contrary to Panthel, found good colonization but an inconsistent vaccine response from experiment to experiment (J.G. Nedrud, H. Ding *et al.*, unpublished data). p19-deficient mice have not yet been evaluated, so it remains unclear whether IL-12, IL-23 or both play a role in vaccine-mediated protective immunity.

IL-23 and IL-17

The IL-23/IL-17 pathway has also been investigated for its potential role in *H. pylori* immunity. Originally associated with autoimmune inflammatory responses, an IL-23/IL-17 axis has been found to play a role in other inflammatory responses such as inflammatory bowel disease (reviewed in Maloy, 2008) and defence against pathogenic microorganisms (reviewed in Curtis and Way, 2009). Luzzza and colleagues reported enhanced levels of both IL-17 mRNA and protein in gastric biopsies from *H. pylori*-infected patients relative to uninfected controls (Luzzza *et al.*, 2000). They also demonstrated that IL-17 production was involved in neutrophil recruitment via stimulation of IL-8. More recently, this group demonstrated that IL-23 was involved in the regulation of IL-17 production in biopsies from *H. pylori*-infected patients (Caruso *et al.*, 2008). Results consistent with this were observed in *H. pylori*-infected wild-type mice, where neutrophil recruitment was shown to be blunted in IL-17 knockout animals, coinciding with a concomitant increase in bacterial load (Shiomi *et al.*, 2008).

These results led other investigators to investigate the role of IL-17 in vaccine-mediated protection from *H. pylori*. One vaccine study demonstrated that T cells from mice immunized intranasally with *H. pylori* produced significant amounts of IL-17 when stimulated with *H. pylori*-pulsed antigen-presenting cells *in vitro* (DeLyria *et al.*, 2009). Gastric tissue from these immunized mice also expressed significant amounts of mRNA for IL-17 and the neutrophil-activating chemokines LIX (lipopolysaccharide-induced CXC), KC (keratinocyte-derived) and MIP-2 (macrophage inflammatory protein-2) after *H. pylori* challenge. An influx of neutrophils

was noted to coincide with a drop in *H. pylori* numbers (protection). Depletion of gastric neutrophils from the immunized mice blunted this protective immunity. These results support a role for the IL-23→Th-17→CXC chemokines→neutrophil pathway of inflammation in vaccine-mediated protection (DeLyria *et al.*, 2009). Anna Muller's laboratory has also suggested that neutrophils may be important in vaccine-mediated protection of mice from *H. pylori* (Sayi *et al.*, 2009). In further work, however, we have shown that immunized IL-17 knockout mice and immunized IL-17 receptor knockout mice both exhibited wild-type levels of protection from *H. pylori* challenge (E.S. DeLyria *et al.*, unpublished data) arguing against a role for this cytokine in vaccine-mediated protection against *H. pylori*. Another group of investigators used a neutralizing anti-IL-17 monoclonal antibody to demonstrate a blunting of vaccine efficacy in *H. felis*-immunized wild-type animals (Velin *et al.*, 2009). Thus, similar to the role for IFN γ detailed above, the role for IL-17 in protective *Helicobacter* immunity remains unresolved. It may be noteworthy, however, that we have observed enhanced neutrophil recruitment into the gastric mucosa of immunized IL-17 receptor knockout mice, suggesting that compensating mechanisms for neutrophil recruitment may operate independently of IL-17 signalling (E.S. DeLyria *et al.*, unpublished data).

9.4.3 The role of mast cells in vaccine-mediated *H. pylori* immunity

Another granulocyte that has been studied as a possible effector cell in *H. pylori* immunity is the mast cell, which has been identified in the inflammatory infiltrate of infected human subjects and mice (Mohammadi *et al.*, 1996; Nakajima *et al.*, 1997). The data, however, are conflicting. Whereas Velin *et al.* (2005) utilized transgenic mast cell-deficient mice and observed that mast cells were essential for protective immunity against *H. felis*, our own results employing an *H. pylori* challenge and a different mast cell-deficient mouse strain demonstrated that the absence of mast cells resulted in only partial ablation of vaccine-

induced protective immunity (Ding *et al.*, 2009).

One unapparent but potentially important laboratory to laboratory difference in the results summarized above on the roles of IFN γ , IL-17, neutrophils and mast cells in protective *H. pylori* immunity may be the resident microbiota within otherwise 'identical' or similar mouse strains within different animal facilities. Different populations of 'normal flora' have the capacity to differentially modulate host immune responses and, similarly, bacterial species can interact with each other to shape the host-microbe outcome (Ivanov *et al.*, 2008; Mazmanian *et al.*, 2008; Willing and Finlay, 2009). Indeed, the immune and inflammatory responses towards *H. pylori* have been shown to be modulated by concurrent infections with intestinal helminths as well as by other *Helicobacter* species that can be unapparent residents in the gastrointestinal tracts of different mouse colonies (Fox *et al.*, 2000; Lemke *et al.*, 2009).

9.4.4 RNA profiling and the role of adipokines in *H. pylori* immunity

The discussion above highlights the difficulties in identifying the effector mechanisms that eradicate *H. pylori* from the gastric mucosa. A relatively recent approach to this problem, microarray analysis of messenger RNA (RNA transcriptional profiling), has been used to identify up-regulated and down-regulated host genes in pathogenesis and vaccine studies for a large number of microorganisms, including *H. pylori* immunization. Two of the published *Helicobacter* host transcriptional profiling studies identified IFN γ and IFN γ -related genes as important in vaccine-mediated immunity against *H. pylori* in mice (Rahn *et al.*, 2004; Sayi *et al.*, 2009). As discussed previously in this chapter, the importance of IFN γ for effective immunity against *H. pylori* has been inconsistent among laboratories. However, to the degree that IFN γ promotes enhanced gastritis, it does appear to play an important but variable role in clearance of *H. pylori* from immunized rodents. Three other studies have identified and expanded upon up-regulation of adipokines and related molecules as a

unique and unexpected signature from the gastric tissues of successfully immunized mice (Mueller *et al.*, 2003; Walduck *et al.*, 2004; Wehrens *et al.*, 2008).

Adipokines are cytokines or cytokine-like hormones produced by adipose tissue. Leptin, one of the most highly studied adipokines, has been shown to regulate appetite, energy metabolism, insulin sensitivity, reproductive fertility and, importantly, T-cell immunity and inflammation (Cava and Matarese, 2004; Fantuzzi, 2005). It has recently been shown that leptin receptor (LepR)-deficient mice were not protected from *H. pylori* challenge after oral vaccination with bacterial lysate and CT (Wehrens *et al.*, 2008). Overall levels of gastritis were not reported, but similar numbers of T cells were shown to infiltrate the stomachs of immunized mice from both groups. Curiously, given the proposed role of post-immunization gastritis in protection, transcriptional profiling of gastric tissue from the non-protected LepR-negative mice showed higher levels of some immune and inflammation-related genes than from the protected LepR-positive mice. The authors suggested that this could represent higher numbers of proliferating regulatory T cells in the LepR-negative mice, since De Rosa and colleagues recently demonstrated enhanced proliferation of regulatory T cells in LepR-negative mice (De Rosa *et al.*, 2007). It also seems likely that leptin signalling may have direct effects on T effector cells, since when CD4⁺CD45^{high} effector T cells from LepR-negative mice were injected into SCID recipients, colitis was blunted when compared with recipients of CD4⁺CD45^{high} cells from wild-type donors (Siegmund *et al.*, 2004). The relationships among *H. pylori* infection, gastritis, vaccine-mediated protective T-cell immunity and adipokines such as leptin are fascinating and complex, and await future research to unravel.

9.5 Bypassing Host Regulatory Immune Response to Induce Protective Immunity

While specific effector mechanisms that contribute to vaccine-induced *H. pylori* eradication remain difficult to identify, it seems

clear from experiments in multiple mouse models that an overall increase in the intensity of the gastric inflammatory response correlates with reductions in bacterial load (Garhart *et al.*, 2002). In fact, most models in which mice have been shown to reduce or eradicate gastric *H. pylori* infections respond to challenge with robust mucosal inflammation that is significantly more severe than that induced by chronic infection of wild-type mice. These models include IL-10^{-/-} mice (Berg *et al.*, 1998; Eaton *et al.*, 2001; Ismail *et al.*, 2003; Matsumoto *et al.*, 2005), NADPH phagocyte oxidase-deficient mice (Blanchard *et al.*, 2003), SCID or rag^{-/-} immunodeficient mice reconstituted with splenic T cells from wild-type donor mice (Eaton *et al.*, 1999; Stuller *et al.*, 2008) and, as discussed above, mice immunized against *H. pylori* by any number of strategies.

Such observations have led investigators to question whether the natural host immune and inflammatory response to *H. pylori* infection might actually be down-regulated. Early studies performed to assess the T-cell recall response of *H. pylori*-infected subjects showed that the host immune response to *H. pylori* is suppressed. Lymphocytes isolated from blood or the gastric mucosa of infected patients have been demonstrated in numerous studies to respond no better than T cells from seronegative patients, as measured by *H. pylori*-induced cytokine production and proliferation *in vitro* (Blanchard *et al.*, 2004). These observations were difficult to put into context knowing that infected subjects routinely displayed significant histological gastritis.

Recent advances in our understanding of regulatory T cells (Tregs) have facilitated investigation of the role suppression may play in *H. pylori* pathogenesis (see Robinson and Atherton, Chapter 6, this volume). Studies in both mice and humans have revealed that *H. pylori*-specific Tregs participate in the host response to infection and actively suppress immunity. Several models exist in which immune dysregulation results in reduced bacterial loads or even eradication of *H. pylori*, suggesting that vaccine-mediated protection may also be due to the ability to induce active immunity instead of the regulatory response.

IL-10, an important product of both Tr1 and Treg cells, is known to down-regulate immunity (Couper *et al.*, 2008). Perhaps more importantly, Rubtsov *et al.* (2008) have elegantly demonstrated that mice in which only cells expressing Foxp3 are deficient in IL-10 production develop inflammation in the colon and lungs in response to normal flora. Similarly, the NADPH phagocyte oxidase knockout mouse model, which can spontaneously clear *H. felis* and *H. pylori* infections (Blanchard *et al.*, 2003), has now also been shown to be compromised in its ability to generate Treg cells (Romani *et al.*, 2008). Reactive oxygen species are required to promote Treg development and, in the absence of functional phagocyte oxidase, an inflammatory effector T-cell response is favoured. Reconstitution of SCID mice with splenic T cells does not exclude Treg participation but any Treg cells that might be transferred fail to prevent newly activated T cells from promoting pronounced and protective gastritis. When transfer is limited specifically to memory cells, however, inflammation is limited and *H. pylori* continues to colonize (Eaton *et al.*, 2001; Stuller *et al.*, 2008). Ultimately, the ability of immunization to induce a protective immune response may be due to its ability to induce an active immune response in the absence of induced Treg cells, possibly due to the site of T-cell activation being lymph nodes instead of the mucosa where down-regulation is favoured.

In addition to the possible role of Tregs in inhibiting an effective immune response towards *H. pylori*, it has been suggested that *H. pylori* may impair the capacity of dendritic cells (DCs) to stimulate a T-cell response. Mitchell *et al.* (2007) demonstrated that, although *H. pylori* treatment of human DCs induced expression of the co-stimulatory molecules CD40 and CD86, the inhibitory member of the B7 family, PD-L1, was also induced. In addition they showed that prolonged (48 h) incubation of DCs, such as might be observed in chronic *H. pylori* infections, leads to DC 'exhaustion'. The exhausted DCs produced low levels of IL-12 and IL-10 and had an impaired capacity to stimulate allogeneic T-cell IFN γ production when compared with DCs incubated with *H. pylori*

for only 8 h (Mitchell *et al.*, 2007). Thus it may be necessary for *H. pylori* vaccine strategies to effectively overcome not only Treg cells but also inhibitory effects on DCs.

9.6 Non-human Primate and Human Clinical Trials

9.6.1 Mucosal vaccines

Non-human primate models have also been used to obtain compelling evidence to proceed with clinical trials. Initially, a therapeutic strategy was employed in a colony of cynomolgus monkeys harbouring an endogenous *Helicobacter heilmannii* infection (Guy *et al.*, 1997). Oral or intranasal immunizations with inactivated whole *H. pylori* plus recombinant urease plus LT were used. These strategies failed to reduce the bacterial load determined 1 month after immunization, but it was difficult to ascribe the lack of efficacy to the immunization strategy or to the use of *H. pylori* antigens to eradicate *H. heilmannii*. Subsequent studies have been performed with rhesus monkeys and models of natural *H. pylori* acquisition. In the first such study, young seronegative animals were given oral doses of either recombinant urease plus LT or placebo and then tested 10 months later for acquisition of *H. pylori* from the greater monkey population (Dubois *et al.*, 1998). Eight of 26 (31%) immunized monkeys remained *H. pylori*-free compared with two of 29 (7%) animals receiving placebo.

Another study using older, naturally infected rhesus monkeys to test the therapeutic potential of oral recombinant urease plus LT found no therapeutic activity (Lee *et al.*, 1999b). These monkeys were then treated with antimicrobial therapy to further test the capacity of the vaccination to prevent re-infection. Treated monkeys received the oral vaccine again and were then challenged with a strain of *H. pylori* previously isolated from one of the monkeys in the study. Although all monkeys became experimentally infected, immunized monkeys had significantly fewer bacteria in the stomach compared with placebo-immunized animals. A separate study by the

same investigators used adult rhesus monkeys that had been treated with antimicrobial therapy to eradicate naturally acquired *H. pylori* and then compared oral immunization with recombinant urease plus LT with either parenteral immunizations or a combination of oral and parenteral immunizations for protection against infectious challenge (Lee *et al.*, 1999a). In this study, no protective efficacy was demonstrated by prophylactic oral immunization, although some success was achieved with parenteral and combination vaccines (discussed in Section 9.6.2). The most recent rhesus *H. pylori* vaccine trial used animals that were removed from the colony at birth and hand reared to ensure absence of endogenous *H. pylori* infection. This study attempted to compare oral and parenteral vaccination using recombinant urease. Because all immunized macaques became infected after challenge, the vaccines appeared to be a failure (Solnick *et al.*, 2000). However, only one of two sham-immunized animals became infected after challenge, making a quantitative determination of possible vaccine efficacy impossible.

Results achieved in murine and non-human primate models have provided the impetus for performing clinical trials in human volunteers. In the first study of note investigators tested recombinant urease as a therapeutic vaccine in *H. pylori*-positive volunteers (Michetti *et al.*, 1999). Various doses of urease were given orally with either 5 or 10 µg LT in four weekly immunizations, with mixed results. No individuals eradicated *H. pylori*, but reductions in bacterial load were measured in the group receiving the lowest dose of urease. Because bacteria were not eradicated, it is not surprising that gastric inflammation was not reduced. Unfortunately, the wild-type LT was not well tolerated by volunteers, with the 10 µg dose of LT being discontinued mid-study, and even the 5 µg dose inducing diarrhoea in many subjects. The results of this study highlight the complications associated with mucosal immunizations, an issue further illustrated by a subsequent study to evaluate limiting amounts of LT in combination with urease administered as a soluble vaccine or in capsules for enteric release (Banerjee *et al.*, 2002). LT was given in doses as high as 2.5 µg and as low as 0.1 µg. Only those subjects

receiving 2.5 µg responded immunologically to immunization, but this dose induced diarrhoea in 50% of the test subjects. The most recent human trial with urease evaluated rectal immunization as an alternative to oral immunization (Sougioultzis *et al.*, 2002). The vaccine, consisting of 60 mg urease and either 5 or 25 µg LT, was given as an enema to *H. pylori*-negative volunteers, three times over the course of 28 days. LT was immunogenic in most subjects but few developed circulating antibodies to the urease protein and no meaningful T-cell recall response could be measured. The use of a soluble, mucosally delivered vaccine against *H. pylori*, therefore, will require additional research into mucosal adjuvants, effective doses and routes of immunization (see Table 9.1 for alternative adjuvants which have been evaluated in mice but generally have not yet been approved for human use).

The second type of *H. pylori* vaccine tested in human subjects has been inactivated whole-cell preparations. Such vaccines have the benefit of multivalency. Kotloff *et al.* (2001) utilized a formalin-inactivated whole-cell vaccine given orally in combination with a mutant form of LT that has reduced susceptibility to trypsin and therefore should possess less toxicity. The vaccine was given to both infected and uninfected subjects. Antibody responses were observed only in infected individuals receiving the highest dose of vaccine. Conversely, *in vitro* recall responses of blood leucocytes were observed only from vaccinated, but uninfected, volunteers. The efficacy of this vaccine as a therapeutic agent was tested in infected subjects given either whole-cell preparation plus mutant LT or one of several placebos. Colonization was assessed by the ¹³C-urea breath test and all subjects were shown to remain infected up to 7.5 months later, irrespective of vaccination. Because bacterial load was not determined, reductions in colonization levels could not be established. Similar to studies described above with wild-type LT, 28% of test subjects and 33% of placebo recipients developed diarrhoea in response to the mutant LT, suggesting that additional mutations or other strategies such as with CTA1-DD (see Section 9.3.1) may be necessary to develop an acceptable mucosal adjuvant from CT or LT.

The last types of mucosal vaccine tested for *H. pylori* in humans are the recombinant bacterial vector vaccines expressing *H. pylori* proteins. The vectors employed have been exclusively strains of *Salmonella*. Two initial studies to assess immunogenicity utilized avirulent strains of *Salmonella enterica* serovars Typhi or Typhimurium, expressing *H. pylori* urease (DiPetrillo *et al.*, 1999; Angelakopoulos and Hohmann, 2000). Only one of the studies elicited any antibodies to urease subunits and this result was not uniform among subjects. Another group performed a series of trials with *Salmonella* vaccine strain Ty21a expressing both subunits of *H. pylori* urease. Their initial safety study demonstrated only weak responses in *H. pylori*-negative volunteers with none of the oral vaccine recipients generating antibodies to the urease subunits, although several subjects did develop T-cell memory to urease (Bumann *et al.*, 2001). A follow-up study was performed to determine if prior exposure to the carrier vector might impact on the host response to the recombinant *H. pylori* vaccine strain (Metzger *et al.*, 2004). Prior immunization with *Salmonella* vaccine strain Ty21a did not impact on the subsequent response to the vaccine, and subjects again failed to develop humoral immunity to urease. However, similar to the initial study, over half of the subjects developed a urease-specific cellular immune response. This vaccine was later used in a human challenge study in which *H. pylori*-negative volunteers were given multiple oral doses of the urease-expressing *Salmonella* strain or a second recombinant strain expressing the HP0231 antigen (Aebischer *et al.*, 2008). Subjects were challenged with a previously characterized human challenge strain of *H. pylori* (Graham *et al.*, 2004), 42 days after the final immunization. Subjects developed immune responses consistent with *H. pylori* infection, including cytokine production and histological gastritis. Immune responses to urease were not detectable until after challenge. The bacterial load was shown to decrease in eight vaccinated subjects, but a similar decrease was also observed in non-immunized controls. What may be instructive here, however, is that, regardless of immunization status, 69% of the subjects who did

clear or reduce *H. pylori* colonization demonstrated T-cell reactivity *in vitro*, compared with only 13% of subjects who did not reduce bacterial load. This result offers partial confirmation of animal studies demonstrating a need for cell-mediated immune responses in *H. pylori* immunity and suggests that future efforts should proceed in this direction. Finally, although these live vector vaccines have met with limited success, they do have the advantage of being well tolerated by test subjects and seem to avoid the undesirable side-effects inherent to enterotoxin mucosal adjuvants.

9.6.2 Parenteral vaccines

Although many studies have reported on the use of non-human primates for *H. pylori* pathogenesis and vaccine development, only a few have evaluated systemic immunization strategies. The earliest study compared oral, parenteral and a combination of both oral and parenteral vaccination with whole killed *H. pylori* and urease as therapeutic immunization strategies against a pre-existing *H. heilmannii* infection in cynomolgus monkeys (Guy *et al.*, 1997). Only limited protection was achieved, but parenteral and combination immunizations induced a higher proportion of animals with reduced bacterial load. A subsequent study in rhesus monkeys compared parental, oral and an oral prime followed by parental boost for efficacy against challenge with *H. pylori* (Lee *et al.*, 1999a). In that study, parenteral immunization failed to induce immunity, although the combination strategy did induce a reduction in bacterial load to one-tenth that of sham-immunized animals. Finally, another study performed in rhesus monkeys employed prophylactic immunization to compare oral immunization to intramuscular immunization with urease in Bay adjuvant, but all animals became infected upon challenge and there were no differences in bacterial load (Solnick *et al.*, 2000).

Rossi *et al.* (2004) used a canine model to test therapeutic vaccination of experimentally infected beagles. Their vaccine, which consisted of purified recombinant VacA, CagA and NAP in combination with aluminium

hydroxide and administered by intramuscular injections, induced a reduction in bacterial colonization and, most encouragingly, gastritis. Results from the study provided the impetus for the same laboratory to test their vaccine formulation for safety and immunogenicity in human volunteers (Malfertheiner *et al.*, 2008). Subjects were given three intramuscular immunizations with the multiunit VacA, CagA and NAP proteins plus aluminium hydroxide. No untoward side-effects were noted beyond what might be observed at the site of injection for well-established aluminium hydroxide-based vaccines. The vaccine proved to be highly immunogenic, inducing strong antibody and cellular recall responses as measured by IFN γ production *in vitro*. Importantly, this response was detectable for several months and booster immunizations applied almost 2 years later induced a strong memory response. These promising results require further study to determine if this might ultimately be a practical approach for vaccination of human subjects against *H. pylori*.

9.7 Concluding Remarks

Many *H. pylori* vaccine formulations have now been tested in several animal models and in various early-stage clinical studies. Although success in terms of acceptable efficacy has not been achieved in humans, a survey of the efforts put forth since the mid-1990s is instructive. It bears noting that few vaccines exist for the large number of enteric infections that are the cause of significant morbidity and mortality worldwide, and many of the problems associated with developing a vaccine against *H. pylori* have confronted the immunological community for decades. Nevertheless, we now know that there is much utility in the use of a subunit vaccine that incorporates multiple antigens. It is also apparent that bacterial enterotoxins, while too toxic in their native form, may after further modification have the potential of inducing acceptable and safe immunity to *H. pylori*. The CTA1-DD construct may prove to be one such improvement.

Other strategies such as the use of attenuated bacterial vectors, though well tolerated, have exhibited generally disappointing immunogenicity in human *H. pylori* trials. A new generation of highly attenuated and highly immunogenic *Salmonella* vectors (see e.g. Kong *et al.*, 2008; Curtiss *et al.*, 2009) may improve the performance of these vectors for *H. pylori* vaccines. There may also be value in either exploring parenteral immunization further or, at the very least, exploring combination vaccines in which mucosal vaccination is administered along with intramuscular or subcutaneous injection. Most importantly, as difficult as it is to immunize a host against a gastrointestinal bacterial infection, it has been amply demonstrated in animal models, and observed within some subjects in several clinical studies, that it is in fact possible to positively influence the host immune response to *H. pylori*. Studies in the mouse model routinely achieve significant reductions in bacterial load and sterilizing immunity has even been noted in several reports. Encouragingly, a reduction in bacterial load was observed in one human therapeutic vaccine trial using recombinant urease with LT adjuvant (Michetti *et al.*, 1999). In addition, in an *H. pylori* challenge human trial, a cell-mediated immune response was associated with clearance or reductions in bacterial load, even though these reductions occurred in both vaccinated and placebo-treated volunteers (Aebischer *et al.*, 2008). Continued innovation in adjuvant and carrier technology, along with novel protocols for administration of prototype vaccines (Table 9.1), also offers the potential for improvements in vaccine efficacy and ultimately achieving the goal of inducing protective immune responses capable of eradicating *H. pylori* from the gastric mucosa.

Acknowledgements

Research in the authors' laboratories was funded by National Institutes of Health (US) grants DK-046461 and AI-055710.

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10 Lipopolysaccharides of *Helicobacter pylori*: Importance in Gastric Adaptation and Pathogenesis

A.P. MORAN

10.1 Introduction

As in other Gram-negative bacteria, *Helicobacter* spp., including *Helicobacter pylori*, contain lipopolysaccharides (LPS) in the outer membrane of the cell envelope (Moran *et al.*, 1992; Moran, 1995a; Hynes *et al.*, 2004). In general, LPS play a central and essential role in the integrity of the cell envelope of Gram-negative bacteria but, despite this, due to their ability to adversely affect a broad range of host immunological and physiological properties, have been considered toxic and have been termed endotoxins (Rietschel *et al.*, 1990; Raetz and Whitfield, 2002; Caroff and Karibian, 2003). Nevertheless, these molecules do not simply reside in the outer membrane; they can be released by multiplying or disintegrating bacteria, as well as by blebbing of outer membrane vesicles from the bacterial cell surface as occurs in *H. pylori* (Hynes *et al.*, 2005; Keenan *et al.*, 2008). Thus, these phosphorylated lipoglycans, which in many Gram-negative bacteria possess potent immune-stimulating and immune-modulating properties, play a central role in the pathogenesis and virulence of these bacteria. In the case of *H. pylori*, although an important pathogenic factor, LPS particularly possesses properties influencing the severity and chronicity of this infection (Moran, 2007; Moran and Trent, 2008).

Like *Escherichia coli*, *H. pylori* is capable of producing high-molecular-mass (smooth-form) LPS composed of an outermost saccharide moiety, divided into the O-polysaccharide or O-specific chain (O-antigen) and core oligosaccharide regions, covalently linked to a lipid moiety, termed lipid A, that anchors the molecule in the outer leaflet of the outer membrane (Rietschel *et al.* 1990; Moran *et al.*, 1992; Moran, 1995b; Caroff and Karibian, 2003) (Fig. 10.1A). This contrasts with the production of the LPS-related, but distinct, low-molecular-mass lipooligosaccharides by certain other mucosal pathogens, e.g. *Campylobacter jejuni*, *Neisseria* spp., *Haemophilus influenzae*, *Bordetella pertussis*, etc. (Rietschel *et al.*, 1990; Moran, 1995b, 2009) (Fig. 10.1B). The latter differ from LPS by the absence of the O-polysaccharide chain and exhibit greater structural variability in their outer core oligosaccharides. In general, each of the domains of LPS has differing structural and biological properties: the O-chain contributes to the antigenicity and serospecificity of the molecule; the core oligosaccharide influences permeation properties of the outer membrane, modulates the immune response to lipid A and can influence virulence; whereas the lipid A moiety interacts with immune receptors and endows the LPS molecule with a range of immunological and potent endotoxic properties (Rietschel *et al.* 1990; Moran *et al.*, 1992;

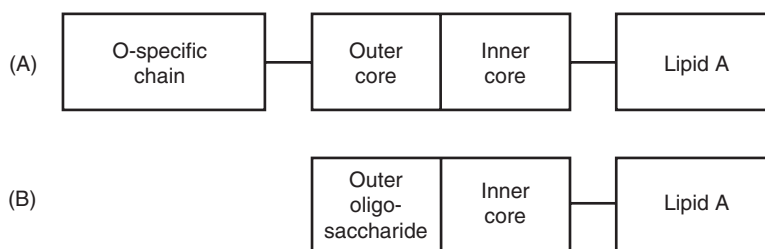


Fig. 10.1. Schematic diagram of the general structures of high-molecular-mass lipopolysaccharide (A) and low-molecular-mass lipooligosaccharide (B).

Moran, 1995b; Caroff and Karibian, 2003). Clinical isolates of *H. pylori* produce smooth-form LPS with O-polysaccharide chains of relatively constant chain length compared with enterobacterial LPS (Moran *et al.*, 1992; Moran, 1995a). Their length is determined by an enzymatic molecular ruler mechanism (Nilsson *et al.*, 2006). As surface antigens (O-antigens), *H. pylori* LPS can induce a specific antibody response, and their structural variation forms the basis for serotyping and lectin typing schemes (Simoons-Smit *et al.*, 1996; Hynes *et al.*, 1999, 2002). However, numerous subcultures on conventional solid media can induce the production of low-molecular-mass, rough-form LPS lacking the O-polysaccharide chain in many strains (Moran, 1995a), which has proven a useful manipulation when studying the structure and properties of the LPS core (Moran, 1999). The ability of such strains to revert to smooth-form LPS production and the influence of environmental factors, such as pH, on LPS expression and antigenic phase variation has been reviewed previously (Moran, 2001b; Moran and Trent, 2008). Moreover, the genetics and characteristics of the enzymes required for biosynthesis of *H. pylori* LPS also have been detailed in a number of reviews (Berg *et al.*, 1997; Moran, 2001a,b, 2008; Ma *et al.*, 2006; Moran and Trent, 2008).

H. pylori has been proposed as a model for investigating and understanding the dynamics of bacterial persistence and parasitism in chronic infections (Blaser and Kirschner, 1999). Likewise, studies on the attributes of *H. pylori* LPS have produced novel insights into the structure and contributing properties of this class of molecule to chronic pathogenesis

(Moran, 2007). Therefore, the present chapter discusses how the structure and properties of *H. pylori* LPS are adapted and modelled to fit the environmental niche of this bacterium, and the pathogenic consequences of these structures. Although the core oligosaccharide contributes to *H. pylori* pathogenesis (the subject of other reviews; see Moran 1996, 1999, 2001a,b), discussion here focuses on the lipid A moiety and O-antigen of this molecule, reflecting the intensive investigation of these portions of the *H. pylori* LPS molecule in recent years.

10.2 Low Immunological Activity of *H. pylori* Lipopolysaccharides

A striking feature of *H. pylori* LPS is the significantly lower endotoxic and immunological activities of these molecules, compared with enterobacterial LPS as the gold standard (reviewed extensively elsewhere; see Moran 1999, 2001a, 2007; Moran and Trent, 2008). The pyrogenicity and mitogenicity of *H. pylori* LPS is 1000-fold lower, lethal toxicity in mice 500-fold lower, and induction of various cytokines and chemokines 1000-fold lower than enterobacterial LPS (Muotiala *et al.*, 1992; Pece *et al.*, 1995). Priming of neutrophils to release toxic oxygen radicals, E-selectin expression on endothelial cells, induction of prostaglandin E_2 and nitric oxide, natural killer-cell activity and abolition of suppressor T-cell activity are also all significantly lower (Baker *et al.*, 1994; Nielsen *et al.*, 1994; Moran, 1999, 2001a; Odenbreit *et al.*, 2006). Similarly, cyclooxygenase-2 induction in gastric cells by

H. pylori LPS is negligible compared with that induced by *Escherichia coli* (Smith, G.V. *et al.*, 2003).

Importantly, early studies that tested chemically modified *H. pylori* LPS-derived components in immunological assays indicated that the molecular basis for these low immunoactivities resided in the lipid A moiety, and is modulated by the saccharide component of *H. pylori* LPS, particularly the core oligosaccharide. For instance, the phosphorylation pattern in lipid A influences cytokine production and induction of pro-coagulant activity from mononuclear leucocytes, as well as *Limulus* amoebocyte lysate activity; nevertheless, the core oligosaccharide modulates these effects (Pece *et al.*, 1995; Semeraro *et al.*, 1996). In contrast, the lipid A acylation pattern has been attributed responsibility for the lack of abolition of suppressor T-cell activity by *H. pylori* LPS (Baker *et al.*, 1994) and the lipid A phosphorylation pattern is considered of lesser importance than acylation for reduced priming of neutrophils to release toxic oxygen radicals (Nielsen *et al.*, 1994). Agreeing with the hypothesis that the structure of lipid A endows *H. pylori* LPS with low immunoactivities (Muotiala *et al.*, 1992), detailed structural studies have found under-phosphorylation, under-acylation and substitution by long-chain fatty acids in this lipid A (Moran *et al.*, 1992, 1997; Moran, 1995a; Suda *et al.*, 2001) compared with enterobacterial-derived lipid A (Moran, 1998). Based on established structure-bioactivity relationships of lipid A molecules, the primary structural characteristics of *H. pylori* are likely to be reflected in reduced immunological activities (Rietschel *et al.*, 1990, 1994).

10.3 *H. pylori* Lipid A Fine Structure and Biosynthesis

10.3.1 Structural analysis of *H. pylori* lipid A

Using two different strains, two independent research groups examined the fine structure of lipid A from *H. pylori* rough-form LPS. One study reported the presence of a tetra-acyl

lipid A (Moran *et al.*, 1997), whereas the other reported a tri-acyl lipid A with an identical phosphorylation pattern to the tetra-acyl form, but lacking acylation at position 3 of the lipid A backbone (Suda *et al.*, 1997). A re-investigation by the latter group of the same strain found the tetra-acyl form of *H. pylori* lipid A (Suda *et al.*, 2001) and thus, potentially, the tri-acyl lipid A may have arisen as a degradation product of the former during chemical isolation of the lipid A (Moran, 2001b). Hence, it may be concluded that the major molecular species in lipid A of *H. pylori* rough-form LPS is composed of a β -(1 $\rightarrow$ 6')-linked D-glucosamine disaccharide backbone, acylated by (R)-3-hydroxyoctadecanoic acid (18:0(3-OH)) and (R)-3-hydroxyhexadecanoic acid (16:0(3-OH)) at positions 2 and 3, with (R)-3-(octadecanoyloxy)octadecanoic acid (3-(18:0-O)-18:0) at the 2'-position, and carries phosphate or phosphoethanolamine (PEtN) groups at position 1 (Moran *et al.*, 1997) (Fig. 10.2B).

Likewise, this mono-phosphorylated tetra-acyl lipid A predominates in *H. pylori* smooth-form LPS, but there is a second, minor constituent consisting of a bis-phosphorylated hexa-acyl lipid A that is distinguished from tetra-acyl lipid A by carrying 3-(12:0-O)-16:0 or 3-(14:0-O)-16:0 at position 3' and an extra phosphate group at position 4' (Moran *et al.*, 1997) (Fig. 10.2A). Overall, whether derived from rough- or smooth-form LPS, compared with the lipid A of *E. coli* (Fig. 10.3) the predominant lipid A molecular species of *H. pylori* lacks the usual 4'-phosphate group, as well as the 3'-ester-linked fatty acyl chains, thus containing only four rather than six fatty acids, which have longer chain length (16–18 carbons versus 12–14 carbons), and is derivatized with a PEtN residue at the C-1 position of the proximal glucosamine (Moran *et al.*, 1997; Moran, 1998). However, because this bacterium can synthesize a bis-phosphorylated hexa-acyl lipid A, it can be deduced that *H. pylori* expresses enzymes (e.g. a 4'-phosphatase and 3'-acyloxyacyl deacylase) capable of remodelling, and hence modifying to a tetra-acyl form, its lipid A domain (Reynolds *et al.*, 2006; Tran *et al.*, 2006) after completion of the conserved hexa-acyl lipid A biosynthetic pathway (Tran *et al.*, 2005; Moran and Trent, 2008).

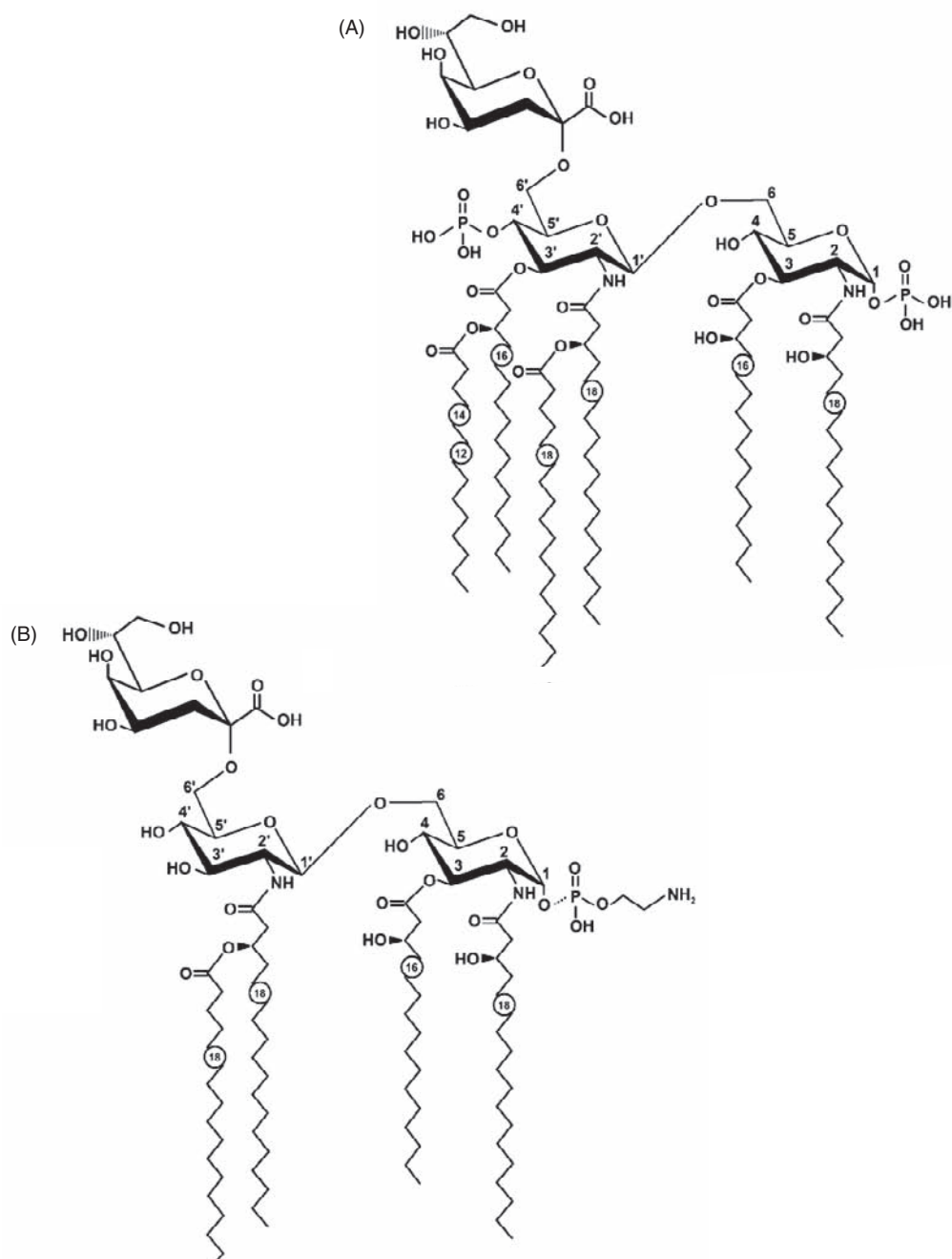


Fig. 10.2. Structures of the minor hexa-acyl lipid A molecular species found in *Helicobacter pylori* smooth-form lipopolysaccharide (LPS) (A) and the predominating tetra-acyl lipid A species found in *H. pylori* rough- and smooth-form LPS (B) (Moran *et al.*, 1997). Numbers in circles refer to the number of carbon atoms in the acyl chains. One 3-deoxy-D-manno-2-octulosonic acid residue, as occurs in the *H. pylori* core oligosaccharide, is shown attached to the 6'-position of lipid A. Compared with the hexa-acyl species (A), the tetra-acyl molecular species (B) lacks 4'-phosphate and is substituted at position 1 by phosphoethanolamine.

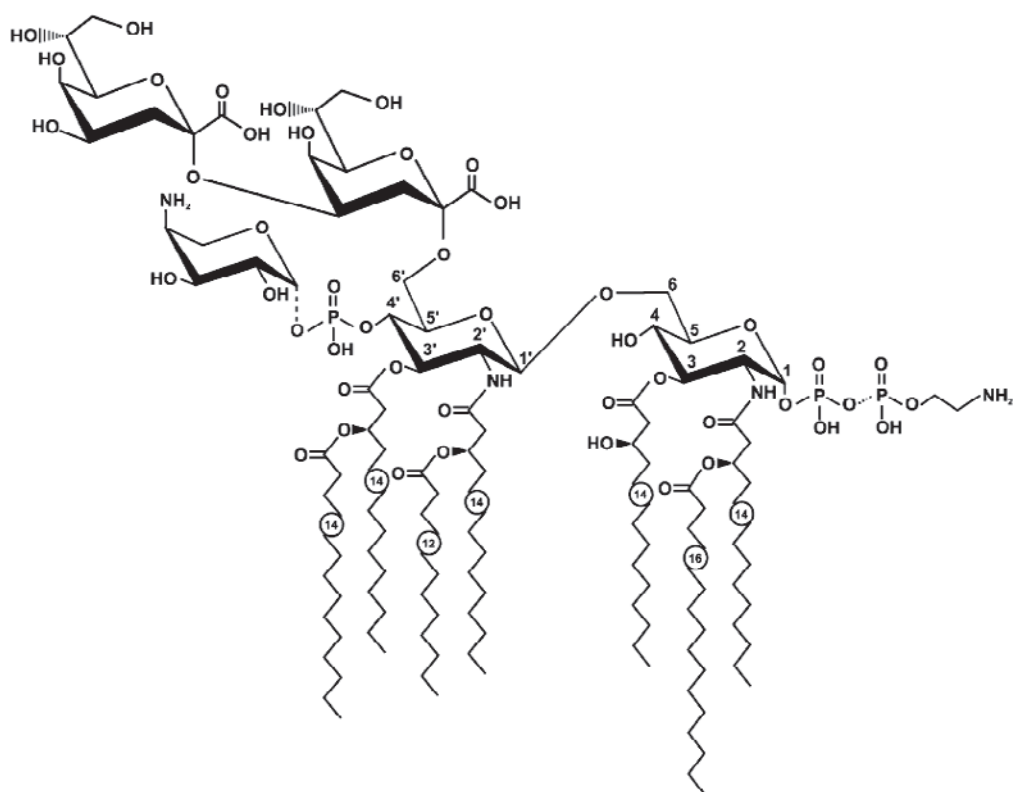


Fig. 10.3. Structure of the major lipid A species of *Escherichia coli* (Rietschel *et al.*, 1990; Raetz *et al.*, 2007). Numbers in circles indicate the number of carbon atoms in the acyl chains. This lipid A is composed of a hexa-acylated D-glucosamine disaccharide that is substituted at the 1- and 4'-positions with phosphate groups and at C-6' with the 3-deoxy-D-manno-2-octulosonic acid residues of the core oligosaccharide. The phosphate groups may be substituted with ethanolamine phosphate (C-1) and 4-amino-4-deoxy-L-arabinopyranose (C-4').

10.3.2 Biosynthetic pathway of *H. pylori* lipid A

The constitutive lipid A biosynthesis pathway is highly conserved, occurs on the cytoplasmic surface of the inner membrane, and generally a single copy of each *lpx* gene can be found in the genomes of virtually all Gram-negative bacteria (reviewed in Raetz *et al.*, 2007). Similarly, bioinformatic analysis of the *H. pylori* 26695 and J99 genomes indicates that the enzymatic machinery for lipid A biosynthesis is conserved in *H. pylori* (Moran and Trent, 2008).

Most enterobacterial lipid A structures are characterized by primary-linked acyl chains of 14 carbons (14:0(3-OH)) in length (Rietschel *et al.*, 1990, 1994; Moran, 1995b; Raetz and Whitfield, 2002; Trent *et al.*, 2006; Raetz *et al.*, 2007). The occurrence of acyl chains of longer length in *H. pylori* compared with *E. coli* lipid A can be attributed to substrate preferences of the primary and secondary acyltransferases. Since the primary-linked hydroxylated acyl chains of *H. pylori* lipid A are either 16 carbons or 18 carbons in length (Fig. 10.2), the early acyltransferases of the *H. pylori* lipid A pathway, LpxA and LpxD, utilize

substrates bearing longer fatty acyl chains when compared with enzymes expressed by most Gram-negative bacteria (see Fig. 10.3 for comparison). The major tetra-acyl lipid A molecular species of *H. pylori* contains a single secondary acyl chain of 18 carbons in length (18:0) located at the 2'-position (Moran *et al.*, 1997; Suda *et al.*, 1997) (Fig. 10.2B), which, again, is longer than the secondary acyl chains found in *E. coli* lipid A. The minor hexa-acyl lipid A molecular species (Fig. 10.2A) bears two secondary acyl chains (at positions 2' and 3') and the primary 16:0(3-OH) chain at the 3'-position is substituted with either a dodecanoyl (12:0) or tetradecanoyl (14:0) group. Only a single secondary acyltransferase, encoded by HP0280 or JHP0265 (Tomb *et al.*, 1997; Alm *et al.*, 1999), was initially identifiable in the sequenced *H. pylori* genomes of 26695 and J99. This led to the inference that either the HP0280-encoded protein is a bifunctional acyltransferase or a further unknown secondary acyltransferase functions to acylate *H. pylori* lipid A. Biochemical characterization of this protein using an *in vitro* assay system has confirmed it to be a monofunctional octadecanoyl (18:0) acyltransferase (Stead *et al.*, 2008). The enzyme utilizes an acyl-acyl carrier protein (acyl-ACP) as its donor, requires 3-deoxy-D-manno-2-octulosonic acid (Kdo) residues attached to the lipid A precursor for activity, and transfers 18:0 to the primary 18:0(3-OH) chain at the 2'-position of *H. pylori* lipid A. Therefore, this acyltransferase functions similarly to *E. coli* LpxL (Clementz *et al.*, 1996) except for acyl chain preference. Additionally, studies assaying *H. pylori* membranes with key lipid A precursors have detected a second acyltransferase, a 3'-secondary acyltransferase, that can utilize 12:0 and 14:0 acyl-ACPs (Stead *et al.*, 2008), and hence functions in a similar manner to *E. coli* LpxM (Clementz *et al.*, 1996). Thus, the latter enzyme transfers 12:0 or 14:0 residues to the primary 16:0(3-OH) chain at position 3' of *H. pylori* lipid A. Concerning the phosphorylation pattern, *lpxH* and *lpxK* are essential genes in *E. coli* encoding the UDP-2,3-diacylglucosamine pyrophosphatase (Babinski *et al.*, 2002) and the lipid A 4'-kinase (Garrett *et al.*, 1997), required for 1- and 4'-phosphorylation, respectively. Of note, only distant orthologues

of LpxH and LpxK are present in *H. pylori* (Moran and Trent, 2008). Nevertheless, the bacterium has the necessary machinery to synthesize a completely phosphorylated hexa-acyl lipid A, although this represents a minor molecular species in the outer membrane.

Transfer of Kdo residues, which link the core and O-antigen carbohydrate domains to the lipid A moiety (Rietschel *et al.*, 1990; Raetz and Whitfield, 2002) (see Fig. 10.3), is catalysed by the glycosyltransferase WaaA (formerly KdtA) in *E. coli* and *Salmonella*, where WaaA is a bifunctional enzyme transferring two sugars to the tetra-acylated lipid A precursor known as Lipid IV_A (Clementz and Raetz, 1991; Belunis *et al.*, 1995). However, not all Gram-negative bacteria contain two Kdo sugars (Isobe *et al.*, 1999; Brabetz *et al.*, 2000) and structural studies have shown that the inner core region of *H. pylori* LPS contains a single Kdo sugar (Aspinall *et al.*, 1994, 1995, 1996, 1997, 1999; Knirel *et al.*, 1999; Monteiro *et al.*, 2000; Moran *et al.*, 2002a), which at first would suggest that *H. pylori* WaaA is a monofunctional transferase. Importantly, Stead *et al.* (2005) demonstrated that *H. pylori* WaaA transfers two Kdo residues to the disaccharide backbone of lipid A, but also reported the occurrence of a Kdo-trimming enzyme, a Kdo hydrolase, that was predicted to function following MsbA-dependent transport (Zhou *et al.*, 1998; Doerrler, 2006) across the inner membrane. Why the bacterium transfers two Kdo residues, but then removes the second, is unclear; it is possible that the charge of the second sugar is necessary for proper functional transport or that secondary acylation of *H. pylori* lipid A precursors may require the presence of two Kdo sugars (Moran and Trent, 2008). After assembly and transport of the *H. pylori* core-lipid A, removal of the outer Kdo residue may benefit the organism by reducing the net negative charge on the bacterial surface, thereby promoting resistance to antimicrobial peptides. After transport across the inner membrane, a number of bacterial species modify the Kdo-lipid A domain of their LPS by modification systems that are found on the periplasmic side of the inner membrane or at the extracellular surface (Raetz *et al.*, 2007). Thus, there is a physical

separation of the constitutive pathway from the modification systems (Trent *et al.*, 2006).

H. pylori lipid A contains a PETN group directly linked to the C-1 hydroxyl group of the proximal glucosamine (Moran *et al.*, 1997; Suda *et al.*, 1997) (Fig. 10.2B), which arises from the removal of the phosphate group at position 1 of lipid A, followed by the addition of PETN to form a glycosidic phosphodiester linkage, and is catalysed by the proteins encoded by HP0021 and HP0022, respectively (Tran *et al.*, 2004, 2006; Moran and Trent, 2008). This modification of the 1-position of *H. pylori* lipid A occurs on the periplasmic side of the inner membrane. Although separated by only 10 bp of DNA sequence, the genes encoding these enzymes appear to form a typical prokaryotic operon as they are unidirectionally transcribed. The HP0021-encoded protein, also known as LpxE, has distant sequence similarity to *E. coli* PgpB, a phosphatidyl glycerolphosphatase (Icho and Raetz, 1983; Ichio, 1988), but is highly selective for the 1-phosphate group of lipid A (Wang *et al.*, 2004). HP0022, an orthologue of *eptA*, is homologous to the lipid A PETN transferases identified in other Gram-negative pathogens including *E. coli*, *Salmonella enterica* serovar Typhimurium and *Neisseria meningitidis* (Tran *et al.*, 2004), but the *H. pylori* EptA contrasts in activity with the EptA of other bacteria, which modifies the lipid A phosphate group directly (Trent and Raetz, 2002; Lee *et al.*, 2004). Disruption of *lpxE* (JHP0019) in *H. pylori* J99 results in the production of a lipid A bearing a single phosphate group at the 1-position, since lack of LpxE function results in loss of PETN transfer as *H. pylori* EptA (HP0022-encoded) requires prior dephosphorylation for activity (Tran *et al.*, 2006). Unexpectedly, it has been observed that disruption of *lpxE* in *H. pylori* 26695 (HP0021) results in the synthesis of lipid A with two phosphate groups and six acyl chains (Tran *et al.*, 2006). The structure is identical to the minor lipid A species previously reported (Fig. 10.2A), thereby confirming the findings of Moran *et al.* (1997).

The highly conserved lipid A kinase, LpxK, phosphorylates the 4'-hydroxyl group of the disaccharide backbone in the constitutive biosynthetic pathway (Garrett *et al.*, 1997) but in some microorganisms, including *H.*

pylori, the 4'-phosphate group is removed subsequently due to phosphatase activity (see Moran and Trent, 2008). Although a 4'-phosphatase, LpxF, has been identified in *Francisella tularensis* (Wang *et al.*, 2006), and is localized to the inner membrane and removes the 4'-phosphate group from lipid A after transport across the inner membrane, there is no identifiable homologue in the genome of *H. pylori* J99 or 26695. Attempts to clone the structural gene encoding *Helicobacter* LpxF have been unsuccessful (Moran and Trent, 2008). Thus, collectively the findings indicate that dephosphorylation of the 1- and 4'-positions of *H. pylori* lipid A occurs within the periplasmic region of the bacterial cell.

The final modification of the *H. pylori* lipid A domain is the removal of the 3'-acyloxyacyl residue, resulting in the production of a tetra-acylated lipid A (consistent with the structural findings above), which is catalysed by the outer membrane protein encoded by HP0694 (JHP0634), also known as LpxR (Rutten *et al.*, 2009). Inactivation of HP0694 by insertion of a selectable marker results in the production of a hexa-acylated lipid A structure, but does not inhibit modification of the phosphate groups. Although LpxR resides in the outer membrane of *Salmonella* under normal growth conditions, the enzyme remains dormant (Reynolds *et al.*, 2006), whereas the LpxR of *H. pylori* is constitutively active since *H. pylori* lipid A is not substituted at the 3'-position (Fig. 10.2B). Despite these findings, the factors that promote enzymatic activation of *H. pylori* LpxR or function of the deacylase to date remain unknown.

Synthesis of the Kdo-lipid A domain of LPS is followed by the attachment of the core oligosaccharide and transport of the core-lipid A across the inner membrane (Raetz and Whitfield, 2002). For this process, MsbA, a conserved ABC transporter that is also found in *H. pylori*, serves as the lipid A flippase (Zhou *et al.*, 1998; Doerrler, 2006). The O-chain of *H. pylori* is then attached on the periplasmic side of the inner membrane to form whole LPS. Additional steps are required for translocation of LPS to the outer leaflet of the outer membrane, but are not yet clearly defined (Doerrler, 2006).

10.3.3 Molecular and supramolecular basis for the low immunological activities of *H. pylori* lipid A

A structural comparison of the predominant *H. pylori* lipid A molecular species with that of *E. coli* (Moran, 1998) shows the absence of 4'-phosphate and the occurrence of four, rather than six, fatty acids whose average chain length is longer (16–18 carbons versus 12–14 carbons) in *H. pylori* lipid A. These structural differences in *H. pylori* lipid A are consistent with the established structure–bioactivity relationships for low immunological activities of lipid A (Rietschel *et al.*, 1990, 1994). Moreover, natural and synthetic *H. pylori* lipid A, with the reported underacylation and under-phosphorylation patterns, have been shown to have low endotoxic potency and immunological activities (Suda *et al.*, 2001; Ogawa *et al.*, 2003) like *H. pylori* LPS, as discussed above. Importantly, these novel attributes not only impact upon the established primary structure–bioactivity relationships but also upon the supramolecular conformation of *H. pylori* lipid A (Schromm *et al.*, 2000) and other biophysical properties of lipid A that have been correlated previously with lower bioactivity (Seydel *et al.*, 2000). These include a higher phase transition temperature and a lower inclination angle of the lipid A diglucosamine backbone to the membrane plane than those encountered with enterobacterial lipid A (Moran *et al.*, 2005), which would influence interaction with immune receptors.

10.4 Relevance of Low Lipopolysaccharide and Lipid A Immunoactivities to *H. pylori* Pathogenesis: a Model

As seen with other chronic bacterial infections or colonizing commensal bacteria, the induction of low immunological responsiveness may aid the prolongation of *H. pylori* infection and aid chronicity (Lee and Moran, 1994). It has been hypothesized that *H. pylori* LPS, and its lipid A component in particular, have evolved their present structure, on the

one hand, to fulfil their role in producing a functional macromolecular matrix for bacterial interaction with its environment and, on the other, to reduce the immune response to these essential molecules of the *H. pylori* outer membrane (Muotiala *et al.*, 1992; Moran, 1995a, 1996). Thus, in producing a functional macromolecular matrix relevant to the microbial niche of *H. pylori*, the lipid A component, which is embedded in the outer membrane, would retain essential structural features to maintain structural integrity of the outer membrane, but undergo modification to allow survival within the specific microbial niche.

An example supporting this view concerns resistance to cationic antimicrobial peptides (CAMPs) produced by the innate immune system (see Kaparakis *et al.*, Chapter 8, this volume). Of note, expression of human cathelicidin LL-37 (Hase *et al.*, 2003) and human β -defensin 2 (Wada *et al.*, 1999; Hamanaka *et al.*, 2001) is up-regulated in the gastric mucosa of *H. pylori*-infected patients. Furthermore, *H. pylori* itself produces a ceropin-like peptide, Hp(2-20), to which it is resistant, that is derived from the amino-terminal part of its ribosomal protein L1 (Putsep *et al.*, 1999). In order to persist in the human gastric mucosa, *H. pylori* must be able to resist the action of CAMPs. Importantly, in some other Gram-negative bacteria, ceropin, as an example of a CAMP, has been shown to bind to bis-phosphorylated lipid A (De Lucca *et al.*, 1995), and masking the negative charge of their phosphate groups on lipid A with positively charged amine-containing substituents (e.g. ethanolamine and 4-amino-4-deoxy-L-arabinose) promotes CAMP resistance. As lipid A phosphate groups are necessary for binding of cationic peptides (Trent *et al.*, 2006), the predominant lipid A molecular species, lacking 4'-phosphate and with PETN substitution at C-1 of the *H. pylori* lipid A backbone, should promote resistance to CAMP during host infection. Experimentally, wild-type strains of *H. pylori* are inherently resistant to polymyxin, a CAMP, but disruption of *lpxE* (JHP0019) in *H. pylori* J99 results in the production of a lipid A bearing a single phosphate group at the 1-position, thereby producing a 25-fold

increase in polymyxin sensitivity (Tran *et al.*, 2006).

In addition, the interactions of *H. pylori* LPS via its lipid A component with a variety of immune recognition molecules and receptors, for example LPS-binding protein, CD14 and Toll-like receptors (TLRs), have consistently been reported to be low (reviewed in Moran, 2001a, 2007; Moran and Trent, 2008). Whether *H. pylori* LPS and lipid A act as TLR4 agonists (see Kaparakis *et al.*, Chapter 8, this volume), as classically seen with the majority of LPS of other Gram-negative bacteria, or as TLR2 agonists has been the focus of much discussion (Smith, M.F. *et al.*, 2003; Mandell *et al.*, 2004; Smith *et al.*, 2006; Moran, 2007; Triantafyllou *et al.*, 2007; Uno *et al.*, 2007; Moran and Trent, 2008). In any case, upon initial infection of the TLR4-expressing but TLR2-deficient gastric mucosa (Ortega-Cava *et al.*, 2003; Mandell *et al.*, 2004), *H. pylori* is weakly recognized or unrecognized by TLR4 and may escape detection and elimination by the immune response initially. Consistent with this evasion of immune detection is the observation that, although functional TLR5 is expressed in the adult stomach (Schmausser *et al.*, 2004), and TLR5 is considered to bind and respond to bacterial flagellins, *H. pylori* flagellins FlaA and FlaB induce a very low activation of TLR5-mediated responses (Lee *et al.*, 2003).

With progression of the immune response in long-term infection, a substantial inflammatory cytokine response to *H. pylori* may develop only after the infiltration of TLR2-expressing granulocytes and monocytes into the infected gastric mucosa. *H. pylori* can activate mononuclear cells by an LPS-independent, as well as a TLR4-independent mechanism (Mai *et al.*, 1991), and hence non-LPS component(s) of the bacterium are the major inflammation-activating molecule(s). In general, because bacterial lipoproteins, lipopeptides and lipoteichoic acids are considered TLR2 ligands, the numerous putative lipoproteins encoded in the *H. pylori* genome (Doig *et al.*, 1999) potentially represent candidate TLR2 ligands (Moran, 2007). An *H. pylori* heat-shock protein (HSP60) has been implicated in activation of TLR2 (Takenaka *et al.*, 2004), but this protein has also been reported

to induce interleukin (IL)-6 production by macrophages via a TLR2-independent mechanism (Gobert *et al.*, 2004). Additionally, recognition of *H. pylori*, which is considered to be predominantly non-invasive, by epithelial cells has been shown to be mediated by Nod1, an intracellular pattern-recognition molecule with specificity for peptidoglycan-derived muropeptides, which are delivered intracellularly by the type IV secretion system-encoding *cag* pathogenicity island (PAI) (Viala *et al.*, 2004). This may be one mechanism by which *cag*PAI-positive strains elicit a more vigorous inflammatory response and, in part, explain why these strains are associated with more aggressive disease symptoms, such as gastric cancer.

Moreover, as a consequence of enzymatic degradation of LPS by human phagocytes, some LPS and/or lipid A partially modified structures can be excreted by exocytosis. Such compounds retaining some immunological activity could play a role as subliminal, low-grade, persistent stimuli involved in *H. pylori* pathogenesis (Jirillo *et al.*, 1999; Moran, 1999). Notably, *H. pylori* LPS can induce nitric oxide synthase in an *in vivo* animal model, thereby contributing to intestinal damage (Lamarque *et al.*, 2000; Kiss *et al.*, 2001), and also influence gastric motor function (Quintana *et al.*, 2005). Although controversial, whether, during long-term chronic infection of the gut mucosa by *H. pylori*, low-grade LPS/lipid A stimuli contribute to extragastric infection sequelae remains an interesting question (Moran, 1999, 2007; Grebowska *et al.* 2006) (see Mitchell *et al.*, Chapter 5, this volume).

10.5 Lewis Antigen Expression

A further striking feature of *H. pylori* LPS is the expression of Lewis and blood-group mimicry in the O-antigen component of this molecule (Moran, 2008). The Lewis (Le) antigens are biochemically related to the ABH blood-group antigens of humans that are formed by the sequential addition of L-fucose, D-galactose and N-acetyl-D-galactosamine to the backbone carbohydrate chains of both lipids and proteins (Fig. 10.4). The structural basis of type 1 and type 2 backbone chains, as

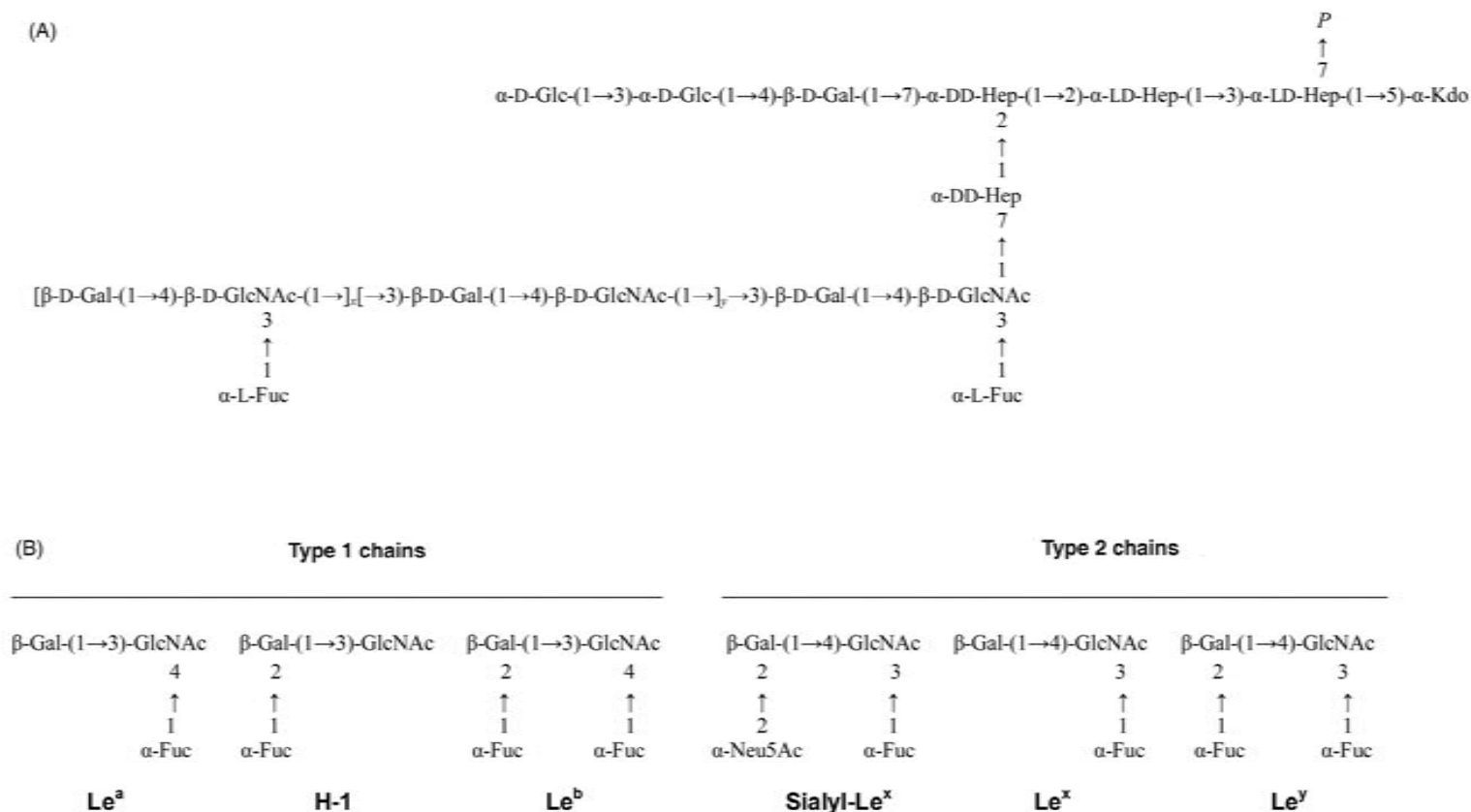


Fig. 10.4. Structure of the saccharide component of lipopolysaccharide of the *Helicobacter pylori* type strain NCTC 11637 (A) and structures of the Lewis antigens expressed in *H. pylori* strains (B) (for details see Aspinall *et al.*, 1996; Moran, 2008). Abbreviations: Fuc, fucose; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetyl-glucosamine; DD-Hep, D-glycero-D-manno-heptose; LD-Hep, L-glycero-D-manno-heptose; Kdo, 3-deoxy-D-manno-2-octulosonic acid; *P*, phosphate; Neu5Ac, *N*-acetyl-neuraminic acid (sialic acid).

well as Le and related antigens (e.g. H-1 and H-2), have been detailed elsewhere (Moran, 2001b, 2008). The mammalian genes controlling the expression and secretion of these molecules have been described (see Green, 1989). Of note, the terms secretor and non-secretor indicate the capacity of an individual to secrete such substances; secretors produce ABH, Le^b and Le^y, whereas non-secretors produce Le^a and Le^x in their saliva (Sakamoto *et al.*, 1984).

Expression of Le^x or Le^y antigens is a common property of *H. pylori* strains, as 80–90% of isolates from various geographical regions worldwide, which have been screened using anti-Le antibodies as probes, express these antigens (Moran, 2008). Nevertheless, serological analysis can underestimate Le^x and Le^y expression in a population of strains (Hynes and Moran, 2000), as some strains that are non-typeable with anti-Le antibodies have been shown to express these antigens when examined in structural studies (Knirel *et al.*, 1999). The genetic determination of Le antigens and their biosynthetic pathways in *H. pylori* have been extensively reviewed (Moran, 2001b, 2008; Ma *et al.*, 2006; Moran and Trent, 2008). Collectively, factors affecting Le antigen expression in *H. pylori*, which can thereby influence the biological impact of this molecular mimicry, include regulation of fucosyltransferase (FucT) genes through slipped-strand mispairing, the activity and expression levels of the functional enzymes, the preferences of the expressed enzyme for distinctive acceptor molecules, and the availability of activated sugar intermediates (Moran, 2008). With the accessibility of the crystal structure of *H. pylori* FucTs, further insights should be gained into the molecular recognition and functioning of these enzymes (Sun *et al.*, 2007).

Structurally, the O-chains of *H. pylori* clinical isolates have a poly-*N*-acetyl-lactosamine (LacNAc) chain decorated with multiple lateral α -fucose residues forming internal Le^x units with terminal Le^x or Le^y units (Fig. 10.4) or, in some strains, with additional D-glucose or galactose residues (Aspinall *et al.*, 1994, 1995, 1996, 1997, 1999; Knirel *et al.*, 1999; Monteiro *et al.*, 2000; Monteiro, 2001; Moran, 2001a,b, 2008; Moran *et al.*, 2002a).

Also, Le^a, Le^b, sialyl-Le^x and H-1 antigens have been structurally described in other strains, as well as the related blood groups A and B, but occur in association with Le^x and LacNAc chains (Moran, 2008). Overall, a mosaicism of Le antigen and blood group expression can occur in the same O-antigen, and hence, along with some variability in the core oligosaccharide of LPS, give rise to antigenic diversity that can be detected by antibody and lectin probing (Simoons-Smit *et al.*, 1996; Hynes *et al.*, 1999, 2002). On the other hand, *H. pylori* strains that do not express Le antigens (Kocharova *et al.*, 2000; Senchenkova *et al.*, 2001; Monteiro *et al.*, 2001; Britton *et al.*, 2005) (Fig. 10.5) have been isolated from clinically asymptomatic individuals. The absence of Le antigen mimicry in these strains, and animal studies in which a genetically modified *H. pylori* strain lacking Le antigen expression failed to induce gastritis compared with the parental strain (Eaton *et al.*, 2004), are consistent with the view that Le antigen-expressing LPS contributes directly to disease development.

The biological and pathogenic roles of Le antigens expressed in the O-antigen of *H. pylori* LPS have been the subject of recent reviews (Moran, 2008, 2009; Moran and Trent, 2008). Briefly, as extensively reviewed, *H. pylori* Le expression has been implicated in evading the immune response upon initial infection and in influencing bacterial colonization and adhesion (see Lindén *et al.*, Chapter 12, this volume). Furthermore and controversially, as chronic infection progresses, Le expression has been suggested to contribute to gastric autoimmunity leading to gastric atrophy (reviewed in Moran, 2008, 2009). As mentioned in Section 10.1, the discussion here focuses on the role of *H. pylori*-expressed Le antigens in fitting the environmental niche of this bacterium.

10.6 Role of *H. pylori*-expressed Lewis Antigens in Gastric Adaptation

In the human stomach, Le^a and Le^b are mainly expressed on the surface and foveolar epithelia, whereas Le^x and Le^y are predominantly

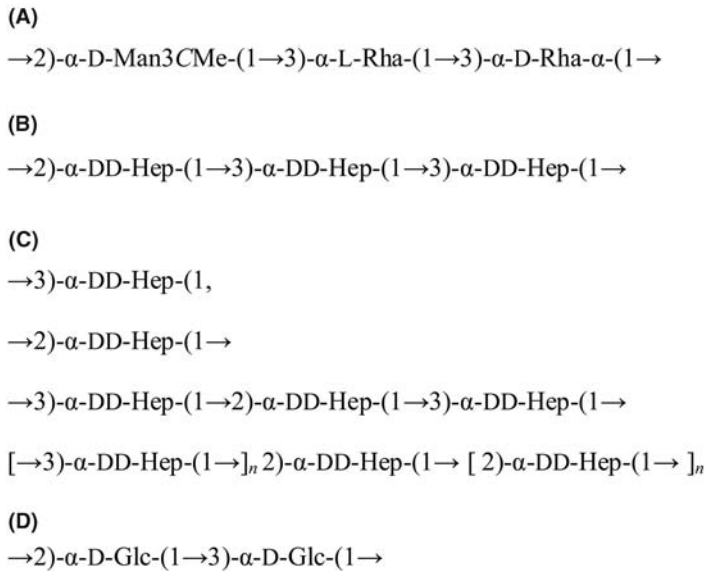


Fig. 10.5. Structures of the non-Lewis mimicking units of O-chains of *Helicobacter pylori* strains: (A) D1, D3 and D6; (B) D2, D4 and D5; (C) Hp1C2, Hp12C2, Hp62C, Hp7A, Hp77C and HpPJ1; (D) serotype O2 (for details see Kocharova *et al.*, 2000; Monteiro *et al.*, 2001; Senchenkova *et al.*, 2001; Britton *et al.*, 2005). Abbreviations: Glc, glucose; DD-Hep, D-glycero-D-manno-heptose; Man3CMe, 3-C-methyl-mannose; Rha, rhamnose.

expressed by the mucous, chief and parietal cells of the gastric glands (Kobayashi *et al.*, 1993), although the physical separation of Le antigens along the gastric pits is not always obvious (Murata *et al.*, 1992). More specifically, in non-secretors the surface and foveolar epithelia express Le^a, whereas these epithelia express Le^b and some Le^y in secretors; but irrespective of secretor status of the individual, the glandular epithelium lacks type 1 antigens (Le^a and Le^b) and expresses type 2 antigens (Le^x and Le^y), but in non-secretors Le^y expression is weaker in the glands (Sakamoto *et al.*, 1989). Hence a variety of Le antigens are expressed within the ecological niche of *H. pylori*. On the basis of this known Le antigen expression in the gastric mucosa, it has been proposed that bacterial molecular mimicry and its adaptation could provide an escape for *H. pylori* from the humoral response by preventing the formation of anti-bacterial antibodies because of cross-reaction with the host (Wirth *et al.*, 1997; Appelmek *et al.*, 2000).

In particular, an initial study that examined the relationship between infecting *H. pylori* isolates and gastric Le expression in the human host, as determined by erythrocyte Le(a,b) phenotype (i.e. secretor status), reported that the relative expression of Le^x or Le^y by *H. pylori* strains corresponded to Le(a+,b-) and Le(a-,b+) blood group phenotypes, respectively, of the hosts from whom the individual strains were isolated (Wirth *et al.*, 1997). Thus, it was inferred that because Le^a and Le^b are isoforms of Le^x and Le^y, and surface and foveolar epithelia express Le^a in non-secretors and Le^b in secretors, that the correlations observed were a form of adaptation or selection of *H. pylori* strains to the gastric mucosa of the individual host (Wirth *et al.*, 1997). A second study, performed in an identical manner but in a different patient population, did not find a correlation between Le^x/Le^y on colonizing *H. pylori* isolates and host Le expression (Heneghan *et al.*, 2000). Likewise, another study, which used the different approach of directly examining Le

expression on gastric epithelial cells of infected human subjects, did not find a correlation (Taylor *et al.*, 1998). Nevertheless, the discrepancies between these patient studies may reflect characteristics of the human populations under investigation and the study procedures employed (Moran, 1999; Broutet *et al.*, 2002).

Of considerable importance is that, in the human study population where the correlation was observed, 26% of individuals were determined to be of the recessive Le phenotype, Le(a-,b-), based on erythrocyte testing (Wirth *et al.*, 1997). This is higher than that usually observed for Caucasian or European populations, from which the two other study populations were drawn (Taylor *et al.*, 1998; Heneghan *et al.*, 2000). Importantly, using salivary testing rather than erythrocyte typing would have allowed the distribution of this phenotypically recessive group of patients to their true secretor/non-secretor phenotype and, based on theoretical calculations, would have influenced the correlative results (Heneghan *et al.*, 1998). Also, in the two other studies, no patients of a recessive phenotype were observed (Taylor *et al.*, 1998; Heneghan *et al.*, 2000). Finally, despite high anti-LPS antibody titres (Appelmek *et al.*, 1996; Heneghan *et al.*, 2001), *H. pylori* eradication and protection is mediated by cellular not humoral immunity (O'Keeffe and Moran, 2008) and this challenges whether anti-Le, and hence anti-LPS, antibodies can act as a selective pressure for *H. pylori* strains in humans (Appelmek *et al.*, 2000).

On the other hand, in an animal model, using experimental infection of rhesus monkeys, an *H. pylori* strain isolated from infected monkeys of the secretor phenotype expressed more Le^y than Le^x; conversely, the same infective strain expressed more Le^x than Le^y when isolated following colonization of non-secretor monkeys (Moran *et al.*, 2002b; Wirth *et al.*, 2006). Therefore, in this experimental system, Le^x/Le^y expression by *H. pylori* depended on the host secretor status. This at first would appear to support the view that Le antigen-based mimicry could provide an escape for *H. pylori* from the host humoral response by a camouflage-type mechanism but, unlike in human subjects (Appelmek *et*

al., 1996; Heneghan *et al.*, 2001), no data are available as to whether the *H. pylori*-infected monkeys formed antibodies against Le^x and Le^y that would have provided a selective pressure (Appelmek *et al.*, 2000). However, the use of transgenic animals as an *H. pylori* infection model, for example Le^b-expressing mice (Guruge *et al.*, 1998), could help to more effectively resolve the question of whether host Le phenotype affects bacterial Le expression.

Of note, it has been demonstrated that *H. pylori* strains expressing Le^x and those expressing Le^y can be isolated from the same host (Wirth *et al.*, 1999), and that extensive diversity in expression of Le^x and Le^y in *H. pylori* O-chains can occur over time and in different regions of the human stomach (Nilsson *et al.*, 2006). Moreover, Nilsson *et al.* (2008) detected genetic modifications in *H. pylori* *fucT* genes that could be attributable to recombination events within and between these genes that creates diversity and which, together with phase variation, contributes to divergent LPS expression within an *H. pylori* colonizing community. The extent of this variation would apparently contradict the hypothesis of bacterial Le antigen adaptation to that of gastric Le expression as determined simply by the secretor status of the host (Wirth *et al.*, 1997).

Notwithstanding this, the diversity of Le expression by *H. pylori* subclonal isolates in one host (Nilsson *et al.*, 2006) may reflect an ability, and potential, of the bacterium to adapt to differing micro-niches and environmental conditions within the human stomach (Moran *et al.*, 2002a; Keenan *et al.*, 2008). For example, pH may vary locally in the stomach and has been shown to influence relative Le^x/Le^y expression by *H. pylori* (Moran *et al.*, 2002a). The pH may vary between the differing regions of the stomach (i.e. the antrum versus the corpus where the stomach's acid-secreting cells are located), as well as varying across the microbial niches of the mucosa (i.e. pH 1–3 on the luminal surface of gastric mucus, about pH 4–5 within the mucus and pH 7 on the epithelial cell surface). Likewise, dietary iron can vary as can accessible iron for *H. pylori* acquisition (Keenan *et al.*, 2004), which in turn affects pathogenesis of *H. pylori*,

and can influence the quantitative expression of LPS and the nature of Le antigen mimicry occurring in *H. pylori* O-antigens (Keenan *et al.*, 2008).

10.7 *H. pylori*-expressed Lewis Antigens and Modulation of the Inflammatory Response

Contributing to the chronicity of *H. pylori* infection by modulation of the immune response, strain-to-strain variability in Le expression can influence the interaction of *H. pylori* LPS with the cellular innate immune receptor, dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), a C-type lectin (Bergman *et al.*, 2004). This binding contributes to a changed T-cell polarization after innate immune activation (Bergman *et al.*, 2006). In the effector phase of an immune response, different T-cell subsets, called T-helper 1 (Th1) and T-helper 2 (Th2) cells, expand; Th1 cells promote proinflammatory cell-mediated immunity whereas Th2 cells promote humoral immunity that induces B cells to produce antibodies (O'Keefe and Moran, 2008). Dendritic cells, in response to *H. pylori*, secrete a range of cytokines, but preferentially IL-12 that induces a Th1 response, and also lesser amounts of IL-6 and IL-10 (Guiney *et al.*, 2003). Since Le antigen expression is subject to phase variation (Appelmek *et al.*, 1999; Ma *et al.*, 2006; Moran, 2008), a significant proportion of Le-negative variants can occur within a population of *H. pylori* cells. Importantly, Le-negative variants of *H. pylori* escape binding by dendritic cells and induce a strong Th1-cell response (Bergman *et al.*, 2004). On the other hand, *H. pylori* variants that express Le^x/Le^y can bind to DC-SIGN on dendritic cells and enhance the production of IL-10, which promotes a Th2-cell response and blocking of Th1-cell activation (Bergman *et al.*, 2006). Hence, a polarized Th1 effect can change to a mixed Th1/Th2-cell response through the extent of Le antigen-DC-SIGN interaction (Bergman *et al.*, 2004). This modulation of the host response allows a switch from an acute infection response to one that

allows development of chronic infection, as the humoral response and antibody production are not associated with *H. pylori* eradication or protection (O'Keefe and Moran, 2008). A similar alteration in the T-cell response, although based on over-expression of IL-10 but induced by polymeric Le^x, has been observed in infection by eggs of the parasitic worm *Schistosoma mansoni* (Velupillai and Harn, 1994; Moran *et al.*, 1996).

Additionally, in patients with more aggressive inflammation and pathologies, the expression of both Le^x and Le^y by *H. pylori* may aid persistence of proinflammatory *cagA*-positive strains (Wirth *et al.*, 1996), i.e. strains carrying the *cagPAI*-encoding type IV secretion system (see Backert *et al.*, Chapter 11, this volume). This hypothesis was based on observations in a sample human population that *H. pylori* isolates positive for Le^x/Le^y were predominantly *cagA*-positive, and that a genetically engineered *cagA*-negative strain had diminished expression of Le^y (Wirth *et al.*, 1996). In contrast, two other studies drawn from a different human study population did not find an association between bacterial *cagA* status and Le^x/Le^y expression (Marshall *et al.*, 1999; Heneghan *et al.*, 2000). The discrepancy between these findings has been attributed to adaptation of *H. pylori* strains with differing properties to the different human populations studied (Moran, 1999), a conclusion that was later supported in a pan-European study examining *cagA* status and Le expression of isolates in differing ethnic populations (Broutet *et al.*, 2002). Thus, Le^x/Le^y expression by *H. pylori* might aid persistence of more aggressive strains depending on the human host population.

10.8 Conclusions

H. pylori has a well-established ability to evade and even subvert innate and adaptive immune responses during long-term infection and, as has been discussed (Moran, 2007, 2008), certain properties of *H. pylori* LPS, particularly those associated with the O-chain and lipid A components, contribute to this. In addition, the properties of *H. pylori* LPS,

which collectively contribute to both chronicity and disease development, conform with the proposal of *H. pylori* as a model for investigating and understanding the dynamics of bacterial persistence and parasitism in chronic infections (Blaser and Kirschner, 1999). In addition to the biological properties detailed above, a number of other bioactivities that have been attributed to *H. pylori* LPS and which influence pathogenesis, such as mucin production and quality, epithelial cell-mucin binding, trefoil factor binding, acid secretion, leptin induction and apoptosis, remain less well characterized (see Brzozowski *et al.*, 2000; Moran 2001b; Durkin *et al.*, 2006; Reeves *et al.*, 2008). The molecular structures within *H. pylori* LPS that are potentially responsible for these properties, and hence the relevant structure-bioactivity relationships, require establishment for unequivocal acceptance but could give further insights into the role of *H. pylori* LPS in pathogenesis.

Finally, despite being at an early stage of investigation, the comparative structural and biological characterization of LPS from different members of the genus *Helicobacter*, whether they colonize the gastric or hepato-intestinal environments (Hynes *et al.*, 2004; Moran *et al.*, 2004; Sterzenbach *et al.*, 2007), is likely to yield new insights into the role of LPS in infection in general, and of *Helicobacter* pathogenesis in particular, in various compartments of the gut.

Acknowledgements

Studies in the author's laboratory are supported by the Science Foundation Ireland grant no. 08/SRC/B1393 and the EU Marie Curie Programme grant no. MTKD-CT-2005-029774.

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11 Virulence Factors of *Helicobacter pylori*

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11.1 Introduction: Gastric Biology of *Helicobacter pylori* Infections

11.1.1 The interplay of multiple virulence and pathogenicity factors

The stomach has long been considered a sterile environment due to its low pH. The discovery of *H. pylori* in gastric biopsies by Barry Marshall and Robin Warren, a quarter of a century ago, radically changed this view and had fundamental consequences for our current understanding and treatment of gastric diseases (Marshall and Warren, 1984). This bacterium lives near the surface of the human gastric mucosa and is one of the most successful microbial pathogens. Half of the world's population carries *H. pylori*, causing chronic gastritis in all infected human subjects, and more severe gastric disease in 10–15% of those infected (Montecucco and Rappuoli, 2001; Peek and Blaser, 2002; Sepulveda and Graham, 2002; Suerbaum and Josenhans, 2007). Infections commonly occur in early childhood and can persist lifelong in the absence of antimicrobial therapy. Although *H. pylori* infection is consistently associated with an intense cellular inflammatory response that is initiated by the innate and adaptive immune systems, the bacteria are

not eliminated. Several mechanisms of evasion have been identified, and *H. pylori* has become a paradigm for a persistent bacterium (Monack *et al.*, 2004; Cover and Blanke, 2005; Rieder *et al.*, 2005b; Wunder *et al.*, 2006; Wilson and Crabtree, 2007). Studies have revealed not only the ability of *H. pylori* to colonize individual hosts for many decades, but also that this bacterium has coexisted with man for a very long time through history. Genetic studies indicate that *H. pylori* spread during human migrations from east Africa around 58,000 years ago (Linz *et al.*, 2007). Because of this long period of co-evolution, it has been proposed that this colonization may have been beneficial for the human carrier and hence provided a selective advantage (Blaser and Atherton, 2004). In the modern world, however, infections with *H. pylori* exert a heavy toll of morbidity and mortality as a consequence of peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma and, the most dangerous complication, gastric adenocarcinoma (Peek and Blaser, 2002; Sepulveda and Graham, 2002; Kusters *et al.*, 2006; Correa and Houghton, 2007).

The clinical outcome of *H. pylori* infection is determined by a complex scenario of host–pathogen interactions (Blaser and Atherton, 2004; Peek and Crabtree, 2006). In

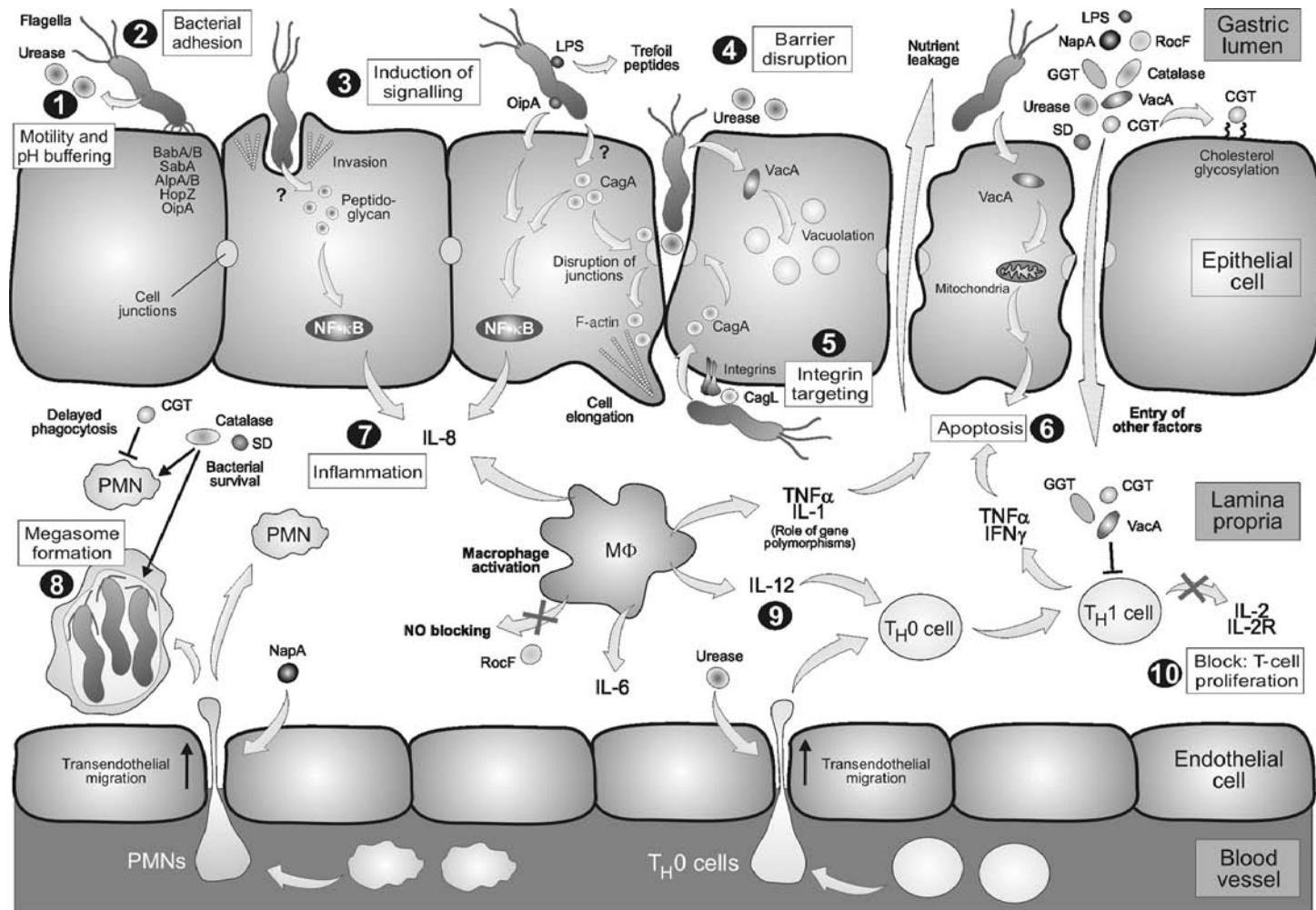
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the last few years, the cellular and molecular mechanisms used by *H. pylori* to subvert host defences have been investigated intensively (Fig. 11.1). Disease outcome is dependent on multiple parameters including both the bacterial genotype and genetic predisposition of the host as well as environmental factors. *H. pylori* isolates are surprisingly diverse in both their genome sequences and their pathogenicity. A myriad of bacterial factors have been shown to influence pathogenesis caused by *H. pylori* and can be classified as virulence factors and pathogenicity-associated factors. While virulence factors, by definition, have direct toxic or other damaging effects, pathogenicity factors are elements without direct damaging activities but which are also important, e.g. for establishment of successful bacterial colonization. There are two classical secreted virulence factors in *H. pylori*: the vacuolating cytotoxin (VacA) and the CagA protein encoded by the *cag* (cytotoxin-associated genes) pathogenicity island (cagPAI). Much of the research interest worldwide is focused on the CagA effector protein because *cagA*-positive *H. pylori* strains are associated with the development of more severe gastric diseases. Other known pathogenicity-associated phenotypes include flagella-driven motility in the stomach mucus layer, acid neutralization via urease (UreA, UreB and accessory proteins), adhesion to gastric epithelial cells mediated by several adhesins (see Lindén *et al.*, Chapter 12, this volume) and adherence of neutrophils to endothelial cells and chemotactic activities induced by the neutrophil-activating protein NapA (Graham and Yamaoka, 2000; Montecucco and Rappuoli, 2001; Aspholm *et al.*, 2006; Dubois and Borén, 2007; Suerbaum and Josenhans, 2007) (Fig. 11.1). In contrast to many other bacteria, *H. pylori* lipopolysaccharide (LPS) has very weak proinflammatory activity, and was recently shown to bind trefoil peptides in the gastric lumen, which probably promotes colonization (Reeves *et al.*, 2008). However, *H. pylori* induces proinflammatory responses by the concerted signalling activities of peptidoglycan, outer inflammatory protein A (OipA) and CagA, which activate the transcription factor nuclear factor- κ B (NF- κ B) (Graham and Yamaoka, 2000; Viala *et*

al., 2004; Brandt *et al.*, 2005). There are also numerous other marker genes for disease development such as *dupA* (duodenal ulcer promoting gene A) or *iceA* (induced by contact with the epithelium gene A), but their molecular functions and biological relevance are as yet unknown (Lu *et al.*, 2005). In addition, specific polymorphisms in human genes involved in proinflammatory and immunoregulatory processes, such as those encoding interleukin (IL)-1 β , IL-8, tumour necrosis factor- α (TNF α), NOD (nucleotide oligomerization domain) and others, have also been linked to an increased risk of developing gastric disease including cancer (Peek and Blaser, 2002; Amieva and El-Omar, 2008; see Sutton *et al.*, Chapter 7, this volume). Moreover, cagPAI-positive *H. pylori* can induce the expression of a DNA-editing enzyme (AID) in host cells, resulting in the accumulation of mutations in the tumour suppressor TP53 (Matsumoto *et al.*, 2007). Thus, induction of AID might be a mechanism whereby gene mutations accumulate during *H. pylori*-associated gastric carcinogenesis.

11.1.2 Animal and *in vitro* infection models

Recent functional studies in animal and cell culture models have provided compelling evidence for the importance of VacA, CagA and the cagPAI in *H. pylori* pathogenesis (Covacci and Rappuoli, 2000; Cover and Blanke, 2005; Rieder *et al.*, 2005b; Backert and Meyer, 2006; Hatakeyama, 2008). A range of mouse, Mongolian gerbil and rhesus monkey models have proven useful for studying *H. pylori*-induced pathology *in vivo* (Hirayama *et al.*, 1996; Watanabe *et al.*, 1998; Ogura *et al.*, 2000; Peek and Crabtree, 2006; Hornsby *et al.*, 2008). For example, mice deficient in protein tyrosine phosphatase receptor type Z (Ptpz, also called PTP- ζ or RPTP β) do not show mucosal damage and ulceration by VacA (Fujikawa *et al.*, 2003). The gerbil is another useful model, as gastric pathology resulting from *H. pylori* infection is similar to that in humans and this animal has been used to evaluate virulence and colonization factors as



well as host responses (Kavermann *et al.*, 2003; Franco *et al.*, 2005; Rieder *et al.*, 2005a). Much effort has been put into identifying the mechanism of *H. pylori*-associated carcinogenesis in gerbils and mice. A first direct causal link between CagA and oncogenesis *in vivo* was identified by the generation of transgenic C57BL/6J mice expressing CagA in the absence of *H. pylori*, some of which developed gastric polyps and adenocarcinomas of the

stomach and small intestine (Ohnishi *et al.*, 2008). Each animal model has distinct advantages and disadvantages and can, therefore, be considered as complementary systems (Rogers and Fox, 2004; Solnick *et al.*, 2004; Rogers and Houghton, 2009).

In addition to these *in vivo* models, the use of several *in vitro* cell culture systems has proven very efficient for studying signalling cascades of relevance to disease development.

Fig. 11.1. Model for *Helicobacter pylori*-induced gastric damage, inflammation and persistence. The interplay between target cells and a variety of pathogenicity factors modulates multiple host responses leading to both chronic gastritis and persistent colonization. (1) *H. pylori* use their flagella to enter the mucous layer and secrete urease for pH buffering. (2) Several adhesins such as BabA/B, SabA, AlpA/B, HopZ and OipA mediate binding to gastric epithelial cells. (3) After adherence, *H. pylori* can translocate effector molecules such as CagA and peptidoglycan into the host cell. Peptidoglycan binds to the intracellular receptor NOD1 and activates transcription factor NF- κ B to induce IL-8 release (Viala *et al.*, 2004). *H. pylori* is also able to occasionally enter host cells (Kwok *et al.*, 2002). Injected CagA can trigger several signalling cascades including the activation of proinflammatory transcription factor NF- κ B, actin rearrangements and disruption of cell-to-cell junctions. (4) Disruption of cell junctions by CagA and cellular effects induced by VacA and urease may contribute to disruption of the epithelial barrier. Internalization of VacA into the cells leads to the formation of large vacuoles and gastric damage, a hallmark of the ulceration process. In addition, host cell functions are subverted by LPS, which can bind to trefoil peptides in the lumen, and the CGT enzyme allows host cholesterol glycosylation. (5) Injection of CagA appears to be dependent on integrins, which are receptors on the basolateral side of the epithelium. (6) VacA can also trigger apoptosis when recruited to the mitochondria to induce the release of cytochrome c. These processes may cause the disruption of epithelial barrier functions, leading to leakage of nutrients into the gastric lumen and to numerous bacterial pathogenicity factors crossing the epithelial layer. (7) *H. pylori* antigens can activate macrophages to release several proinflammatory cytokines, such as IL-8, IL-6, IL-1 and IL-12. This and the aforementioned induction of IL-8 by epithelial cells constitute crucial determinants for *H. pylori*-triggered inflammatory responses. These cytokines attract immune cells such as PMNs to infiltrate into the bloodstream into the gastric mucosa. Bacterial NapA seems to play a role in this process (Brissler *et al.*, 2005). (8) A number of indicated *H. pylori* factors have been shown to play a role in oxidative defence and are particularly important for bacterial survival when *H. pylori* comes in contact with PMNs. Antiphagocytosis, delayed phagocytosis and megasome formation are also observed. (9) The IL-12 micro-environment induced by activated macrophages is particularly important for shifting the CD4⁺ helper response into a prominent Th1 type. Pathogenicity factors such as urease and as yet unidentified *cagPAI* components appear to enhance the recruitment of T cells from the bloodstream (Enarsson *et al.*, 2005). The production of TNF α , IFN γ and IL-1 from T cells and macrophages amplifies the VacA-induced apoptosis of epithelial cells. (10) *H. pylori* modulates the immune response by blocking T-cell proliferation using VacA based on down-regulation of IL-2 and IL-2R transcription (Boncristiano *et al.*, 2003; Gebert *et al.*, 2003). Inhibition of T-cell proliferation also occurs by the activities of CGT and GGT. Each of these activities is believed to contribute to *H. pylori* persistence and eventually to development of gastric disease. Abbreviations: AlpA/B, adherence-associated lipoproteins A and B; BabA/B, blood group antigen-binding adhesins A and B; CagA, cytotoxin-associated gene A protein; CD4⁺ cells, cluster of differentiation 4-positive cells; CGT, cholesterol-glucosyltransferase; F-actin, filamentous actin; GGT, γ -glutamyl transpeptidase; Hop, *Helicobacter* outer-membrane porin; IFN γ , interferon- γ ; IL, interleukin, IL-2R, interleukin-2 receptor; LPS, lipopolysaccharide; M Φ , macrophage; NapA, neutrophil-activating protein A; NF- κ B, nuclear factor- κ B; NO, nitric oxide; NOD1, nucleotide oligomerization domain protein 1; OipA, outer inflammatory protein A; PMN, polymorphonuclear neutrophil; RocF, protein with arginase activity; SabA, sialic acid-binding adhesin A; SD, superoxide dismutase; T_H cell, T-helper cell; TNF α , tumour necrosis factor- α ; VacA, vacuolating cytotoxin. (Adapted from Backert *et al.* (2006) with kind permission from Elsevier.)

In particular, gastric epithelial and colonic cell lines (e.g. AGS, Caco2, KATO-III, AZ-521, HEP-2, MKN28 and MKN45), primary gastric epithelial cells and professional phagocytes, including human polymorphonuclear neutrophils (PMNs) and human or murine macrophage cell lines (e.g. JoskM, THP-1, U937, RAW264.7 and J774A.1), have been utilized. A series of well-studied phenotypic responses elicited during infection of both gastric epithelial cells and phagocytes *in vitro* is shown in Fig. 11.2. This includes induction of cell scattering, cell motility and dramatic cytoskeletal modifications in gastric epithelial cells resulting in the so-called 'hummingbird' or elongation phenotype, where the *cagPAI* and *CagA* have been shown to play a role (see Section 11.3.7). In addition, the induction of vacuolation and apoptosis can also be induced by *VacA* in these cells (see Sections 11.2.1 and 11.2.7). Each of these phenotypes probably contributes to *H. pylori*-induced disruption of epithelial barrier functions *in vivo* (Fig. 11.1). Such lesions are regularly seen in biopsies from patients with severe gastric disease, and suggest that *H. pylori* has direct contact with a variety of immune cells. Thus, long-term survival of the bacterium in the human stomach requires mechanisms to evade killing by the host immune system. Indeed, a multitude of such mechanisms have been identified using *in vitro* cell systems. For example, *H. pylori* actively retards uptake by macrophages and subsequently persists within large vacuoles called megasomes that result from phagosome fusion (Allen *et al.*, 2000). Regulation of local actin polymerization and phagocytosis by atypical protein kinase C (PKC)- ζ plays a role in the delay of bacterial uptake (Allen and Allgood, 2002). In studies using human blood monocytes and PMNs, *H. pylori* has been shown to actively block its own phagocytosis (Ramarao *et al.*, 2000b). Each of these phenotypes depends on the presence of a functional *cagPAI* and an unknown factor which appears not to be *CagA*. *VacA* also exhibits immune-suppressive effects (see Section 11.2.8) and arginase (encoded by *rocF*) that is expressed by *H. pylori* can down-regulate eukaryotic nitric oxide (NO) production (Gobert *et al.*, 2001). The antimicrobial effect of NO is an essential part of innate immunity, thus down-

regulation of NO may allow *H. pylori* to survive (Fig. 11.1). Other described pathogenicity-related factors include catalase or superoxide dismutase, with proposed functions in the oxidative defence of *H. pylori*, as well as cholesterol- α -glucosyltransferase and γ -glutamyl transpeptidase, which inhibit T-cell proliferation (Ramarao *et al.*, 2000a; Wunder *et al.*, 2006; Schmees *et al.*, 2007; Carpenter *et al.*, 2009). In addition, infection of JoskM and U937 cells with *cagPAI*-positive *H. pylori* is accompanied by the formation of large homotypic cell aggregates (Fig. 11.2). This occurs through up-regulation and recruitment of ICAM-1 (intracellular adhesion molecule-1) to the cell surface of infected cells, which then mediates aggregation via its ligand LFA-1 (lymphocyte function-associated molecule-1), a signalling pathway that may regulate cell-cell interactions, inflammatory responses and also inhibit bacterial uptake (Moese *et al.*, 2002). In the following sections we describe in detail the epidemiology, function and signalling activities of the two major virulence constituents of *H. pylori*, *VacA* and *CagA*.

11.2 The Vacuolating Cytotoxin, VacA

11.2.1 Discovery of VacA

Soon after the discovery of *H. pylori*, Leunk and co-workers (1988) reported that broth-culture supernatants of approximately 55% of *H. pylori* isolates contained a proteinaceous cytotoxin that induced cytoplasmic vacuolation in eukaryotic cells *in vitro*. Two other early studies indicated that the prevalence of infection with toxin-producing *H. pylori* is higher among infected persons with peptic ulceration than among infected persons with gastritis alone (Figura *et al.*, 1989; Goossens *et al.*, 1992). Multiple cell types from man and animal species were susceptible to its toxic activity. Subsequently, a protein with vacuolating activity was purified from *H. pylori* broth-culture supernatants, suggesting that it was secreted as a soluble toxin (Cover and Blaser, 1992). Under denaturing conditions, the molecular mass of the purified cytotoxin was ~87 kDa and the non-denatured form was estimated to be about 972 kDa in size,

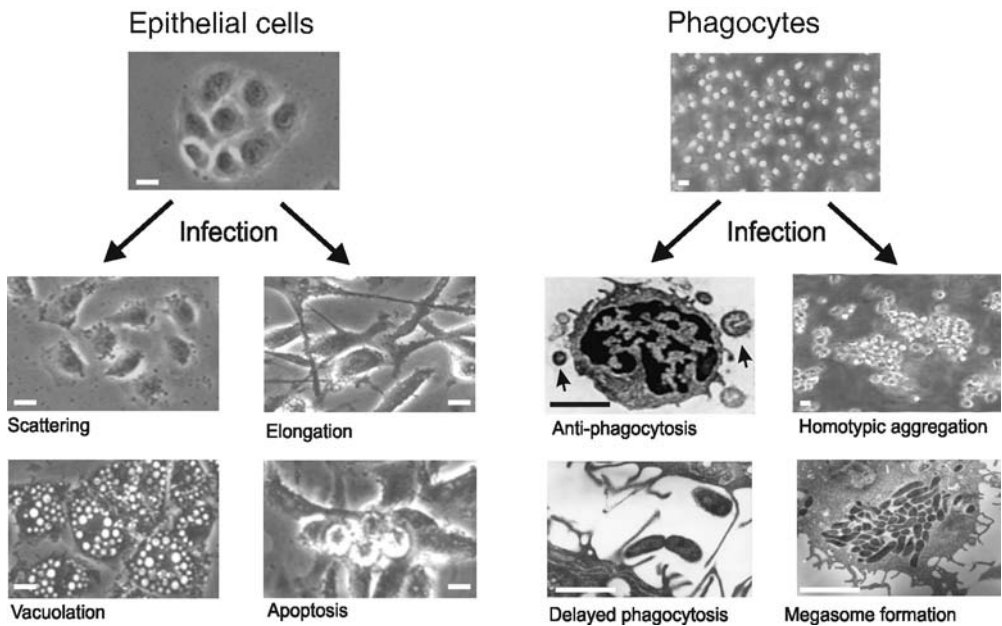


Fig. 11.2. Phenotypic responses during infection of AGS gastric epithelial cells and immune cells with *Helicobacter pylori* *in vitro*. Infection of cultured AGS cells with *H. pylori* results in different phenotypes that can be observed in a time- and strain-dependent manner. First, AGS infected with cytotoxin-associated genes pathogenicity island (*cagPAI*)-positive *H. pylori* exhibit a strong motility response (after 1–2 h) followed by elongation of the cells (after 3–4 h) (Moese *et al.*, 2004). Vacuole formation can be observed during infection with toxin-producing *H. pylori* (after 3–6 h). Vacuolating cytotoxin (*VacA*) is also implicated in triggering apoptosis of infected epithelial cells (after 24–48 h). Infection of phagocytes by *cagPAI*-positive *H. pylori* can induce delayed phagocytosis (Allen *et al.*, 2000) or anti-phagocytosis (Ramarao *et al.*, 2000b) and homotypic aggregation of the infected cells (Moese *et al.*, 2002). Bars, 5 μ m. (Adapted from Backert *et al.* (2006) with kind permission from Elsevier and Blackwell Publishing. The pictures for delayed phagocytosis and megasome formation were originally published by Allen *et al.* (2000) and were kindly provided by Lee-Ann Allen with permission of *The Journal of Experimental Medicine*.)

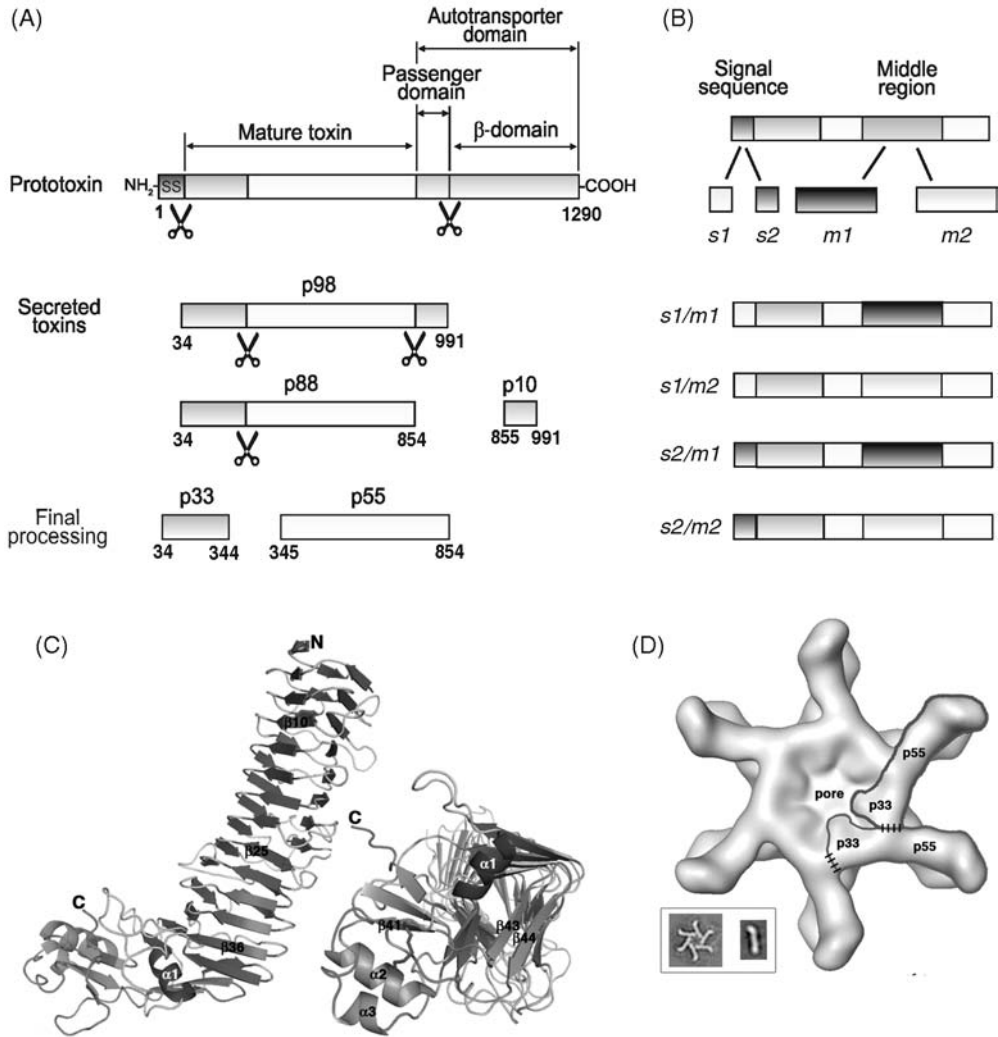
suggesting that *VacA* can assemble into large oligomeric structures (Cover and Blaser, 1992). Antiserum raised against the purified cytotoxin completely neutralized vacuolating activity. Similarly, cytotoxin activity was neutralized by serum immunoglobulin (Ig) G antibodies from a subset of *H. pylori*-infected persons, but not by serum IgG from uninfected persons. The gene encoding the vacuolating cytotoxin was termed *vacA* and has been cloned from several strains (Cover *et al.*, 1990; Phadnis *et al.*, 1994; Schmitt and Haas, 1994; Telford *et al.*, 1994). The derived protein sequence of *VacA* does not show striking sequence similarity to any other known

bacterial or eukaryotic protein, but exhibits short stretches homologous to ion channel or transport proteins (Cover and Blaser, 1992). However, the structural organization of *VacA* closely resembles the IgA protease type of exoproteins produced by pathogenic *Neisseria* and *Haemophilus* (Schmitt and Haas, 1994) that are today known as the autotransporter or type-V secretion family of proteins (Backert and Meyer, 2006). In response to acidic pH, *VacA* undergoes a structural change that increases its activity and renders it resistant to proteolysis by pepsin (de Bernard *et al.*, 1995). Thus, *VacA* is ideally suited to activity in the acidic gastric milieu.

11.2.2 VacA expression, secretion, maturation and allelic variation

Although *vacA* is predicted to encode a potential 135–145 kDa protein depending on the strain, VacA proteins of this size have never been reported (Cover and Blanke, 2005). Like other classical autotransporters, VacA undergoes cleavage during the transport via bacterial membranes (Fig. 11.3A), and VacA contains a 33-amino-acid signal sequence. Three VacA protein spots were identified by two-dimensional gel electrophoresis (2-DE) in the TIGR strain 26695. The most dominant

VacA species have apparent molecular masses of approximately 95.7 kDa and 87.9 kDa, found both in the *H. pylori* membrane and in culture supernatant fractions (Bumann *et al.*, 2002; Backert *et al.*, 2005). In addition, a 10.5 kDa VacA spot was found in the secreted protein fraction (Bumann *et al.*, 2002). Mapping data of the identified peptides are consistent with a model where the 95.7 kDa VacA protein is the secreted toxin, which is then cleaved into an 87.9 kDa mature protein (p88) and a 10.5 kDa passenger domain (p10, also called the α -protein) (Fig. 11.3A). The exact cleavage site between p88 and the



β -domain was detected between amino acids 991 and 992 (Bumann *et al.*, 2002). The processed β -domain of the autotransporter domain was not detected by 2-DE but has been observed in total cell lysates (Telford *et al.*, 1994). However, the mature, secreted p88 subunit can undergo further limited proteolytic cleavage to yield two fragments, p33 and p55 (Telford *et al.*, 1994; Blaser and Atherton, 2004; Cover and Blanke, 2005; Rieder *et al.*, 2005b) (Fig. 11.3A, bottom). The latter two processing products are considered to represent two functional domains or subunits of VacA. Cell binding is mediated by the p55 fragment of mature VacA (Reyrat *et al.*, 1999) while both p33 and p55 have diverse functions (see Sections 11.2.3 to 11.2.9).

The *vacA* gene is present among virtually all *H. pylori* strains worldwide, but exhibits considerable sequence variation. In some studies, VacA and its subunits have slightly different sizes, but we will use the p33 and p55 nomenclature throughout this chapter. The basis of this sequence variation was found in several *vacA* alleles that have been identified in the signal region (genotypes *s1* and *s2*) and in the mid-region (genotypes *m1* and *m2*), occurring in all possible combinations (Fig. 11.3B). VacA molecules of the *s2* genotype are inactive in assays for vacuolating cytotoxicity, genotype *s1/m1* produces an extensive vacuolation phenotype in a large variety of cell types, and genotype *s1/m2* produces detectable vacuolation in a more restricted range of

Fig. 11.3. Processing, allelic variation and molecular structure of vacuolating cytotoxin (VacA). (A) The amino-terminal signal sequence (SS) and carboxy-terminal domain are cleaved from the 135–145 kDa VacA protoxin to yield a 98 kDa secreted toxin (p98) that is transported into the extracellular space via an autotransporter mechanism (Cover and Blanke, 2005). Proteins of this autotransporter family are able to mediate their own secretion without the need of additional bacterial proteins. The 33 kDa carboxy-terminal β -barrel domain of VacA is predicted to insert into the outer membrane and to form a channel through which the mature VacA toxin is secreted. The secreted toxin can be processed into the mature toxin (p88) and p10. The mature, secreted p88 subunit can undergo further limited proteolytic cleavage to yield two fragments, p33 and p55 (Telford *et al.*, 1994; Blaser and Atherton, 2004; Cover and Blanke, 2005), believed to represent two functional domains or subunits of VacA. (B) A high level of sequence diversity occurs among *vacA* alleles from different *H. pylori* strains. The *vacA* gene is a polymorphic mosaic with two possible signal regions (designated *s1* and *s2*) and two possible mid-regions (designated *m1* and *m2*). Mosaic forms of *vacA* are thought to arise via homologous recombination among *vacA* alleles from different strains and different combinations can occur as indicated. The *s1* signal region is fully active, but the *s2* region encodes a protein with a different signal-peptide cleavage site, resulting in a short amino-terminal extension to the mature toxin that blocks vacuolating activity and attenuates pore-forming activity. The mid-region encodes a cell-binding site, but the *m2* type binds to and induces the vacuolation phenotype in fewer cell lines *in vitro*. (C) Crystal structure of the p55 subunit. p55 adopts a β -helix structure composed of three parallel β -sheets connected by loops of varying length and structure (Gangwer *et al.*, 2007). The α 1-helix is contained within one of the loops to exemplify how it caps the end of the β -helix. The carboxy-terminal domain has a mixture of α/β secondary structure elements and contains a disulfide bond, not previously observed in an autotransporter passenger domain structure. The carboxy-terminus of the β -helix shown to the right is capped by a β -hairpin from the carboxy-terminal domain and the α 1-helix located in one of the long helix loops. This view represents a rotation of the molecule on the left side by $\sim 90^\circ$ into the plane of the page. (D) Docking p55 into a 19 Å cryo-EM (electron microscopy) map of the VacA dodecamer results in a model for oligomerization. Twelve p55 subunits are shown docked into a 19 Å cryo-EM map of a VacA dodecamer (El-Bez *et al.*, 2005). Gangwer *et al.* (2007) proposed an oligomerization model in which p33 interacts with the amino-terminal portion of p55 from the neighbouring subunit. Regions of contact between p33 and p55 are depicted with dashed lines (right structure). From EM images, the shape of a VacA hexamer (inset, left) is similar to the shape of a single layer within the dodecamer (El-Bez *et al.*, 2005). The rod-like shape of the p88 VacA monomer (inset, right) supports a model in which the β -helix observed in p55 will extend into p33. (Parts C and D were originally published in Gangwer *et al.* (2007) and were kindly provided by Borden Lacy with permission of the National Academy of Sciences, USA.)

cell types in agreement with its location in p55 being the VacA binding domain (Montecucco and Rappuoli, 2001; Blaser and Atherton, 2004; Cover and Blanke, 2005; Rieder *et al.*, 2005b). Most of the research has focused on the most interactive (vacuolating) type, *s1/m1*. In addition, two intermediate region variants (*i1* and *i2*) have been identified as important determinants of VacA toxicity (Rhead *et al.*, 2007).

11.2.3 Molecular structure of VacA

The secreted p88 toxin can assemble into water-soluble oligomeric structures (Lupetti *et al.*, 1996) and can insert into planar lipid bilayers to form anion-selective membrane channels (Czajkowsky *et al.*, 1999; Iwamoto *et al.*, 1999; Tombola *et al.*, 1999). These structures resemble 'flowers' in which a central ring is surrounded by peripheral 'petals' (Fig. 11.3D). The first reported high-resolution images were 19 Å cryo-EM (electron microscopy) maps of VacA dodecamers (El-Bez *et al.*, 2005), and atomic-force microscopy and electrophysiological studies suggest that membrane-associated VacA channels are single-layered structures (Czajkowsky *et al.*, 1999; Iwamoto *et al.*, 1999). When the p33 and p55 subunits are expressed independently and then mixed, p33 and p55 physically interact and reconstitute vacuolating activity (Ye *et al.*, 1999; Torres *et al.*, 2004, 2005). The p33 domain contains a hydrophobic sequence (residues 6–27) involved in pore formation (Vinion-Dubiel *et al.*, 1999; McClain *et al.*, 2003), whereas p55 contains one or more cell-binding domains (Reyrat *et al.*, 1999; Wang and Wang, 2000). The 2.4 Å crystal structure of p55, which has an important role in mediating VacA binding to host cells, has recently been reported (Gangwer *et al.*, 2007). The structure is predominantly a right-handed parallel β -helix, a feature that is characteristic of autotransporter passenger domains but unique among known bacterial toxins (Fig. 11.3C). Notable features of p55 include disruption of β -sheet contacts that result in five β -helix subdomains and a carboxy-terminal domain which contains a disulfide bond. Interestingly, p55 contains the *m1* and *m2*

alleles, and investigation of protein sequences from unrelated *H. pylori* strains allowed the identification of structural features of the VacA surface that may be important for interactions with host receptors (Gangwer *et al.*, 2007).

11.2.4 VacA targets multiple host surface molecules of epithelial and immune cells

In an effort to understand how VacA contributes to *H. pylori* colonization of the stomach and the development of gastroduodenal disease, the cellular effects of VacA have been investigated both *in vivo* and *in vitro*. A model for the complex interactions of VacA with multiple cell types and downstream signaling events is shown in Fig. 11.4 and interaction partners are summarized in Table 11.1. Like many bacterial toxins, VacA interacts with the plasma membrane of susceptible cells as a first step in intoxication (Cover and Blanke, 2005). Subsequent to binding, VacA is internalized by a pinocytic-like mechanism (Blaser and Atherton, 2004; Cover and Blanke, 2005; Rieder *et al.*, 2005b). A remarkable feature of VacA is that it can target multiple cell-surface components *in vitro* or on epithelial cells, including RPTP β (receptor protein-tyrosine phosphatase β) (Yahiro *et al.*, 1999; Fujikawa *et al.*, 2003), RPTP α (Yahiro *et al.*, 2003), fibronectin (Hennig *et al.*, 2005), the epidermal growth factor (EGF) receptor (Seto *et al.*, 1998), heparin sulfate (Utt *et al.*, 2001), various lipids (Moll *et al.*, 1995; Molinari *et al.*, 1998b; Czajkowsky *et al.*, 1999) and sphingomyelin (Gupta *et al.*, 2008), as well as CD18 (β_2 -integrin) on T cells (Sewald *et al.*, 2008). Determining which of these factors is/are required for internalization and subversion of cellular signalling has been a challenging topic of research and is still not fully elucidated.

The role of RPTPs for VacA-dependent activities has been studied in great detail. For example, VacA causes gastric injury in wild-type mice but not in RPTP β -knockout mice, which indicates that interaction of VacA with RPTP β on the surface of gastric epithelial cells is mechanistically important in VacA-induced gastric injury (Fujikawa *et al.*, 2003).

Table 11.1. Interaction partners of vacuolating cytotoxin (VacA) and proposed roles in *Helicobacter pylori* infection.

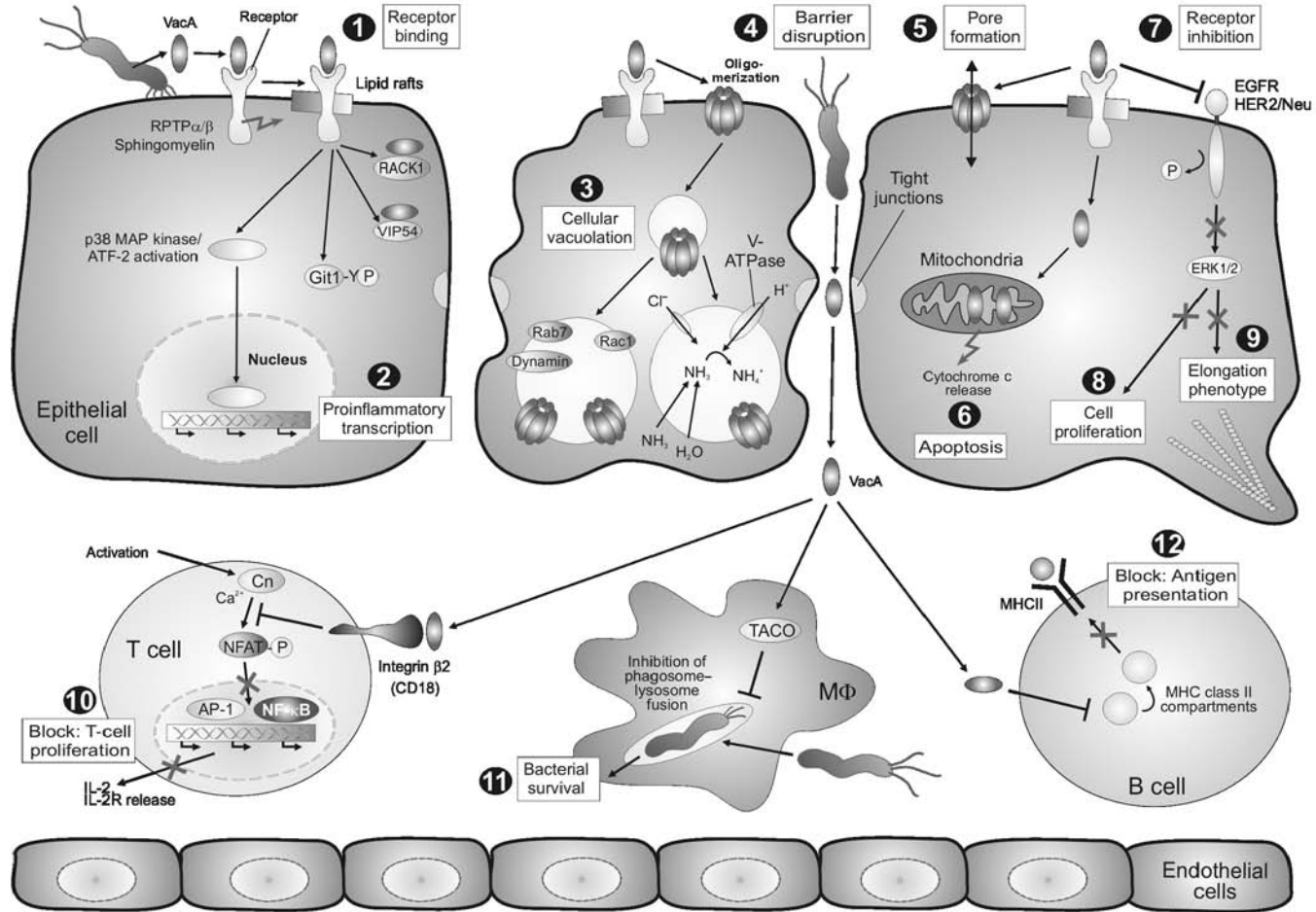
Interaction partner ^a	Proposed function	Experimental evidence ^c	Applied methods ^d	Reference(s)
Anionic lipid bilayers	Low pH-triggered pore formation	Binding <i>in vitro</i>	AFM	Czajkowsky <i>et al.</i> (1999)
CD18 (β_2 -integrin)	Receptor on T cells	Infection <i>in vitro</i> Treatment of cells <i>in vitro</i>	IF, live cell imaging, use of knockout T cells, flow cytometry	Sewald <i>et al.</i> (2008)
EGFR	Receptor on epithelial cells	Infection <i>in vitro</i>	IP, antibody blocking	Seto <i>et al.</i> (1998)
Fibronectin	Binding of <i>H. pylori</i> to host cells	Binding <i>in vitro</i>	Cell adhesion and binding assays	Hennig <i>et al.</i> (2005)
Glycosphingolipids	Binding partner for VacA internalization	Binding <i>in vitro</i>	Chromatography, MS, binding assays	Roche <i>et al.</i> (2007)
Heparin sulfate	Receptor/co-receptor on epithelial cells	Binding <i>in vitro</i>	SPR-based biosensor studies	Utt <i>et al.</i> (2001)
Lipid vesicles	Binding and host cell entry of the toxin	Binding <i>in vitro</i>	Light scattering and energy transfer studies	Moll <i>et al.</i> (1995)
Lipid rafts	Putative docking and entering sites on cells	Treatment of cells <i>in vitro</i>	Cell fractionation, IF, MCD treatment, flow cytometry	Schraw <i>et al.</i> (2002); Kuo and Wang (2003); Nakayama <i>et al.</i> (2006)
Phospholipids	Binding and host cell entry of the toxin	Binding <i>in vitro</i>	Photolabelling and ANS-binding studies	Molinari <i>et al.</i> (1998b)
RPTP α	Receptor on epithelial cells	Infection and treatment of cells <i>in vitro</i>	IP, MS, antisense silencing	Yahiro <i>et al.</i> (2003); De Guzman <i>et al.</i> (2005)
RPTP β	Receptor on epithelial cells, receptor for GIt1 ^b phosphorylation	Infection of mice and cells Binding <i>in vitro</i>	IP, IF, siRNA, flow cytometry, use of knockout mice	Yahiro <i>et al.</i> (1999); Padilla <i>et al.</i> (2000); Fujikawa <i>et al.</i> (2003)
RACK-1	Not determined	Binding <i>in vitro</i>	Y2H, PD	Hennig <i>et al.</i> (2001)
Sphingomyelin	Receptor on epithelial cells	Binding and treatment of cells <i>in vitro</i>	Binding studies, flow cytometry, ELISA, treatment with sphingomyelinase	Gupta <i>et al.</i> (2008)
VIP54	Interaction between intermediate filaments and late endosomes	Infection <i>in vitro</i> Treatment of cells <i>in vitro</i>	Y2H, IP, IF	de Bernard <i>et al.</i> (2000)

^aAbbreviations: EGFR, epidermal growth factor receptor; RACK-1, receptor for activated C-kinase 1; RPTP α/β , receptor protein-tyrosine phosphatase α/β ; VIP54, VacA-interacting protein of 54 kDa.

^bGIt1, G-protein-coupled receptor-kinase-interacting protein-1.

^cInfection *in vitro*, *H. pylori* infection of cultured host target cells; Infection of mice, infection of wild-type and knockout mice; Binding *in vitro*, purified or recombinant VacA was incubated with the factor of interest *in vitro*; Treatment of cells *in vitro*, purified or recombinant VacA was applied externally to cultured host cells *in vitro* and then binding of the factor of interest was investigated.

^dAbbreviations: AFM, atomic force microscopy; ANS, 1-anilino-8-naphthalene sulfonate; IF, co-localization by immunofluorescence; IP, co-immunoprecipitation; MCD, methyl- β -cyclodextrin; MS, mass spectrometry; PD, pull-down experiments; siRNA, silencing RNA knockdown; SPR, surface plasmon resonance; Y2H, yeast two-hybrid screening.



Interestingly, VacA can still induce vacuolation in cultured RPTP $\beta^{-/-}$ gastric epithelial cells and in G401 human kidney cells, which do not express RPTP β (Fujikawa *et al.*, 2003; Yahiro *et al.*, 2003), but down-regulation of RPTP β by silencing oligonucleotides in HL-60 cells inhibited VacA-induced vacuolation (Padilla *et al.*, 2000). These data indicate that RPTP β has an important role in the process of VacA-induced signalling but not necessarily vacuolation per se. VacA has also been shown to induce tyrosine phosphorylation of the G-protein-coupled receptor-kinase-interacting protein-1 (Git1) (Fujikawa *et al.*, 2003) and lipid raft-dependent signalling (Nakayama *et al.*, 2006). In line with these observations, VacA was shown to bind to a QTTQP motif (positions 747–751) in the extracellular domain of RPTP β which was also necessary for inducing Git1 phosphorylation (Yahiro *et al.*, 2003). Another reported consequence of

VacA binding to RPTP β is detachment of primary murine gastric epithelial cells from matrices (Fujikawa *et al.*, 2003). In addition to the RPTPs, VacA has been shown to interact with lipid preparations and artificial membranes, but the specificity and importance of VacA–lipid interactions for toxin function was unknown for a long time. Recently, evidence was provided that sphingomyelin modulates the sensitivity of epithelial cells to VacA (Gupta *et al.*, 2008). Sphingomyelin is a key structural component of the cell membrane and, in particular, lipid rafts, while at the same time serving a functional role as the parent compound of several lipid mediators. Sphingomyelin was shown to be important for VacA binding to cells and its association with lipid rafts on the plasma membrane, suggesting that sphingomyelin functions as a receptor for VacA on epithelial cells (Gupta *et al.*, 2008). However, VacA

Fig. 11.4. Multiple activities of secreted VacA contribute to altered host cell signalling and *Helicobacter pylori* colonization of the stomach. (1) Secreted VacA forms monomers and oligomers. The monomeric form binds to epithelial cells, both non-specifically and through specific receptor binding, i.e. to RPTP α/β , which may also modulate cell signalling. A series of experiments has demonstrated the potential for VacA to specifically bind RACK1 or VIP54. VacA can also trigger phosphorylation of Git1 and p38 MAP kinase, the latter being implicated in (2) ATF-2 transcription factor activation resulting in the induction of proinflammatory gene expression. (3) Following endocytosis, internalized VacA can also trigger cell vacuolation, depending on cellular factors such as the V-ATPase, three GTPases and others. Although the vacuolation phenotype is easily detected *in vitro*, vacuoles are rarely seen *in vivo*. (4) In polarized host cell monolayers, VacA can decrease transepithelial resistance significantly, but the involved mechanism is not well understood. (5) Membrane-bound VacA can oligomerize and form pores. (6) VacA also induces apoptosis, in part by forming pores in mitochondrial membranes, allowing cytochrome c egress. (7) VacA exhibits suppressive effects on the activation of the EGFR and HER2/Neu receptors by an as yet unknown mechanism. However, inactivation of these growth factor receptor tyrosine kinases leads to suppression of the MAP kinase ERK1/2, and consequently (8) down-regulation of cell proliferation and (9) CagA-induced host cell elongation *in vitro*. (10) VacA can interact with the receptor integrin β_2 (CD18) on T cells and block the transcription factor NFAT. This results in inhibition of IL-2 secretion resulting in a blockade of T-cell activation and proliferation. (11) VacA can also inhibit phagosome–lysosome fusion in macrophages by recruiting the coat protein TACO (coronin-1). (12) VacA inhibits antigen presentation in B cells, possibly by blocking the maturation of endosomes to MHC class II compartments where antigen loading normally occurs. However, the relative importance of the latter effects on *H. pylori*–host cell interaction and bacterial persistence remains unclear. Abbreviations: AP-1, activator protein 1; ATF-2, activating transcription factor 2; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinases 1 and 2; Git1, G-protein-coupled receptor-kinase-interacting protein-1; HER2/Neu, human epidermal growth factor receptor 2, another member of the EGFR family; IL-2(R), interleukin-2 (receptor); MHC, major histocompatibility complex; M Φ , macrophage; NFAT, nuclear factor of activated T cells; NF- κ B, nuclear factor- κ B; p38 MAP kinase, 38 kDa mitogen-activated protein kinase; Rab GTPases, Ras superfamily of small GTPases; RACK1, receptor for activated C-kinase 1; RPTP α/β , receptor protein-tyrosine phosphatase α/β ; TACO, tryptophan aspartate-containing coat protein; VacA, vacuolating toxin; VIP54, VacA-interacting protein of 54 kDa.

interacts not only with gastric epithelial cells but also with immune cells including macrophages, B cells and T cells (Fig. 11.4). For example, VacA efficiently enters activated, migrating primary human T lymphocytes by binding to the CD18 receptor and exploiting the recycling of LFA-1 (Sewald *et al.*, 2008). LFA-1-deficient Jurkat T cells were resistant to vacuolation, and genetic complementation restored sensitivity to VacA. Interestingly, VacA targeted human but not murine CD18 for cell entry, consistent with the species-specific adaptation of *H. pylori* (Sewald *et al.*, 2008).

11.2.5 VacA-dependent effects on endosomal maturation and vacuolation of epithelial cells

The first identified effect of internalized VacA was its ability to induce cell vacuolation in many cell types (Leunk *et al.*, 1988). A current model for vacuole formation proposes that VacA binds to the plasma membrane of target cells, is internalized, forms anion-selective channels in endosomal membranes and the vacuoles arise due to swelling of endosomal compartments (Fig. 11.4, middle). VacA-induced vacuoles are hybrid compartments of late endosomal origin containing some lysosomal markers (Molinari *et al.*, 1997). Vacuole production depends on the presence and activity of a number of cellular factors including V-ATPase (Papini *et al.*, 1993) and three GTPases, Rab7 (Papini *et al.*, 1997), Rac1 (Hotchin *et al.*, 2000) and dynamin (Suzuki *et al.*, 2001). Protein kinase PIKfyve is also necessary for vacuole formation, and micro-injection of its substrate PIP2 (phosphatidylinositol-3,5-bisphosphate) is sufficient to induce vacuoles (Ikonomov *et al.*, 2002), whereas involvement of the SNARE protein syntaxin-7 is more controversial (de Bernard *et al.*, 2002; Suzuki *et al.*, 2003). When expressed intracellularly, the minimum portion of VacA required for cell-vacuolating activity comprises the entire p33 domain and about 110 amino acids from the amino terminus of p55 (de Bernard *et al.*, 1998; Ye *et al.*, 1999). Vacuolating activity also depends on VacA di- or oligomerization (Willhite *et al.*, 2002; Ye

and Blanke, 2002) and requires the hydrophobic amino terminus (~30 amino acids) containing three tandem GXXXG motifs, which are transmembrane dimerization sequences (McClain *et al.*, 2003; Kim *et al.*, 2004). These requirements indicate that channel formation is a prerequisite for vacuolation, which is also supported by the fact that pharmacological anion-channel inhibitors block vacuole formation (Szabó *et al.*, 1999).

11.2.6 VacA-induced effects on epithelial monolayer permeability

One of the characteristics of functional epithelia is the development of transepithelial electrical resistance (TER), a measurable indicator of epithelial integrity. In polarized host cell monolayers, VacA was shown to decrease TER significantly (Papini *et al.*, 1998). This effect was observed in several different types of polarized epithelial cells including MDCK, T84 and EPH4. Interestingly, this effect was independent of VacA's vacuolating activity as also observed with *vacA m2* alleles (Pelicic *et al.*, 1999). It was proposed that selective permeabilization of epithelial monolayers by VacA results in the release of molecules such as Fe^{3+} , Ni^{2+} , sugars and amino acids, which might support the growth of *H. pylori* within the gastric mucus layer (Papini *et al.*, 1998; Pelicic *et al.*, 1999). As yet, the mechanism by which VacA alters paracellular permeability is not well understood. It has also been noted that VacA increases the transepithelial flux of certain molecules such as urea – the substrate of *H. pylori* urease (Tombola *et al.*, 2001). The release of urea from cells, however, is attributed to the formation of VacA channels in the plasma membrane. Further studies have indicated that disruption of cell–cell junctions in polarized epithelia by *H. pylori* is not dependent on VacA as a sole factor, but also requires injected CagA, which targets both tight and adherens junctions (see Section 11.3.8). More complexity arises from another recent study showing that *H. pylori* induced a progressive loss of barrier function in MKN28 gastric epithelial cells and hypergastrinaemic INS-GAS mice, which was attenuated by inactivation of the *ureB* gene, but not *vacA* or

cagPAI genes (Wroblewski *et al.*, 2009). Interestingly, in that study *H. pylori* induced the deregulation of specific tight-junction proteins, including occludin internalization and phosphorylation of MLC (myosin regulatory light chain) by MLC kinase. Thus, epithelial barrier disruption by *H. pylori* is highly complex, requiring multiple bacterial factors and signalling pathways (Wessler and Backert, 2008).

11.2.7 Internalized VacA targets mitochondria to induce apoptotic cell death

Infection with *H. pylori* has been associated with both increased and reduced levels of apoptosis in the gastric epithelium (Blaser and Atherton, 2004). *In vitro*, *H. pylori* reproducibly stimulates apoptosis in infected gastric epithelial cells, and this has also been seen in tissues isolated from infected patients or animal models (Montecucco and Rappuoli, 2001; Peek and Blaser, 2002). Early studies indicated that purified VacA induced mitochondrial damage, indicated by a dramatic decrease in cellular ATP levels (Kimura *et al.*, 1999). In transfected HEp-2 cells, p33 was found to localize specifically to mitochondria, whereas p55 was cytosolic (Galmiche *et al.*, 2000). When purified VacA was incubated with purified mitochondria, p33 (but not p55) was translocated into these organelles. Transient expression of p33–green fluorescent protein (GFP) or VacA–GFP in HeLa cells induced the release of cytochrome c from mitochondria and activated caspase-3, as determined by the cleavage of poly (ADP-ribose) polymerase (PARP). PARP cleavage was antagonized specifically by co-transfection of DNA encoding Bcl-2, known to block mitochondria-dependent apoptotic signals (Galmiche *et al.*, 2000). These findings are supported by *in vitro* infection studies using wild-type and *vacA* mutant *H. pylori* strains (Kuck *et al.*, 2001; Cover *et al.*, 2003), which show that the *s1/m1* type of VacA induces high levels of apoptosis, while *s2/m1* toxin or a *vacA* mutant lacking the hydrophobic region near the amino terminus does not.

These results indicate that VacA induces gastric epithelial cell apoptosis and suggest

that differences in levels of gastric mucosal epithelial apoptosis among *H. pylori*-infected persons may result from strain-dependent variations in VacA structure. However, for gastric cell apoptosis *in vivo*, several other parameters are likely to be important. Normally, rapid self-renewal of gut epithelia, which occurs by a balance of progenitor proliferation and pit-cell apoptosis, serves as a host defence mechanism to limit bacterial colonization. It was demonstrated that *H. pylori* inhibits the apoptotic loss of pit cells in Mongolian gerbils (Mimuro *et al.*, 2007). Suppression of apoptosis contributed to pit hyperplasia and persistent bacterial colonization of the stomach, and this was mediated by CagA, which stimulated the pro-survival mitogen-activated protein (MAP) kinase members ERK1/2 and anti-apoptotic protein MCL-1 in the gastric pits. Thus, CagA counteracts VacA effects and activates host cell survival and anti-apoptotic pathways to overcome self-renewal of the gastric epithelium and help sustain *H. pylori* infection (Mimuro *et al.*, 2007).

11.2.8 VacA exhibits suppressive effects on immune cells

Research since the late 1990s has provided compelling evidence that, during evolution, *H. pylori* has accumulated multiple activities to suppress immune cell functions. Some of these functions have been attributed to VacA (Fig. 11.4). A very early study demonstrated that VacA can inhibit processing and presentation of antigenic peptides to human CD4⁺ T cells (Molinari *et al.*, 1998a) and suggested for the first time that VacA may contribute to the persistence of *H. pylori* by interfering with protective immunity (Molinari *et al.*, 1998a). In infected professional phagocytes, such as human THP-1 and mouse RAW 264.7 macrophage cell lines, wild-type *H. pylori* displayed an enhanced survival compared with *vacA* deletion mutants (Zheng and Jones, 2003). Thus, it appears that VacA arrests phagosome maturation by recruiting and retaining tryptophan aspartate-containing coat protein (TACO or coronin-1). However, increased survival of isogenic *vacA* mutants was not seen in two other reports when freshly

isolated human monocytes were infected (Ramarao *et al.*, 2000b; Rittig *et al.*, 2003). These differences may be due to different *vacA* alleles, infective dose and/or cell types used in the different studies.

Infection or co-incubation of purified VacA with T lymphocytes yields multiple effects. VacA specifically blocks the antigen-dependent proliferation of Jurkat T cells by interfering with IL-2-mediated signalling (Boncristiano *et al.*, 2003; Gebert *et al.*, 2003). As discussed above, *H. pylori* targets CD18 (β_2 -integrin) as a VacA receptor on human T lymphocytes. After entry, VacA inhibits Ca^{2+} mobilization and, subsequently, down-regulation of the activity of the Ca^{2+} -dependent phosphatase calcineurin. This in turn inhibits activation of the transcription factor NFAT (nuclear factor of activated T cells). Thus, NFAT target genes such as those encoding IL-2 and the high-affinity IL-2 receptor (IL-2R α) are not expressed (Fig. 11.4). VacA, however, exerts a different effect on primary human CD4 $^{+}$ T cells, whose proliferation is inhibited through the T-cell receptor CD28, and eventually by other VacA activities (Sundrud *et al.*, 2004). In the latter study, VacA was shown to suppress IL-2-induced cell-cycle progression and proliferation of primary T cells without affecting NFAT. Thus, VacA may inhibit the clonal expansion of T cells that have been activated by bacterial antigens, thereby allowing *H. pylori* to evade the adaptive immune response, resulting in chronic infection.

11.2.9 Other VacA-induced effects on cellular signal transduction pathways

Besides the VacA-induced activities described above, several other features have been reported. Yeast two-hybrid studies and other experiments have demonstrated VacA's potential for specific binding to RACK-1 (receptor for activated C-kinase 1) (Hennig *et al.*, 2001) or to the intermediate filament-binding protein VIP54 (de Bernard *et al.*, 2000), but its importance for infection remains unknown. Moreover, purified VacA or VacA-containing culture supernatants exhibit suppressive effects on proliferation, migration or wound healing of gastric epithelial cells *in vitro* (Ricci

et al., 1996; Pai *et al.*, 1998, 2000; Tabel *et al.*, 2003). In these studies it was shown that VacA can block the activity of externally added EGF, suggesting that VacA interferes with EGF receptor (EGFR) signalling. Indeed, VacA preparations or infection with VacA-expressing *H. pylori* exhibit(s) suppressive effects on phosphorylation of EGFR (Pai *et al.*, 1998; Tegtmeyer *et al.*, 2009) and HER2/Neu, another member of the EGFR family (Tegtmeyer *et al.*, 2009). Immunofluorescence data showed internalized EGFR, possibly due to endocytosis, in infections with VacA-expressing *H. pylori*, providing a possible mechanism for how significant portions of EGFR are inactivated by VacA (Tegtmeyer *et al.*, 2009). Interestingly, RPTP α but not vacuolation per se plays a role in this process. Further investigation is thus necessary to reveal which mechanism is involved in VacA-dependent inactivation of EGFR and HER2/Neu.

The effect of VacA on endosomal and lysosomal functions has also been studied by following procathepsin D maturation and EGF degradation in HeLa cells exposed to the toxin. VacA inhibited the conversion of procathepsin D (53 kDa) into both the intermediate (47 kDa) and the mature (31 kDa) form, and intracellular degradation of EGF was also suppressed (Satin *et al.*, 1997). This suggests that VacA can also impair the degradative power of late endosomes and lysosomes. Further studies indicated that addition of VacA to AZ-521 cells activates two classes of MAP kinases (p38 and ERK1/2) and the activating transcription factor 2 (ATF-2) signalling pathway (Nakayama *et al.*, 2004). VacA has also been reported to induce activation of p38 in several other types of cells (Boncristiano *et al.*, 2003). Pharmacological inhibition of p38 kinase activity, however, did not block the vacuolation phenotype nor mitochondrial damage, which indicates that VacA-induced activation of the p38/ATF-2 signalling cascade is independent of endosomal and mitochondrial pathways of VacA (Nakayama *et al.*, 2004). In another cell culture model system, RBL-2H3 mast cells, binding of VacA results in a rapid change in cytosolic calcium concentrations (de Bernard *et al.*, 2005). This is accompanied by stimulation of proinflammatory cytokines, including TNF α

and IL-6, resulting in chemotaxis and degranulation of these mast cells (Supajatura *et al.*, 2002; de Bernard *et al.*, 2005). Finally, VacA treatment stimulates expression of cyclooxygenase-2 (COX-2), a proinflammatory enzyme in neutrophils and macrophages (Boncristiano *et al.*, 2003). Thus, VacA is a remarkable bacterial virulence factor with multiple activities on epithelial and immune cells.

11.3 The Cytotoxin-associated Genes Pathogenicity Island (*cagPAI*)

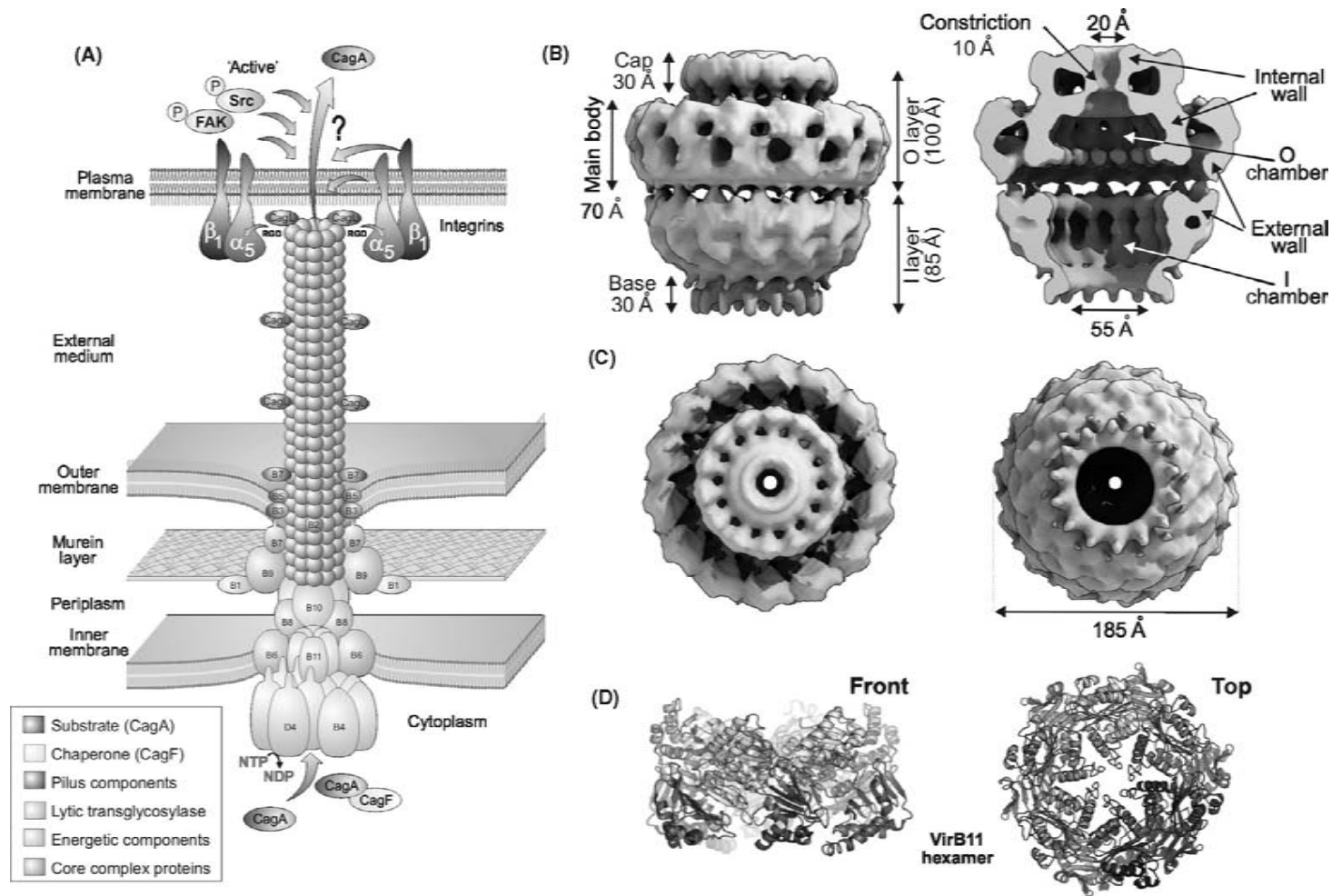
11.3.1 Discovery of *CagA* and *cagPAI* genes

CagA was discovered in the early 1990s by pioneering work in the labs of Martin Blaser (Cover *et al.*, 1990), Jean Crabtree (Crabtree *et al.*, 1991) and Antonello Covacci (Covacci *et al.*, 1993). Initially, a strong association between serological responses to *CagA* and peptic ulcer disease was reported (Cover *et al.*, 1990), which led to cloning of the *cagA* gene (Covacci *et al.*, 1993; Tummuru *et al.*, 1993) as well as identification of adjacent *cagPAI* genes and their role in inflammation (Tummuru *et al.*, 1995). One of these genes was *picB* (*cagE*) with homology to VirB4 family proteins of a putative type IV secretion system (T4SS) (Tummuru *et al.*, 1995). A few years later the entire *cagPAI* was sequenced from different *H. pylori* isolates and found to represent a 40 kb DNA insertion element, which carries up to 32 genes flanked by 31 bp direct repeats (Censini *et al.*, 1996; Akopyants *et al.*, 1998). The *cagPAI* was acquired by a horizontal DNA transfer event from an as yet unknown ancestor and integrated into the chromosomal glutamate racemase gene. *CagA* is recognized as a marker for the *cagPAI* region, which is found in more virulent strains but is typically missing in less virulent *H. pylori* isolates (see Correa and Piazuelo, Chapter 3, this volume). Although the function of *CagA* and the *cagPAI* remained unknown for several years, a major breakthrough in the study of *CagA* came when five groups independently reported that the *cagPAI* encoded a T4SS which injects *CagA* into target cells (Covacci and Rappuoli, 2000).

11.3.2 Assembly and structure of the type IV secretion system

T4SSs, a large group of transmembrane transporters found in many Gram-negative bacteria, are ancestrally related to conjugation systems (Covacci *et al.*, 1999; Cascales and Christie, 2003; Backert and Meyer, 2006). These T4SSs are functionally diverse both in terms of the transported substrate (proteins or DNA-protein complexes) and the recipients, which can be either bacteria of the same or different species, or organisms from a different kingdom (e.g. fungi, plants or mammalian cells). In addition to *H. pylori*, T4SSs have been found in *Agrobacterium*, *Legionella*, *Bordetella* and other pathogens. Figure 11.5A shows a hypothetical model of the assembled T4SS in *H. pylori* based on the prototype from *Agrobacterium tumefaciens*, which delivers oncogenic nucleoprotein particles (T-DNA) into plant cells, resulting in the growth of crown gall tumours (Cascales and Christie, 2003). These transporters typically consist of 11 VirB proteins (encoded by *virB1* to *virB11*) and the so-called coupling protein (VirD4, an NTPase). The agrobacterial VirB proteins can be grouped into three classes: (i) the putative channel or core components (VirB6 to VirB10); (ii) the energetic components (the NTPases VirB4 and VirB11); and (iii) the pilus-associated components (VirB2, and possibly VirB3 and VirB5) (Fig. 11.5A). VirB1 is a transglycosylase for localized lysis of the murein layer at the site of T4SS assembly (Backert and Selbach, 2008).

The *H. pylori* T4SS is a needle-like pilus protruding from the bacterial surface and is induced upon contact with host cells (Rohde *et al.*, 2003; Tanaka *et al.*, 2003; Kwok *et al.*, 2007). These T4SS pili are mainly located at one cell pole and consist of the VirB2 pilin orthologue *CagC* as the major subunit (Andrzejewska *et al.*, 2006). Immunogold-labelling studies indicate that the pilus is covered locally or completely by VirB10 (*CagY*) (Rohde *et al.*, 2003) and *CagL* (Kwok *et al.*, 2007). VirB10 is a very large protein (about 250 kDa) that contains two transmembrane domains. Remarkably, VirB10 proteins can differ in size, due to in-frame deletions or duplications, resulting in reduced host



antibody recognition that may allow immune evasion (Aras *et al.*, 2003a). The T4SS needle base is covered with VirB7 (CagT) and VirB9 (CagW) proteins (Rohde *et al.*, 2003; Tanaka *et al.*, 2003). Immunogold-staining indicates the presence of CagA at the tip of the needle, providing the first direct evidence that CagA can be delivered through these surface appendages, a finding not yet shown for any other T4SS (Kwok *et al.*, 2007). Delivery of CagA is proposed to proceed in an energy-dependent manner driven by the NTPases VirD4, VirB4 and VirB11 (Fig. 11.5).

11.3.3 Function of the type IV secretion system: the receptor hypothesis

For several years it was believed that CagA was randomly injected into epithelial cells. This is obviously not the case, as integrin receptors are required for injection of CagA (Kwok *et al.*, 2007). An important role is played by CagL, a T4SS-pilus-covering protein and VirB5 orthologue (Backert *et al.*, 2008), which acts as a specialized adhesin bridging the T4SS to β_1 -integrin on target cells (Kwok *et al.*, 2007). In addition, it was found that CagL activates the host kinases FAK (focal adhesion kinase) and Src ((sarcoma) intracellular tyrosine kinase) to ensure phosphorylation of CagA directly at

the injection site. This was the first study to directly demonstrate that a bacterial T4SS protein targets a host receptor for its function. Binding of CagL to integrins induces local membrane ruffling, indicative of a general effect on membrane dynamics, and is the first T4SS effect on host cells. Once the T4SS injection needle is constructed, *H. pylori* modulates the actin cytoskeleton, transcriptional responses and cell-cell junctions. Interestingly, CagL contains an arginine-glycine-aspartate (RGD) motif that mediates contact of the T4SS to integrin- $\alpha_5\beta_1$ (Kwok *et al.*, 2007). CagL can also bind in an RGD-independent manner to another integrin member ($\alpha_v\beta_5$) and fibronectin (Urman, 2007). Thus, CagL can trigger signalling by RGD-dependent and RGD-independent pathways. Furthermore, integrins are not found at the apical membrane but at the basal membrane of polarized cells. This suggests a sophisticated control mechanism through which *H. pylori* injects CagA (Kwok *et al.*, 2007). The basal injection model of CagA can also explain why *H. pylori* does not cause more damage and may only inject virulence proteins into target cells under certain circumstances. A likely scenario based on these findings is that *cagPAI*-independent factors such as the known adhesins BabA/B, SabA, HopZ and AlpA/B as well as VacA and OipA loosen local intercellular epithelial junctions before a limited number of bacteria gain

Fig. 11.5. Model for the assembled type IV secretion system (T4SS), cryo-EM (electron microscopy) structure of a T4SS core and crystal structure of the *Helicobacter pylori* VirB11 ATPase complex. (A) Hypothetical model of the T4SS machinery. The T4SS is a multicomponent protein complex spanning the inner and outer membranes of *H. pylori*. Current knowledge of T4SS functions and cellular localization of its components is shown in a simplified manner. The coupling protein VirD4 and structural components (VirB1 to VirB11) are typically required for secretion and are positioned according to their proposed functions. This transporter enables secretion of substrates (CagA, peptidoglycan) from the bacterial cytoplasm directly into the cytoplasm of infected host cells. The CagL protein interacts with integrin receptors to deliver CagA across the host cell membrane and to activate the Src tyrosine kinase for CagA phosphorylation. (B) The cryo-EM structure of the TraN/VirB7, TraO/VirB9 and TraF/VirB10 core complex from the T4SS of plasmid pMK101 (Fronzes *et al.*, 2009). Side view (top left) and cutaway side view (top right) are shown. (C) Top view (view from outside the cell) on the left side and bottom view (view from the cytoplasm) on the right side. (D) Ribbon diagrams of the *H. pylori* VirB11 (HP0525), a hexameric traffic ATPase, from two different views (Yeo *et al.*, 2000). Abbreviations: CagA/F/L, proteins encoded by the *cag* (cytotoxin-associated genes) pathogenicity island; FAK, focal adhesion kinase; RGD, arginine-glycine-aspartate motif; Src (short for sarcoma), intracellular tyrosine kinase. (Part A was adapted from Backert and Selbach (2008) with kind permission from Blackwell Publishing. Parts B to D were kindly provided by Remi Fronzes and Gabriel Waksman with kind permission of *Science* and Elsevier.)

access to integrins and inject CagA (Wessler and Backert, 2008). In line with this hypothesis, inactivation of the *oipA* gene also has an effect on FAK activity (Tabassam *et al.*, 2008). The importance of β_1 -integrin for *H. pylori*-induced host cell motility and elongation (Kwok *et al.*, 2007) and bacterial adherence and invasion (Su *et al.*, 1999) was also reported, and this signalling is associated with the phosphorylation of paxillin and the MAP kinase JNK (Jun N-terminal kinase) (Snider *et al.*, 2008).

11.3.4 Injection of CagA requires accessory proteins

As mentioned above, the *cagPAI* contains up to 32 genes encoding 11 VirB proteins and VirD4 as well as several auxiliary factors. Mutagenesis of all other *cag* genes revealed that they are fully, partially or not required for injection of CagA (Covacci and Rappuoli, 2000; Fischer *et al.*, 2001). While the roles of most of the T4SS-specific accessory factors are unknown, the function of CagF and CagD was recently elucidated. CagF is a chaperone-like protein that binds close to the carboxy-terminal secretion signal of the CagA effector protein and is crucial for its translocation through the T4SS (Couturier *et al.*, 2006; Pattis *et al.*, 2007). CagD serves as a multifunctional component of the T4SS that may be involved in CagA secretion at the inner membrane and may localize outside the bacteria to promote additional effects on the host cell (Cendron *et al.*, 2009).

11.3.5 Crystal structures of several *cagPAI* proteins

A major contribution to our current understanding of T4SS transporters in bacteria came from resolution of a 15 Å crystal structure of a T4SS core from IncN plasmid pKM101 (Fronzes *et al.*, 2009). This core complex is composed of three proteins (VirB7, VirB9 and VirB10), each present in 14 copies and forming a 1.1 MDa, two-chambered, double membrane-spanning channel (Fig.

11.5B and C). The structure is double-walled, with each component apparently forming a major part of the channel. The complex is open on the cytoplasmic side and constricted on the extracellular side. In addition to this major finding, the crystal structures of four *H. pylori* *cagPAI* proteins have been solved. The structure of VirB11 reveals a hexameric ring complexed with the regulator protein HP1451 and functions as a gating molecule at the inner membrane (Hare *et al.*, 2007), which is proposed to cycle through closed and open forms as regulated by ATP binding/hydrolysis (Fig. 11.5D). The crystal structures of CagZ, a 23 kDa protein involved in the translocation of CagA, and CagS, a 23 kDa protein coded by a well-conserved *cagPAI* gene whose function remains elusive, have also been reported (Cendron *et al.*, 2004, 2007). In addition, the structural characterization of CagD revealed that this protein is a covalent dimer in which each monomer folds as a single domain that is composed of five β -strands and three α -helices (Cendron *et al.*, 2009). Finally, the structure of CagL was modelled based on the crystal structure available from another VirB5 orthologue, TraC from pKM101 (Backert *et al.*, 2008). CagL forms a three α -helical bundle plus an exposed domain carrying the RGD motif available for binding of integrins. This is concordant with the published circular dichroism spectrum of CagL, which revealed 65% helical sequences (Kwok *et al.*, 2007).

11.3.6 Phosphorylation of CagA by Src and Abl oncoproteins

As CagA proteins vary in size from about 120 to 145 kDa and exhibit no sequence homology to any pro- or eukaryotic proteins, the evolutionary origin of CagA is unknown. However, one important clue that identified CagA as an injected effector protein was the observation that it undergoes tyrosine phosphorylation (CagA^{PY}) by host cell kinases (Covacci and Rappuoli, 2000). The phosphorylation sites are Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs in the carboxy-terminal half of CagA (Backert *et al.*, 2001b; Stein *et al.*, 2002). Four distinct EPIYA sites have been described, EPIYA-A, -B,

-C and -D, each of which is conserved in sequence. Interestingly, while EPIYA-A and EPIYA-B are present in strains throughout the world, EPIYA-C is predominantly found in strains from Western countries (e.g. Europe, North America and Australia), while EPIYA-D is predominantly found in strains from Eastern countries (e.g. Japan, Korea and China). These EPIYA repeats are flanked by repetitive DNA sequences involved in recombination that could explain the variability in the number of these motifs in CagA variants (Aras *et al.*, 2003b; Kim *et al.*, 2006), as well as strain-specific differences in pathogenicity (Hatakeyama, 2008). Phosphorylation of CagA appears to be highly dynamic in infected cells (Tammer *et al.*, 2007) and 2-DE analyses of CagA protein species from different strains showed that only two EPIYA motifs can be phosphorylated at a given time, both separately and simultaneously (Backert *et al.*, 2001a). The EPIYA region also contains a multimerization motif (Ren *et al.*, 2006) and represents a membrane-targeting signal (Higashi *et al.*, 2005), although in another study CagA-EPIYA deletion mutants still localized to the membrane (Mimuro *et al.*, 2002). Perhaps significantly, the kinases responsible for phosphorylation of CagA are known oncoproteins. Src family kinases (SFKs) that control actin cytoskeletal processes, proliferation and differentiation of normal cells are also key players in carcinogenesis, and have been found to phosphorylate CagA (Selbach *et al.*, 2002; Stein *et al.*, 2002). Following this discovery, Abl kinase members c-Abl and Arg were identified to directly phosphorylate CagA (Poppe *et al.*, 2007; Tammer *et al.*, 2007). Interestingly, *H. pylori* controls the activity of SFKs and Abl in a very specific and time-dependent manner. While Src is activated only during the initial stages of infection (0.5–2 h) and then is rapidly inactivated, Abl is continuously activated by *H. pylori* with strongly enhanced activities at later time points (2–8 h), supporting a model for the successive phosphorylation of CagA by Src and Abl (Tammer *et al.*, 2007). Upon injection or transfection, CagA can then interact with multiple host cell factors in both a phosphorylation-dependent and -independent manner (Table 11.2).

11.3.7 Intracellular functions of CagA: phosphorylation-dependent signalling

An important hallmark of eukaryotic phosphotyrosine signalling is the recruitment of proteins with specific phosphotyrosine-binding modules such as Src homology 2 (SH2) domains. To date, nine SH2-domain-containing host cell proteins are known to bind CagA in a phosphorylation-dependent manner: the tyrosine phosphatases Shp1 and Shp2, the tyrosine kinase Csk, phosphoinositide-3 kinase (PI3K), Ras GTPase activating protein (Ras-GAP), and the adaptor proteins Crk, Grb2 and Grb7 (Higashi *et al.*, 2002; Tsutsumi *et al.*, 2003; Suzuki *et al.*, 2005; Selbach *et al.*, 2009). All nine interaction partners play complex roles in *H. pylori*-induced actin cytoskeletal rearrangements, scattering and elongation of infected host cells in culture, a scenario involving multiple signalling events as summarized in Fig. 11.6.

Gastric epithelial cells cultured *in vitro* with *H. pylori* elongate and scatter, a morphology referred to as the 'hummingbird' phenotype (Segal *et al.*, 1999). This phenotype results from two successive events: (i) the induction of cell motility leading to scattering; and (ii) cell elongation (Moese *et al.*, 2004). CagA^{PY} can induce cellular elongation by triggering a cell retraction defect in focal adhesions, but this is still not completely elucidated (Bourzac *et al.*, 2007). Transfection studies showed that CagA^{PY}-Shp2 interaction contributes to cell elongation by direct dephosphorylation and inactivation of FAK (Tsutsumi *et al.*, 2006) and by stimulating the Rap1→B-Raf→ERK signalling pathway (Higashi *et al.*, 2004), respectively. CagA^{PY}-induced cell elongation also involves tyrosine dephosphorylation of the actin-binding proteins cortactin, ezrin and vinculin (Selbach *et al.*, 2003, 2004; Selbach and Backert, 2005; Moese *et al.*, 2007). The phosphatases involved in this scenario remain unknown. Theoretically, interaction of CagA^{PY} with the tyrosine phosphatase Shp2 could explain the latter events, but cortactin dephosphorylation does not require Shp2 (Selbach *et al.*, 2003). Instead, CagA^{PY} can inhibit Src both directly and via recruitment of Csk, a negative regulator of Src (Selbach *et al.*, 2003; Tsutsumi *et al.*, 2003). As Src is the

Table 11.2. Proteins that interact with cytotoxin-associated gene A protein (CagA) and proposed roles in *Helicobacter pylori* infections.

Interaction partner ^a	Proposed function ^b	Requirement of CagA ^{PY}	Experimental evidence	Applied methods ^c	Reference(s)
Abl	Phosphorylation of CagA and Crkl adapter proteins	n.d. ^g	Infection, transfection of CagA	IP, IF	Brandt <i>et al.</i> (2007); Poppe <i>et al.</i> (2007); Tammer <i>et al.</i> (2007)
c-Met ^d	Cell scattering, activation of PI3K, ERK, β -catenin and NF- κ B	No	Infection Transfection of CagA	IP, IF	Churin <i>et al.</i> (2003) Suzuki <i>et al.</i> (2009)
Crkl, CrklI, CrkL	Cell scattering, loss of AJs, MAPK signalling	Yes	Infection, transfection of CagA	IP	Suzuki <i>et al.</i> (2005)
Csk	Src inactivation, loss of CagA–Shp2 interaction	Yes	Transfection of CagA	IP	Tsutsumi <i>et al.</i> (2003)
E-cadherin	Dephosphorylation of cortactin Destabilization of AJs, β -catenin signalling	No	Proteomics Transfection of CagA	SILAC/MS IP	Selbach <i>et al.</i> (2009) Murata-Kamiya <i>et al.</i> (2007)
Grb2 ^e	Cell scattering, activation of Ras–MAPK signalling	No	Transfection of CagA	PD, IP, IF	Mimuro <i>et al.</i> (2002)
Grb7	Unknown	Yes	Proteomics	SILAC/MS	Selbach <i>et al.</i> (2009)
PAR1	Inhibition of PAR1 activity, disruption of apical junctions	No	Infection Transfection of CagA	IP IP, IF	Saadat <i>et al.</i> (2007); Zeaiter <i>et al.</i> (2008)
PI3K	Unknown	Yes	Proteomics Infection	SILAC/MS IP	Selbach <i>et al.</i> (2009)
PLC- γ^f	Cell scattering	No	Infection	IP	Churin <i>et al.</i> (2003)
Ras-GAP	Unknown	Yes	Proteomics	SILAC/MS	Selbach <i>et al.</i> (2009)
Shp1	Unknown	Yes	Proteomics Infection	SILAC/MS IP	Selbach <i>et al.</i> (2009)

Shp2	Cell scattering, activation of ERK	Yes	Transfection of CagA	IP	Higashi <i>et al.</i> (2002, 2004)
	Tyrosine dephosphorylation of FAK	Yes	Transfection of CagA	IP	Tsutsumi <i>et al.</i> (2006)
Src ZO-1, JAM	Phosphorylation of CagA	n.d.	Infection	IP	Selbach <i>et al.</i> (2003)
	Disruption of lateral junctions and cell motility	No	Infection, transfection of CagA	IF	Amieva <i>et al.</i> (2003); Bagnoli <i>et al.</i> (2005)

^aAbbreviations: Abl, Abelson non-receptor tyrosine kinase; c-Met, hepatocyte growth factor receptor; Crk, SH2/SH3-domain-containing adapter protein; Csk, carboxy-terminal Src kinase; Grb, growth factor receptor-bound protein; JAM, junctional adhesion molecule; PAR1, partitioning-defective 1 kinase; PI3K, phosphoinositide-3 kinase; PLC- γ , phospholipase c gamma; Ras, v-Ha-ras Harvey rat sarcoma viral oncogene homologue; Ras-GAP, Ras GTPase activating protein; Shp1/2, SH2-domain-containing protein-tyrosine phosphatase-1/2; Src (oncogene of the Rous sarcoma virus), intracellular tyrosine kinase; ZO-1, zonula occludens 1.

^bAbbreviations: AJ, adherens junction; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; NF- κ B, nuclear factor- κ B; Ras-MAPK, Ras mitogen-activated protein kinase.

^cAbbreviations: IF, co-localization by immunofluorescence; IP, co-immunoprecipitation; PD, pull-down experiments; SILAC/MS, stable isotope labelling with amino acids in cell culture combined with mass spectrometry of bound proteins.

^dActivation of CagA-induced NF- κ B activation and interleukin-8 secretion was not observed when CagA was co-transfected with dominant-negative c-Met in AGS cells (Brandt *et al.*, 2005).

^eAn interaction of non-phosphorylated CagA or CagA^{PY} with Grb2 was reported (Mimuro *et al.* 2002; Selbach *et al.*, 2009) but none of these interactions was found by Tsutsumi *et al.* (2003) and Churin *et al.* (2003) using transfected CagA.

^fPLC- γ IP revealed a strong background signal for non-injected CagA in a translocation-defective *virb11* mutant.

^gn.d., not determined.

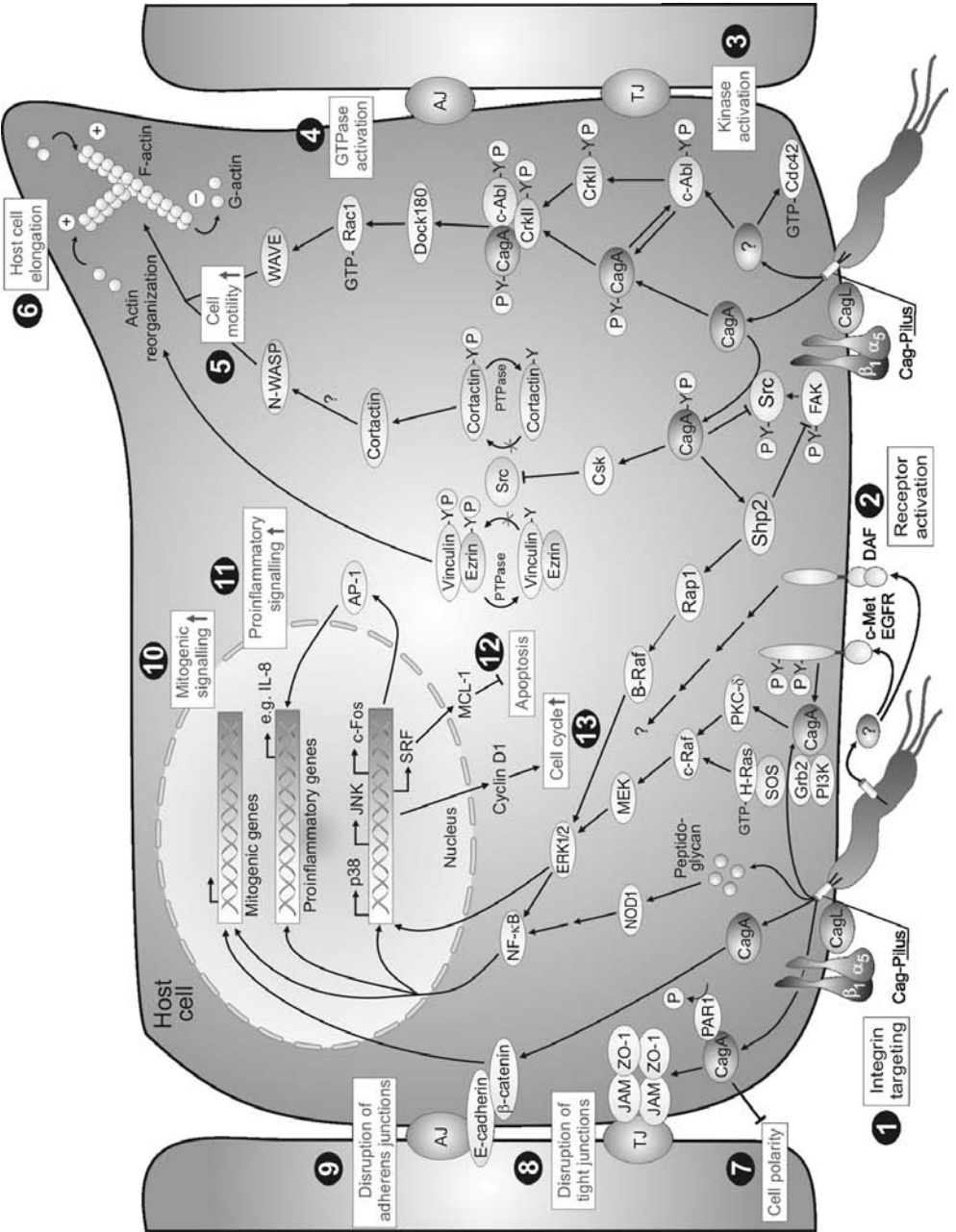


Fig. 11.6. A model for the role of *Helicobacter pylori* type IV secretion system (T4SS) effectors CagL, peptidoglycan and CagA in host cell interaction and signal transduction. T4SS effectors alter different cellular processes in gastric epithelial cells. During infection, *H. pylori* injects effector molecules into gastric epithelial cells associated with integrin $\alpha_5\beta_1$ receptor (1), induces activation of receptor tyrosine kinases EGFR, HER2/Neu, c-Met and receptor DAF (2) and activation of intracellular tyrosine kinases Src and Abl which phosphorylate injected CagA (3). Phosphorylated CagA can influence GTPase activation (4), cell motility (5) and host cell elongation *in vitro* (6). Injected or transfected CagA modulates various

primary kinase activated by CagA, inhibition of Src by CagA^{PY} generates a negative feedback loop that carefully controls the amount of intracellular CagA^{PY}. Similarly, Src inhibition could occur via Shp2 and FAK (Fig. 11.6). Cortactin, ezrin and vinculin are all Src substrates, and Src inactivation causes dephosphorylation of these proteins that is essential for the elongation phenotype (Fig. 11.6). Interaction of CagA^{PY} with CrkII can activate Rac1 via Dock180 (Suzuki *et al.*, 2005). Activation of Abl by CagA^{PY} amplifies this effect as it enhances phosphorylation of the Abl substrate CrkII (Brandt *et al.*, 2007; Poppe *et al.*, 2007; Tammer *et al.*, 2007). Finally, a recent systematic proteomics-based approach verified most but not all of the latter CagA^{PY}-interacting partners and even identified additional binding partners including Grb2, Grb7, Shp1, Ras-GAP and PI3K, but their specific role in signalling needs to be clarified in future studies (Selbach *et al.*, 2009). Taken together, CagA^{PY} interferes with a surprisingly high number of cellular factors and signalling cascades to induce cell scattering, elongation and likely other responses.

11.3.8 Intracellular functions of CagA: phosphorylation-independent signalling

Intracellular CagA functions do not always depend on its tyrosine phosphorylation. The cell adhesion protein E-cadherin, the hepatocyte growth factor receptor c-Met, the phospholipase PLC- γ , the adaptor protein Grb2 and the kinase PAR1 have all been reported to interact with CagA in its non-phosphorylated form (Mimuro *et al.*, 2002; Churin *et al.*, 2003; Murata-Kamiya *et al.*, 2007; Saadat *et al.*, 2007; Zeaiter *et al.*, 2008), and phosphorylation-independent CagA interactions induce pro-inflammatory and mitogenic responses as well as the disruption of cell-cell junctions and loss of cell polarity (Fig. 11.6). Tight and adherens junctions are essential for the integrity of the gastric epithelium. CagA interferes with these intercellular junctions via several pathways. Injected CagA associates with the epithelial tight-junction scaffolding protein ZO-1 (zonula occludens 1) and the transmembrane protein JAM (junctional adhesion molecule), causing an ectopic assembly of tight-junction components at sites of bacterial

signalling cascades involved in altering cell polarity (7), disruption of cell-cell junctions (8, 9), cell proliferation (10), proinflammatory responses (11), suppression of apoptosis (12) and cell cycle (13). The receptor tyrosine kinases (EGFR, HER2/Neu and c-Met), ERK1/2 and small Rho GTPases Rac1 and Cdc42 can also be activated by an unknown T4SS factor. In addition, translocated peptidoglycan appears to activate the intracellular receptor NOD1 which activates NF- κ B in a T4SS-dependent manner. Abbreviations: Abl, Abelson non-receptor tyrosine kinase; AJ, adherens junction; AP-1, activator protein 1 (a transcription factor); CagA/L, proteins encoded by the *cag* (cytotoxin-associated genes) pathogenicity island; c-Fos, transcription factor; c-Met, hepatocyte growth factor receptor; Crk, SH2/SH3-domain-containing adapter protein; Csk, carboxy-terminal Src kinase; cyclin D1, cell-cycle regulator; DAF, decay-accelerating factor; Dock180, dedicator of cytokinesis (a 180 kDa guanine exchange factor for GTPase Rac1); EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinases 1 and 2; FAK, focal adhesion kinase; Grb2, growth factor receptor-bound protein 2; F-actin, filamentous actin; G-actin, globular actin; H-Ras, member of the Ras superfamily of GTPases; IL-8, interleukin-8; JAM, junctional adhesion molecule; JNK, Jun N-terminal kinase; MCL-1, myeloid cell leukaemia 1 protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; N-WASP, neural Wiskott-Aldrich syndrome protein; NF- κ B, nuclear factor- κ B; NOD1, nucleotide oligomerization domain protein 1; P, phosphate group; p38 MAP kinase, 38 kDa mitogen-activated protein kinase; PAR1, partitioning-defective 1 kinase; PKC- δ , protein kinase C- δ ; PI3K, phosphoinositide-3 kinase; PTPase, phosphotyrosine phosphatase; Rac1, Ras substrate of C3 toxin; Raf, serine/threonine kinase; Rap, serine/threonine kinase; Shp2, SH2-domain-containing protein-tyrosine phosphatase 2; SOS, son of sevenless (a guanine exchange factor for Ras); Src kinase, oncogene of the Rous sarcoma virus; SRF, serum response factor; TJ, tight junction; WAVE, WASP family Verprolin-homologous protein; ZO-1, zonula occludens 1 (a tight junction protein). For more details see text. (Adapted from Backert and Selbach (2008) with kind permission from Blackwell Publishing.)

attachment (Amieva *et al.*, 2003; Bagnoli *et al.*, 2005). Very recently PAR1, a central regulator of cell polarity, was found to play a role in this process. CagA directly binds PAR1 and inhibits its kinase activity to promote loss of cell polarity (Saadat *et al.*, 2007; Zeaiter *et al.*, 2008). CagA was also reported to interact with the transmembrane cell–cell junction protein E-cadherin (Murata-Kamiya *et al.*, 2007). However, many of the more recent findings are based on vector-based expression of CagA constructs. Since integrins as T4SS receptors are protected by the junctions, the crucial question arises about the *in vivo* relevance of these processes for *H. pylori* pathogenesis. Thus, more detailed studies are necessary to clarify the intracellular function of CagA in infection models.

H. pylori infection also affects host cell gene transcription, and recent data have established a direct role of CagA in signalling to the nucleus (Fig. 11.6). The first direct evidence came from microarray studies investigating host cell gene expression after infection of target cells with wild-type *H. pylori* and *cagA* mutants (Guillemin *et al.*, 2002; El-Etr *et al.*, 2004). Subsequently, CagA was found to potentiate the activation of proinflammatory transcription factor NF- κ B via the Ras→Raf→MEK→ERK pathway (Brandt *et al.*, 2005). This process appears to involve binding of CagA to Grb2, an upstream effector of the small GTPase H-Ras (Mimuro *et al.*, 2002; Selbach *et al.*, 2009) as well as PKC- δ (Brandt *et al.*, 2009). In line with these findings, CagA was shown to function as a prokaryotic mimic of the eukaryotic Grb2-associated binder (Gab) adaptor protein in transgenic *Drosophila* (Botham *et al.*, 2008). Besides NF- κ B, another transcription factor aberrantly activated by CagA is β -catenin, the nuclear accumulation of which leads to transcriptional up-regulation of mitogenic genes implicated in carcinogenesis (Franco *et al.*, 2005). This can be attributed, at least in part, to the disruption of the adherens junction complex that releases β -catenin and increases nuclear and cytoplasmic β -catenin pools (Murata-Kamiya *et al.*, 2007). In addition, non-phosphorylated CagA was shown to interact with activated c-Met leading to sustained activation of PI3K/Akt signalling in

response to *H. pylori* infection (Suzuki *et al.*, 2009). This in turn led to the activation of β -catenin and NF- κ B signalling, which respectively promote proliferation and inflammation. Interestingly, the phosphorylated EPIYA-B of CagA can interact with the SH2-domain-containing regulatory subunit of PI3K (Selbach *et al.*, 2009). Further complexity arises from the observation that VacA can also activate the PI3K/Akt signalling pathway (Nakayama *et al.*, 2009). These findings further highlight the enormous complexity in *H. pylori*–host cell cross-talk.

CagA was recently reported to activate matrix metalloproteinase-1 (MMP-1) secretion (Pillinger *et al.*, 2007) and serum response element/serum response factor-driven gene transcription, which up-regulates expression of the anti-apoptotic myeloid cell leukaemia sequence-1 (MCL-1) protein (Mimuro *et al.*, 2007). Similar to proinflammatory signalling, MCL-1 up-regulation involves the above-mentioned ERK pathway via CagA binding to Grb2 (Fig. 11.6). In line with these observations, *cagA*-positive *H. pylori* strains have been reported to affect the cell cycle during infection by stimulating cyclin D1 expression, G₁–S phase progression and host cell survival (Chang *et al.*, 2006).

11.3.9 Type IV secretion system-dependent but CagA-independent cellular signalling

Until recently, CagA was the only known effector protein to be translocated through the T4SS. Early reports indicated that VirB structural components of the T4SS but not CagA itself were required for the induction of proinflammatory signalling (Tummuru *et al.*, 1995; Censini *et al.*, 1996) including activation of NF- κ B (Sharma *et al.*, 1998), AP-1 and the proto-oncogenes c-Fos and c-Jun (Meyer-ter-Vehn *et al.*, 2000). This suggested that the T4SS might inject factors in addition to CagA. The T4SS was also found to activate the Rho-GTPases Rac1 and Cdc42 (Churin *et al.*, 2001), and to stimulate EGFR, ERK1/2 and expression of the early growth response gene *Egr1* (Keates *et al.*, 2005) independently of CagA. Despite intensive efforts including a

systematic mutagenesis of all *cagPAI* genes, the hypothetical additional effector remained unknown (Fischer *et al.*, 2001). A possible candidate, however, was identified when Viala and co-workers (2004) reported that proinflammatory signalling of *H. pylori* involved NOD1, an intracellular pathogen-recognition molecule with specificity for Gram-negative peptidoglycan. These observations suggest that T4SS-dependent delivery of peptidoglycan is responsible for activation of NOD1→NF-κB-dependent proinflammatory responses such as secretion of IL-8 (Viala *et al.*, 2004) or β-defensin-2 (Boughan *et al.*, 2006).

Finally, there are other cellular phenotypes that depend on a functional *cagT4SS* but which are independent of CagA. These include anti-phagocytic effects on professional phagocytes (Ramarao *et al.*, 2000b) and the homotypic aggregation phenotype of macrophage-like cells (Moese *et al.*, 2002). In addition, megasome formation has been observed with *cagPAI*-positive but not *cagPAI*-negative *H. pylori* strains (Allen *et al.*, 2000). *H. pylori* has been also described to bind decay-accelerating factor (DAF) as a mediator of gastric inflammation, but the responsible bacterial factor remains elusive (O'Brien *et al.*, 2006). Whether peptidoglycan is involved in targeting DAF, activation of EGFR, Rho-GTPases and Egr-1 is unclear and remains a pressing question. Alternatively, CagL-induced integrin signalling might also play a role in some of the latter activities.

appeared in PubMed since its discovery. However, *H. pylori* infection occurs in approximately half of the world population, with disease being an exception rather than the rule. Understanding how this organism interacts with its host is essential for formulating an effective strategy to deal with its most important clinical consequences. Study of host-bacterial interactions and bacterial virulence factors has provided important insights for clinical practice. Research on VacA, CagA and the T4SS has provided us with many fundamental insights into *H. pylori* biology and function, but many open questions remain. It is now clear that each of these factors plays a central role in pathogenesis and several of the involved mechanisms have already been elucidated. However, much remains to be elucidated regarding how and when VacA and CagA are transported into the host. Both proteins have emerged as extremely versatile effector proteins that interfere with multiple host cell functions. It will be of interest to determine if the T4SS translocates other substrates in addition to CagA. Transfer of peptidoglycan is an intriguing possibility but, to date, there are no confirmatory studies. Finally, the evolutionary advantage of the T4SS for *H. pylori* needs to be investigated. Potentially, reduction of gastric epithelial cell turnover could be an adaptive advantage for *H. pylori*, but this should be studied in more detail. It appears that *H. pylori*'s virulence factors will continue to be a fascinating and rewarding research subject in the future.

11.4 Summary and Outlook

H. pylori is the most common chronic bacterial infection in the world and, although asymptomatic in the majority of infected subjects, it is also the cause of significant human disease. In the nearly 30 years since *H. pylori* was first discovered, a complete paradigm shift has occurred in our clinical approaches to a number of gastric diseases. From a medical point of view, *H. pylori* is a remarkable pathogen responsible for significant morbidity and mortality worldwide. The interest in this field is underlined by more than 27,000 scientific publications that have

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12 *Helicobacter pylori* Adhesion to the Gastric Surface

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12.1 Introduction

To colonize mucosal surfaces and invade the host, microbes commonly exploit host cell structures. Bacterial adhesion to host cells is thought to be mediated by hydrophobic interactions, cation bridging (i.e. divalent cations counteracting the repulsion of the negatively charged surfaces of bacteria and host) and receptor–ligand binding. One of the most extensively studied mechanisms of adhesion is the binding of lectins to corresponding glycosylated receptors. Bacteria may have multiple adhesins with different carbohydrate specificities, and modulation of surface receptor density, kinetic parameters or topographical distributions of these receptors on cell membranes regulates adhesion. While individual adhesin–receptor binding is usually of low affinity, clustering of adhesins and receptors can cause multivalency effects resulting in strong attachment. Fimbriae (or pili), outer membrane proteins and cell wall components, for example lipopolysaccharides (LPSs), may all function as adhesins (Salyers and Whitt, 1994). Adhesion can affect bacteria by stimulation/inhibition of growth, as well as induction of other adhesive structures and proteins required for invasion, whereas the effects of adhesion on host cells can be altered morphology, fluid loss, induction of cytokine

release, up-regulation of adhesion molecules and apoptosis. The first part of this chapter discusses the adhesins and other structures that *Helicobacter pylori* uses to adhere, as well as how these adhesins are regulated. The second part describes the host structures to which *H. pylori* binds.

12.2 *H. pylori* Adhesins and Mode of Adhesion

The genome of *H. pylori* codes for a large number of outer membrane proteins (Tomb *et al.*, 1997) and at least five different lectin-like adhesins have been implicated in *H. pylori* adherence. Different adhesins may mediate adherence to various niches in the stomach or in other potential reservoirs, such as the oral cavity.

The blood group-binding adhesin (BabA) recognizes fucosylated structures such as Lewis (Le) b (Le^b) and H-type-1 antigen (Borén *et al.*, 1993). The majority of *H. pylori* strains investigated carry the *babA* gene (Ilver *et al.*, 1998; Gerhard *et al.*, 1999) and colonization with *H. pylori* strains expressing the BabA adhesin correlates with development of severe gastric disease (Ilver *et al.*, 1998; Gerhard *et al.*, 1999; Prinz *et al.*, 2001; Yamaoka *et al.*, 2006). Studies investigating the *babA*

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gene as a pathogenic factor have produced contradicting results. A plausible explanation for this is that, while some of these studies are based solely on PCR amplification of the *babA* gene (present in the majority of strains), others have included assays that distinguish BabA-expressing strains from BabA-non-expressing strains, such as reverse-transcription PCR that measures *babA* mRNA expression, immunoblot that detects expressed BabA protein or a measurement of Le^b-receptor binding activity. The potential correlation of BabA as a pathogenic factor has been recently discussed (Fujimoto *et al.*, 2007).

The sialic acid-binding adhesin (SabA) is encoded by the JHP662 gene (Mahdavi *et al.*, 2002). SabA recognizes sialyl-Le^x and sialyl-dimeric-Le^x structures, and 43% of the SabA-positive strains also bind sialyl-Le^a. Of 95 Swedish clinical isolates tested, 39% bound sialyl-Le^x, and the prevalence of this adhesin was higher in cytotoxin-associated gene product A (CagA)-positive than in CagA-negative strains (Mahdavi *et al.*, 2002). Most (80%) SabA-positive strains are also BabA-positive. Strains with a SabA-positive and BabA-negative phenotype adhere to inflamed tissue, but not to healthy gastric mucosa (Mahdavi *et al.*, 2002), which is perhaps explained by the observation that sialyl-Le antigens are preferentially expressed in inflamed gastric mucosa, with only minute levels present in healthy epithelium (Madrid *et al.*, 1990; Mahdavi *et al.*, 2002; Lindén *et al.*, 2008a). Furthermore, SabA expression is associated with gastric cancer, intestinal metaplasia and corpus atrophy (Yamaoka *et al.*, 2006).

The *H. pylori* neutrophil-activating protein (NAP) binds to sulfated carbohydrate structures such as sulfo-Le^a, sulfated glycosphingolipids, the high-molecular-mass salivary mucin and the Le^x, Le^y and H-type-1 structures as well as to sulfo-Le^a (Huesca *et al.*, 1996; Teneberg *et al.*, 1997; Namavar *et al.*, 1998). *H. pylori* NAP induces neutrophil adhesion to endothelial cells, and has been demonstrated to be an immunomodulating agent (Polenghi *et al.*, 2007; Wang *et al.*, 2008).

Adherence-associated lipoprotein A and B (AlpA and AlpB) have a function in adherence as mutations/deletions in *AlpA* or *AlpB*

reduce binding to gastric sections (Odenbreit *et al.*, 1999). Although no receptor for AlpA/B has been identified, it has been demonstrated that AlpA and AlpB aid *H. pylori* in colonizing the gerbil stomach (De Jonge *et al.*, 2004).

Furthermore, *H. pylori* has the ability to bind to additional host structures via mechanisms that do not involve the above adhesins, although the *H. pylori* gene(s) for these have not been identified. One example is the low pH-binding mode, an activity present in all *H. pylori* strains investigated, that confers adherence to charged, preferably sialylated structures at acidic pH (Lindén *et al.*, 2004a, 2008c). *H. pylori* can also bind to heparan sulfate and dextran sulfate (Utt and Wadström, 1997). Additionally, a subset of *H. pylori* strains possess a saliva-specific adhesion mode, allowing attachment to structures present on salivary mucins, but not to any of the mucins produced in either a healthy or a gastritis-affected stomach (Lindén *et al.*, 2008c). This indicates that this binding mode may be especially adapted for the oral cavity, possibly because of the presence of sulfated structures that are present on oral mucins but absent on gastric mucins.

12.2.1 Regulatory mechanisms to alter adherence properties

To achieve successful establishment of persistent bacterial infection, optimal expression of virulence factors in concordance with the continuously changing environment is essential. To balance expression of gene activities, *trans*-acting elements that affect transcription via positive and negative regulatory proteins constitute common mechanisms for many bacterial species. In Gram-negative bacteria, alternative sigma factors play an important role in the regulation of gene expression, but in *H. pylori*, only the housekeeping sigma factors of the RNA polymerase (i.e. $\sigma 80$, $\sigma 54$ and $\sigma 28$) have been found (Tomb *et al.*, 1997). In addition, bioinformatic analysis of the two first *H. pylori* genomes sequenced (26695 and J99) has suggested that only a few regulatory networks operate and that only a limited number of two-component signal transduction systems are present (Alm *et al.*, 1999).

Bacterial fitness is a key factor for establishment of a persistent infection, but this requires the generation of populations with high genetic diversity. A multitude of pathogenic bacteria use phase variation mechanisms to quickly turn genes on and off. Since phase shift variation enables a frequent and reversible on/off shift, and ensures the occurrence of dynamic populations with numerous clones with variable phenotypes, subclones of any given phenotype will always be present and ready to adapt to continuous alterations in the environment during persistent *H. pylori* infection (Kuipers *et al.*, 2000; Blaser and Berg, 2001; Aras *et al.*, 2003; Nilsson *et al.*, 2008). Populations with high diversity are also beneficial for evasion of the host immune response. Phase variation can occur via a number of mechanisms including slipped-strand mispairing (SSM), where mutations in a stretch of repetitive DNA sequences bring the open reading frame in and out of frame, DNA methylation and DNA rearrangements (e.g. by homologous recombination). The remainder of this section describes current knowledge of how expression of *H. pylori* adhesins is regulated via phase variation.

Phase variation of BabA via homologous recombination

Annotation of the two first sequenced *H. pylori* genomes revealed the presence of a large family of genes that encodes for outer membrane proteins, termed HOPs. This family shares extensive homologies in their N- and C-terminal regions (Alm *et al.*, 2000). The HOPs constitute porins, adhesins like BabA and SabA, and proteins associated with adherence such as AlpA, AlpB and OipA (outer inflammatory protein A). The localization of *hop* genes on the chromosome varies between strains. The *babA* gene and the highly related *babB* gene are located in reversed loci in *H. pylori* strains 26695 and J99 (Tomb *et al.*, 1997; Alm *et al.*, 1999; Oh *et al.*, 2006). Thus, in strain J99 the *babA* gene is placed in the same locus as the 26695 *babB* gene and the J99 *babB* gene is placed in the same locus as the 26695 *babA* gene. The third sequenced HPAG1 strain carries *babA* in the same locus as strain 26695 (Oh *et al.*, 2006). A third gene with high homol-

ogy to both *babA* and *babB*, called *babC*, is present in strains 26695 and HPAG1, albeit in different loci (Colbeck *et al.*, 2006). Thus, the genomic locations of the *babA*, *babB* and *babC* genes are different between strains 26695, J99 and HPAG1 (Alm *et al.*, 1999; Oh *et al.*, 2006). Further studies of the localization of the *babA* gene in additional strains have indeed confirmed that the localization of the three *bab* genes varies. The majority (54–57%) of the *babA* genes are placed in the locus corresponding to the 26695 *babA* locus. The second most common *babA* localization corresponds to the 26695 *babB* locus and the third commonest corresponds to the 26695 *babC* locus (Ilver *et al.*, 1998; Colbeck *et al.*, 2006; Hennig *et al.*, 2006). In addition, the *babA* gene has occasionally been found in another, as yet unidentified locus. Most strains carry one *babA* gene but far from all express the BabA adhesin (Ilver *et al.*, 1998; Yamaoka *et al.*, 2002; Aspholm-Hurtig *et al.*, 2004).

Several studies have highlighted BabA to be subject to phase variation via homologous recombination. Pride and Blaser (2002) first described the occurrence of *babA/B* chimeric genes, which were suggested to be the result of homologous recombination, i.e. translocation of the *babB* gene into the *babA* locus and a subsequent turn-off of BabA expression. Similar chimeras were later described by Solnick and co-workers (2004), who experimentally infected rhesus macaques with a BabA-expressing/Le^b-binding strain. Most of the recovered output clones had lost BabA expression and subsequent Le^b-binding properties. In some of the BabA down-regulated clones, the change in phenotype was due to translocation of the *babB* gene into the *babA* locus, which resulted in a BabA/B protein that was devoid of Le^b-binding activity (Solnick *et al.*, 2004).

Although most strains carry the *babA* gene, it is not always expressed (Ilver *et al.*, 1998). In such BabA-negative, Le^b-non-binding strains, occasional Le^b-binding cells have been observed at an average frequency of 1:75,000. The switch of some clones into a more virulent phenotype, i.e. by the acquisition of Le^b binding, has been modelled in a defined genetic system. Strain CCUG 17875 carries two *babA* genes: *babA2* that is expressed

and *babA1* that is silent. A *babA2*-knockout mutant that only carried the silent *babA* gene was used to analyse the underlying mechanism of gain of Le^b binding. Clones of the Le^b-binding phenotype were shown to carry a *babB/A* chimeric gene. A copy of the silent *babA* gene had been translocated into the *babB* expression locus via homologous recombination (Bäckström *et al.*, 2004). The isolated Le^b-binding clones exhibited the same Le^b-binding affinity as the CCUG 17875 wild-type, although expression of the BabB/A protein was lower than BabA of CCUG 17875. This was probably due to a weaker promoter in the *babB* expression locus and, therefore, it was suggested that translocation via homologous recombination may not only be a mechanism to change expression from on to off and vice versa, but also to change the expression levels (Bäckström *et al.*, 2004). Variation in BabA expression levels has been studied in a series of strains (Hennig *et al.*, 2004; Fujimoto *et al.*, 2007), but the impact of BabA high-expressing versus BabA low-expressing strains in disease outcome is not yet clear.

Phase variation of BabA via slipped-strand mispairing

Phase variation can also occur via SSM, which is a mutation process that occurs during DNA replication. In this process, DNA strands are misaligned, sometimes resulting in an inability to replicate nucleotide repeat motifs properly due to genes being in or out of frame. Comparison of the 26695 and J99 genome sequences indicated the presence of repeat motifs in the promoter region of some *hop* genes, indicating that phase variation mechanisms could control expression of these genes. In *H. pylori*, 46 genes with homopolymeric tracts and/or dinucleotide repeats, typical for genes regulated by SSM, have been noted and among them are some *hop* genes, including *babA*, *babB*, *sabA* and *sabB* (Salaun *et al.*, 2004). BabA was first demonstrated to be subjected to phase variation by Solnick and co-workers (2004). Other studies have described *babA* also to carry CT repeats (Colbeck *et al.*, 2006; Hennig *et al.*, 2006). The *babA* genes with CT repeats appear to occur more often when *babA* is located in the 26695 *babB* locus and is

thus very similar to a chimeric *babB/A* gene. Phase shift variation and switch of adhesion-expressing phenotype resulting from SSM occurs at a higher frequency than shifts caused by homologous recombination (Pride and Blaser, 2002; Bäckström *et al.*, 2004).

In addition to on and off regulation via homologous recombination and phase shift variation, gene expression may also be affected by point mutations (in locations other than polynucleotide tracts) that bring the gene in or out of frame. The frequency of such events is, however, lower than for SSM mechanisms (Hennig *et al.*, 2006).

Phase variation of SabA expression

An instability in the sialyl-Le^x-binding ability of *H. pylori* strains together with the finding that its cognate adhesin, SabA, carries a CT repeat motif has been described (Mahdavi *et al.*, 2002). These changes in sialyl-Le^x-binding phenotype were related to modifications in the number of CT repeats of the *sabA* gene that were later found to bring the *sabA* gene in and out of frame (Sheu *et al.*, 2006; Yamaoka *et al.*, 2006). Sheu and co-workers (2006) also identified that a high proportion of *H. pylori*-infected individuals carry strains where the *sabA* gene lacks CT repeats. Thus, it is tempting to speculate that the *sabA* gene and the highly related *sabB* gene are translocated and exchange locations, similar to that observed with the *babA* and the *babB* genes, even though there are currently no data that confirm such a hypothesis.

pH and effects on SabA expression

Reduced acid secretion resulting from chronic *H. pylori* infection is strongly associated with an increased susceptibility to gastric cancer (see Correa and Piazuelo, Chapter 3, this volume). Infection with *H. pylori* strains expressing SabA has been correlated to decreased acid secretion in patients (Yamaoka *et al.*, 2006), suggesting a very important role for this adhesin in disease progression. Global gene expression analyses with cDNA arrays have found that *sabA* mRNA levels are down-regulated in response to acidic conditions (Merrell *et al.*, 2003). The same repressing

effect on SabA expression in response to low pH was also found *in vitro* (Yamaoka *et al.*, 2006). Nevertheless, both of these studies included only a few strains and the down-regulation of SabA expression was not consistent for all strains tested. The *arsRS* operon encodes a two-component signal transduction system that responds to acidic conditions (Dietz *et al.*, 2002; Pflock *et al.*, 2004; Sachs *et al.*, 2006). A more recent study has suggested that the acid responsiveness of SabA expression is controlled via the *arsRS* operon (Goodwin *et al.*, 2008). A J99 Δ *arsS* derivative, which exhibits increased *sabA* mRNA levels compared with the J99 wild-type strain, showed increased binding to AGS cells compared with wild-type J99 at neutral pH. Adherence to host cells was not analysed under acidic conditions. In addition, a J99 Δ *arsS* Δ *sabA* strain still bound AGS cells at similar levels as the J99 wild-type at neutral pH. This indicates that additional adhesins are involved in mediating binding of strain J99 to AGS cells.

12.2.2 *H. pylori* lipopolysaccharide-mediated adhesion

O-antigen in *H. pylori* binding

The O-antigen components of LPSs from many *H. pylori* strains express Le antigens, particularly Le^x, Le^y, Le^a and Le^b (Moran, 2008, 2009; see Moran, Chapter 10, this volume). Preliminary evidence (Osaki *et al.*, 1998) showed that an anti-*H. pylori* LPS monoclonal antibody, later shown to be anti-Le^x specific (Gerhardt *et al.*, 2001), was capable of inhibiting the adhesion of *H. pylori* to human gastric cancer (MKN45) cells. Using fluorescently labelled bacteria, it was demonstrated that polymeric Le^x expression by *H. pylori* mediated, at least in part, adhesion to human antral gastric biopsies (Edwards *et al.*, 2000). Further emphasizing the role of Le^x in adhesion-recognition phenomena, fluorescently labelled latex beads bearing polymeric Le^x demonstrated the same tropic binding to the apical surface of mucosal epithelial cells and to cells lining the gastric pits, as fluorescently labelled *H. pylori* bacteria (Edwards *et al.*,

2000). As noted elsewhere in this chapter, *H. pylori* adhesion to the mucosa can be mediated by a number of lectin-like adhesins, but the expression of polymeric Le^x by *H. pylori* is considered to play a distinct adhesion role based upon findings with knockout mutants in binding studies (Mahdavi *et al.*, 2003). Collectively, these results predict the existence of a molecule or receptor in the gastric epithelia, or a gastric Le^x-binding lectin, that recognizes polymeric Le^x borne on *H. pylori*.

Consistent with a role of Le^x-mediated adhesion in colonization, an isogenic *H. pylori* *galE* mutant, with affected galactose incorporation and possessing a truncated LPS without an O-antigen, was unable to colonize a number of mouse strains compared with the Le^x-expressing parental strain (Moran *et al.*, 2000). Likewise, knockout mutation of a β (1,4)-galactosyltransferase gene, which affects synthesis of the O-antigen backbone, resulted in less efficient colonization of the murine stomach (Logan *et al.*, 2000). Inactivation of *rfbM*, which encodes a GDP-D-mannose pyrophosphorylase required for GDP-fucose synthesis, resulted in a mutant with an i-antigen-expressing O-antigen lacking fucose, which exhibited reduced mouse colonization (Moran *et al.*, 2000) and ablated interaction with the human gastric mucosa of biopsy specimens *in situ* (Edwards *et al.*, 2000). Although one study reported no significant changes in mouse colonization by *H. pylori* mutants with ablated Le^x/Le^y expression (Takata *et al.*, 2002), that study used C3H/HeJ (TLR4^{-/-}) mice, so-called LPS non-responder mice, which may not be the most appropriate animal model of infection, since *H. pylori* LPS may induce Toll-like receptor (TLR) 4 signalling (see Kaparakis *et al.*, Chapter 8, this volume). In another study using a more appropriate model with LPS-responsive mice, however, a mutated *H. pylori* strain with a double knockout in both α -(1 $\rightarrow$ 3)-fucosyltransferases did not colonize, in contrast to the Le^x/Le^y-expressing parental strain (Appelmek *et al.*, 2000). Taken together, these data indicate that Le^x expression, rather than O-antigen occurrence alone, is required for colonization.

Initially, it was suggested that *H. pylori*-expressed Le^x might interact with the host via

a homotypic (Le^x-Le^x) interaction, as occurs in the Ca²⁺-dependent homotypic interaction in embryogenesis and tumour cell-cell interaction. However, while soluble Le^x can bind to liposome-incorporated Le^x, attempts to show an interaction between soluble Le^x and *H. pylori* have been unsuccessful (Ilver *et al.*, 1998). Also, anti-Le^x immunoglobulin (Ig) M monoclonal antibodies increased the adherence of *H. pylori* to AGS cells when tested *in vitro* by forming an interaction bridge between bacterial-borne Le^x and that on the host cells (Sheu *et al.*, 2007). Although anti-Le^x IgM antibodies may be produced naturally in humans (Chmiela *et al.*, 1999), whether these occur in the local gastric environment of *H. pylori* remains an open question. On the other hand, Fowler *et al.* (2006) identified the galactoside-binding lectin galectin-3 as the gastric receptor for *H. pylori* Le^x. Expression of galectin-3 was found to be up-regulated by gastric epithelial cells following adhesion of *H. pylori* and subsequently influenced the host response to infection. Overall, the lectin-based interaction of galectin-3, rather than a homotypic interaction, appears the most likely mechanism for recognition of *H. pylori*-expressed Le^x by the gastric epithelium.

Consistent with an adhesion role, high expression of Le^x by *H. pylori* strains has been correlated with a higher colonization density in chronic gastritis patients than that by strains with weaker expression (Heneghan *et al.*, 2000). By assisting bacterial adhesion and interaction with the gastric mucosa, like other adhesins, bacterial Le^x expression may enhance delivery of secreted products into the gastric mucosa (Rieder *et al.*, 1997; Moran, 1999), such as those delivered through the type IV secretion system encoded by the cytotoxin-associated genes (*cag*) pathogenicity island (see Backert *et al.*, Chapter 11, this volume). This would in turn promote chemotaxis and leucocyte infiltration, as observed in an adherence-dependent mouse model of *H. pylori*-induced disease where Le^x expression correlated with inflammation (Guruge *et al.*, 1998). It is important to consider the range of pH that exists within mucus and on the gastric cell surface, i.e. from pH 2 on the luminal side of the gastric mucus layer to almost pH 7 on the cell surface, and that environmental pH

influences Le^x expression by *H. pylori* (Moran *et al.*, 2002). Optimal expression of Le^x by *H. pylori* occurs at neutral pH, as found at the gastric epithelial cell surface, but reduced expression occurs at lower pH, as in the mucus layer, thereby modulating expression of the Le^x-adhesin and allowing free-swimming *H. pylori* in the mucus layer. Potentially, bacteria in the mucus layer may act as a reservoir for continued infection of the mucosa. The observed on/off switching of fucosyltransferase activities, and resulting phase variation in Le^x expression (Appelmek *et al.*, 1999), would be consistent with this mechanism of adhesin molecular modulation (Moran, 2008).

Additionally, *H. pylori* binds surfactant protein D (SP-D), which is up-regulated in the vicinity of *H. pylori* during infection *in vivo* (Murray *et al.*, 2002). *H. pylori* LPS, by interaction with the O-antigen moiety, mediates binding of SP-D, resulting in bacterial immobilization and aggregation (Murray *et al.*, 2002; Khamri *et al.*, 2005). To evade this potentially important mechanism of innate immune recognition of *H. pylori*, escape variants can arise within the bacterial cell population with modifications in O-antigen glycosylation, including greater expression of polymeric Le^x, which has lesser affinity for SP-D and thereby decreases their interaction with SP-D (Khamri *et al.*, 2005; Moran *et al.*, 2005b). Notably, *in vivo* SP-D-binding organisms predominate in mucus, rather than on the epithelial cell surface, where escape variants predominate and expression of polymeric Le^x is optimized (Khamri *et al.*, 2005).

Lipopolysaccharide core and H. pylori binding

The core of LPS from *H. pylori* has also been implicated in adhesion. Together with a 25 kDa sialic acid-specific lectin on *H. pylori* (Valkonen *et al.*, 1997), which is produced *in vivo* (Moran *et al.*, 2005a), the LPS core mediates binding to the extracellular matrix glycoprotein laminin. Although independent of Le antigen agglutination of red blood cells, depending on whether *H. pylori* strains are haemagglutinating or poorly haemagglutinating, two differing mechanisms of interaction

of the LPS core with amino acid sequences within laminin have been observed: a phosphorylated core structure mediates the interaction of haemagglutinating strains, whereas a non-phosphorylated structure is involved in poorly haemagglutinating strains (Valkonen *et al.*, 1994). Despite this variation, the 25 kDa protein adhesin of *H. pylori* that recognizes the sialylated sugar chain of laminin is conserved in both haemagglutinating and poorly haemagglutinating strains (Valkonen *et al.*, 1997). Thus, the binding of laminin by *H. pylori* illustrates a novel dual-recognition system, whereby sugars in the core of LPS interact with a peptide region in laminin and the 25 kDa adhesin of *H. pylori*, with lectin-like properties, recognizes a sialylated trisaccharide in laminin (Moran, 1996; Valkonen *et al.*, 1997). Initial binding to laminin is believed to be mediated by the LPS core structures, and subsequently by the 25 kDa lectin (Valkonen *et al.*, 1997; Moran, 1999). Despite a number of studies showing that *H. pylori* strains exhibit high-affinity binding to laminin, this interaction is likely to be a secondary event after initial colonization of the mucosa (see Moran, 1999). Nevertheless, as a proportion of colonizing *H. pylori* bacteria (5%) are observed associated with intercellular junctions, laminin binding may, in part, explain the association with this microniche. Moreover, this binding to laminin may have pathological consequences since *H. pylori* LPS interferes with the interaction between laminin and a 67 kDa protein receptor (integrin) on gastric epithelial cells (Slomiany *et al.*, 1991), thus potentially contributing to disruption of gastric mucosal integrity.

12.3 *H. pylori* Interaction with Host Structures

12.3.1 The first barrier – mucus

The first barrier *H. pylori* encounters when colonizing the stomach is the highly hydrated mucus gel that covers the mucosal surface and protects the epithelial cells against chemical, enzymatic, microbial and mechanical insult. In addition, mucus acts as an 'unstirred

layer' in which bicarbonate ions secreted by the surface epithelium counteract protons diffusing from the lumen into the gel, causing the pH in the mucus layer to range from acidic in the lumen to neutral at the cell surface. The mucus gel is formed by high-molecular-mass oligomeric glycoproteins (mucins) and protection is reinforced by a number of 'defence factors' (such as defensins, trefoil factors and secretory IgA) trapped in the gel matrix. The mucins are produced by cells in the epithelial surface and/or by glands located in the submucosal connective tissues, and secretion occurs via both constitutive and regulated pathways. The constitutive pathway continuously secretes a small amount of mucin to maintain the mucus layer, whereas the regulated pathway affords a massive discharge of mucus as a response to environmental and/or pathophysiological stimuli. Stimulated mucin release occurs rapidly and is accompanied by a 100-fold or so expansion of secretory granules (reviewed in Lindén *et al.*, 2008b). In the stomach, MUC5AC and MUC6 are the major secreted mucins, the former being produced by the surface epithelium and the latter by the glands (De Bolos *et al.*, 1995).

Mucin polymorphism and glycosylation

Each mucin gene contains unique tandem repeat (TR) motifs coding for regions with a high density of serine, threonine and proline. The TR varies in length between mucins, and there is a genetic polymorphism in the number of repeats referred to as the variable number of tandem repeats (VNTR) polymorphism. VNTR polymorphisms cause mucin size to differ between individuals. The serine and threonine residues can be O-glycosylated and more than 50% (often 70–80%) of the mucin molecular mass is composed of carbohydrate. Each mucin carries of the order of 100 different oligosaccharide structures (Klein *et al.*, 1993). These carbohydrate chains are often clustered into highly glycosylated domains, giving the mucin a 'bottle-brush' appearance. The massive O-glycosylation of the mucins protects them from proteolytic enzymes and induces a relatively extended conformation (Jentoft, 1990). This extended conformation

causes the molecules to occupy large volumes, and secreted oligomeric mucins occupy domains equivalent in size to small bacteria (Jentoft, 1990).

The O-linked glycans contain one to 20 residues, which occur both as linear and branched structures. The carbohydrate chain is initiated with an N-acetyl-galactosamine (GalNAc) linked to serine or threonine and is elongated by the formation of the so-called core structures followed by the backbone region. The chains are terminated by for example fucose (Fuc), galactose (Gal), GalNAc or sialic acid (N-acetyl-neuraminic acid; Neu5Ac) residues in the peripheral region, forming histo-blood-group antigens such as A, B, H, Le^a, Le^b, Le^x and Le^y, as well as sialyl-Le^a and sialyl-Le^x structures. The carbohydrate structures present depend on which glycosyltransferases are expressed in the cells, i.e. by the genotype of the individual. The H1 structure is made by the *secretor* gene product; the majority of individuals (80% of Caucasians, all South American Indians and Orientals) carry this structure and are thus referred to as 'secretors' (Oriol *et al.*, 1986; Oriol, 1995). Individuals may also express the *Lewis* gene (90% of the Caucasian population) and, provided that they are also secretors, will then express the Le^b structure on the H1 antigen (Oriol *et al.*, 1986; Oriol, 1995). If they are non-secretors, Le^a will be expressed on type-1 chains (Oriol *et al.*, 1986; Oriol, 1995). The terminal structures of mucin oligosaccharides are highly heterogeneous, and vary between/within species and even with tissue location within a single individual. The Le^a and Le^b blood-group antigens mainly appear on the surface epithelium (where MUC5AC is produced), whereas the Le^x and Le^y antigens are expressed in mucous, chief and parietal cells of the glands, co-localized with MUC6 (Murata *et al.*, 1992; De Bolos *et al.*, 1995). The Le^a and Le^b structures can be found on the MUC5AC mucin, whereas Le^x and Le^y structures are found on the MUC6 mucin (Lindén *et al.*, 2002; Nordman *et al.*, 2002). The mucins from a healthy human stomach express low levels of sialic acid and sulfate and are, therefore, predominantly neutral.

In gastric precancerous lesions and cancer, altered expression of MUC5AC,

MUC6, MUC2 and MUC5B has been described (Buisine *et al.*, 2000), with MUC2 being a marker for intestinal metaplasia, a precursor of gastric adenocarcinoma. In *H. pylori*-infected adults, aberrant MUC6 expression in the surface epithelium has been observed (Byrd *et al.*, 1997). Mucins appear to be the major carrier of altered glycosylation in carcinomas (Saez *et al.*, 2001) and incomplete glycosylation, leading to expression of Tn and T antigens, and/or sialylation/sulfation are common (Saez *et al.*, 2001).

H. pylori and the mucus layer

H. pylori is well adapted to the mucus niche, having long, whip-like flagella facilitating locomotion through the mucus layer. Flagella, urease, adhesins and genes encoding proteins with a predicted function in chemotaxis are essential for *H. pylori* colonization of laboratory animals (Kavermann *et al.*, 2003). *H. pylori* has been detected in the oral cavity of individuals with and without gastric *H. pylori* infection (Song *et al.*, 2000a; Dowsett and Kowolik, 2003) and more than one strain may exist in the stomach and saliva simultaneously (Song *et al.*, 2000a,b; Wang *et al.*, 2002). Although oral infection could be transient (Czesnikiewicz-Guzik *et al.*, 2004), the oral cavity may serve as a reservoir and portal of entry. Mother-child and sibling-sibling transmission have been indicated as the primary pathways of *H. pylori* infection/colonization, and the incidence of *H. pylori* is high in regions where mothers chew the food for their children (Kivi *et al.*, 2003). While a limited number of studies have suggested that, clinically, the oral cavity may represent a possible source of *H. pylori* re-infection in individuals in whom gastric *H. pylori* has been eradicated due to the fact that *H. pylori* eradication therapy is less effective for the mouth than for the stomach (Miyabayashi *et al.*, 2000; Gebara *et al.*, 2006), this view is controversial. Colonization of the oral cavity may provide the bacterium multiple chances to colonize the stomach. Individuals are likely to be frequently challenged by *H. pylori*, but may only be vulnerable to infection when protective factors are not functioning optimally. Significantly, 1 to 2 l of saliva, which has

excellent *H. pylori*-binding ability, are swallowed every day (Lindén *et al.*, 2008c).

In both the oral and gastric compartment, *H. pylori* are found in the 'stationary' niche of the dental/gingival plaque and gastric epithelial cell surface, as well as in the constantly shedding mucus secretions (Czesnikiewicz-Guzik *et al.*, 2004; Lindén *et al.*, 2004a, 2009). It is reasonable to believe that it is more beneficial for the bacterium to adhere in the 'stationary' niche where they are protected against being flushed away. In contrast, it is in the host's interest to inhibit *H. pylori* interactions with the cell surface, as adhesion is probably of significant importance for efficient delivery of key virulence factors such as the vacuolating cytotoxin (see Backert *et al.*, Chapter 11, this volume). Further, *in vitro* studies show that *H. pylori* requires direct contact with the AGS cell line to induce an invasive phenotype (Prinz *et al.*, 2001). The secreted mucins are likely to function as decoys for *H. pylori* interaction/binding (Lindén *et al.*, 2004a), as they are produced in large amounts and constantly wash the mucosal surfaces. Patients with primary Sjogren's syndrome, which is characterized by autoimmune destruction of exocrine glands and low levels of saliva, have a higher prevalence of *H. pylori* infection, supporting the view that the saliva/mucus layers play a role in protection against this bacterium (El Miedany *et al.*, 2005). Furthermore, in the human-like rhesus monkey infection model of *H. pylori*, animals with mucins that more effectively bound *H. pylori* had a lower density of *H. pylori* in the stomach (Lindén *et al.*, 2008a). Of note, *H. pylori* LPS decreases mucin synthesis at least *in vitro* (Slomiany and Slomiany, 2006), and the BabA and SabA adhesins are known to undergo phase variation and change expression during infection (Mahdavi *et al.*, 2002; Solnick *et al.*, 2004), events that may aid *H. pylori* to evade this host defence.

H. pylori binding to mucins along the oro-gastric infection route

The highly diverse mucin glycosylation motifs present in the oro-gastric tract present multiple potential binding motifs for *H. pylori*. Binding to mucins occurs via four distinct

modes of adhesion: (i) BabA to fucosylated structures; (ii) SabA to sialylated structures; (iii) a charge/low pH-dependent mechanism; and (iv) via an adhesin specific for salivary structures (Lindén *et al.*, 2008c) (see Fig. 12.1). No binding to mucins has been detected to occur via AlpAB (Lindén *et al.*, 2008c). *H. pylori* binding to mucins differs substantially with the anatomical site, mucin type, pH, gastritis status and *H. pylori* strain (Lindén *et al.*, 2008c).

Salivary mucins display a range of ligands to which *H. pylori* can bind via a range of factors including BabA, SabA, the charge/low pH-dependent mechanism and the saliva-specific adhesin (Lindén *et al.*, 2008c). However, as the pH in the oral cavity niche is predominantly neutral, mucin binding is likely to occur at this site mainly via the SabA, BabA and saliva-specific adhesin. *In vitro* binding studies demonstrate that SabA is the adhesin that interacts with the most ligands in saliva (Lindén *et al.*, 2008c). The ligands for *H. pylori* adhesion are present on MUC5B, MUC7 and the salivary agglutinin (Lindén *et al.*, 2008c).

From the oral cavity, saliva containing *H. pylori* is swallowed and ends up in the lumen of the gastric compartment. In the gastric lumen, the mucins MUC5B, MUC7 and MUC5AC, as well as salivary agglutinin, are present and display ligands for the SabA, BabA, saliva-specific adhesin, and additionally for the charge/low pH-dependent mechanism (Lindén *et al.*, 2008c). In this niche, the pH varies due to circadian variations in acid secretion, food and drug intake, as well as disease and stress; all adhesins thus have the potential to be functional at different times/stages of infection. *In vitro* binding studies demonstrate that, at acidic pH, the charge/low pH-dependent mechanism is the most efficient at binding *H. pylori*, whereas at a more neutral pH the SabA-dependent mechanism may be more efficient (Lindén *et al.*, 2008c).

In the adherent gastric mucus layer, the pH ranges from acidic in the lumen to neutral at the cell surface. On the mucins present in this layer in healthy individuals, only ligands for BabA and a very small number of ligands for the charge/low pH-dependent mechanism of binding are present (Lindén *et al.*, 2002,

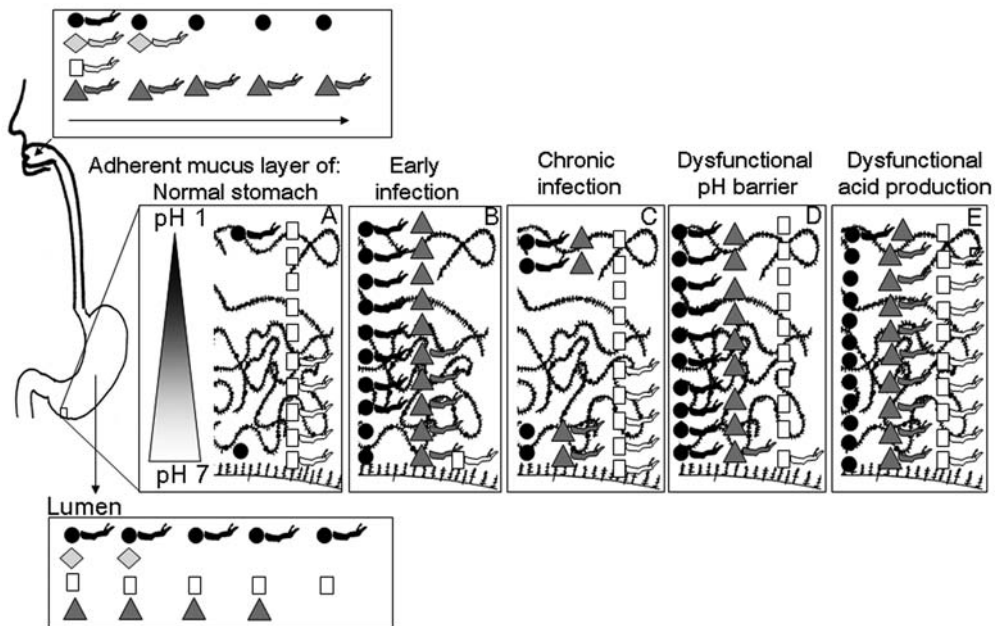


Fig. 12.1. A diagram summarizing locations where the pH and host ligands permit binding of *Helicobacter pylori* to mucins. The four adhesion modes are depicted in the locations where binding to mucins via these modes has been identified: □, BabA-dependent adhesion mode; ▲, SabA-dependent adhesion mode; ◆, saliva-specific adhesion mode; ●, low pH adhesion mode. In the location where the pH is likely to permit binding via this mode, an *H. pylori* in the same colour is attached to the symbol. The number of symbols reflects the level of binding. In the oral cavity, all four binding modes are functional, albeit as the pH is predominantly neutral, the low pH-binding mode may only occur locally and temporarily (Lindén *et al.*, 2008c). In the gastric juice in the gastric lumen, the host ligands for the four binding modes are present, but the acidic pH is likely to prevent binding with all except the low pH-binding mode. In the gastric adherent mucus layer, the BabA-dependent, SabA-dependent and low pH-binding modes occur to a varying degree depending on disease status of the host: (A) in a healthy stomach, mainly BabA-dependent binding occurs; (B) in early infection (as sialylation increases whereas Lewis fucosylation and pH decreases; Lindén *et al.*, 2008a), the BabA-dependent binding decreases and the SabA-dependent and low pH-binding modes increase. (C) In chronic infection, ligands for the BabA-dependent binding increase again to normal levels whereas the ligands for the SabA- and low pH-dependent binding modes decrease compared with early infection, although they are still higher than in an uninfected stomach (Lindén *et al.*, 2008a). Theoretical effects of disease-related changes in pH on the binding modes are also depicted: (D) dysfunctional pH barrier; (E) dysfunctional acid production.

2004a). *H. pylori* binding to MUC5AC occurs at neutral pH and requires Le^b to be displayed on the mucin (Lindén *et al.*, 2002).

In the adherent mucus layer of gastritis-affected stomachs, the ligands for BabA on MUC5AC remain constant, while there is an increase in the ligands for SabA and the charge/low pH-dependent mechanism of binding (Lindén *et al.*, 2008c). *H. pylori*-infected individuals have increased sialylation of their gastric epithelial cells, which return to normal

levels following successful treatment (Ota *et al.*, 1998; Mahdavi *et al.*, 2002). The gastric glycosylation pattern in the rhesus monkey closely mimics that of man (Lindén *et al.*, 2004b) and persistent *H. pylori* infections in these animals up-regulate sialyl-Le^x expression, thereby providing receptors that can be used for *H. pylori* adherence to the gastric surface epithelium (Mahdavi *et al.*, 2002; Lindén *et al.*, 2008a). A strong increase in sialylated Le antigens, as well as SabA-binding

ability, occurs within 1 week of *H. pylori* infection in the rhesus monkey (Lindén *et al.*, 2008a). The majority of the changes that occur following acute infection in this model return to baseline levels by 10 months post-infection, indicating that this adhesin may play a more important role in early rather than chronic infection (Lindén *et al.*, 2008a).

The BabA-positive strains thus bind to mucins from saliva, gastric juice and the adherent gastric mucus layer, demonstrating that this adhesin is the least dependent on anatomical site of the four different binding properties important in mucin binding (Lindén *et al.*, 2008c). However, BabA binding is dependent on the blood group of the host (Lindén *et al.*, 2002), and MUC5AC is also produced in several glycoforms within each individual, affecting the ability of this mucin to interact with BabA due to the steric presentation of the glycans involved (Lindén *et al.*, 2002). BabA, the salivary-binding mode, and possibly also SabA, show their strongest mucin binding at neutral pH. Hence, these adhesins would bind tightly to ligands in the oral cavity and gastric epithelium, facilitating colonization of those niches. However, bacteria swallowed with saliva, or shed from the adherent gastric mucus layer, that arrive in the gastric lumen would be exposed to low pH. This reduced pH would reduce adhesin binding strength, releasing the bacteria from mucins and allowing them to swim through the mucus layer towards the protected niche of the gastric epithelium compatible with *H. pylori* survival. However, when highly charged moieties on mucins are present in the vicinity of these bacteria the low pH-binding mode may take over the *H. pylori*-binding role, thereby acting as a potential host mechanism for aiding in the removal of bacteria from the stomach (Lindén *et al.*, 2004a, 2008c).

Underneath the mucus layer, the cells present a dense forest of highly diverse glycoproteins and glycolipids, which form the glycocalyx. The membrane-associated mucins that are also part of this glycocalyx are larger than most other membrane-bound glycoproteins. Due to their size, cell surface mucins are therefore likely to be the first point of direct contact between host tissue and organisms that penetrate the secreted mucus layer.

MUC1 is the most highly expressed cell surface mucin in the stomach (Packer *et al.*, 2004). MUC1 can carry the Le^b, sialyl-Le^x and sialyl-Le^a structures and bind *H. pylori* via the BabA and SabA structures (Lindén *et al.*, 2009). MUC1 protects the gastric epithelial cells by steric hindrance as well as by acting as a releasable decoy (Lindén *et al.*, 2009). Mice deficient in Muc1 are more susceptible to infection by *H. pylori* (McGuckin *et al.*, 2007). Moreover, short VNTR alleles for MUC1 are associated with increased risk of *H. pylori*-induced gastritis and gastric cancer (see Sutton *et al.*, Chapter 7, this volume).

12.3.2 *H. pylori* binding to host glycosphingolipids

Below the mucus layer is another group of molecules to which *H. pylori* can adhere: the glycosphingolipids. Adherence of *H. pylori* to glycosphingolipids located in the plasma membrane of epithelial cells is likely to confer a close bacterial-epithelial cell association due to the small size of the extracellular part of the molecule. However, little is known about the events resulting from *H. pylori* binding to glycosphingolipids. The first indication that they may be important came early in the history of *H. pylori*, when it was reported that these bacteria bind to the two major acid glycosphingolipids of the human stomach, i.e. the GM₃ ganglioside [α -Neu5Ac-(2→3)- β -Gal-(1→4)- β -Glc-(1→1)-Cer] and sulfatide [(SO₃→3)- β -Gal-(1→1)-Cer] (Saitoh *et al.*, 1991). Since then, a large number of *H. pylori*-binding glycosphingolipids have been reported (summarized in Table 12.1). In some cases the binding-active carbohydrate sequences are also found on glycoproteins, as in the Le^b sequence [α -Fuc-(1→2)- β -Gal-(1→3) [α -Fuc-(1→4)-] β -GlcNAc], the sialyl-neolacto sequence [α -Neu5Ac-(2→3)- β -Gal-(1→4)- β -GlcNAc] and the neolacto sequence [β -Gal-(1→4)- β -GlcNAc], while in other cases the carbohydrate sequences have hitherto only been identified in glycosphingolipids, such as the lactose sequence [β -Gal-(1→4)-Glc] of lactosylceramide and the α -Gal-(1→4)-Glc sequence of galabiosylceramide. In the following text (and Table 12.1), the various

Table 12.1. *Helicobacter pylori*-binding glycosphingolipids.

Glycosphingolipid	Ligand ^a	Reference(s)
Sulfatide	HSP70, NAP	Saitoh <i>et al.</i> (1991)
Neolactoseries gangliosides	SabA	Evans <i>et al.</i> (1988); Hirno <i>et al.</i> (1996); Miller-Podraza <i>et al.</i> (1997); Mahdavi <i>et al.</i> (2002); Roche <i>et al.</i> (2004)
Ganglioseries glycosphingolipids	Not defined	Lingwood <i>et al.</i> (1992); Angstrom <i>et al.</i> (1998)
Fucosylated blood-group antigen-carrying glycosphingolipids	BabA	Borén <i>et al.</i> (1993); Aspholm-Hurtig <i>et al.</i> (2004)
α -Neu5Ac-(2→3)- β -Gal-(1→4)- β -GlcNAc-(1→3)- β -Gal-(1→4)-GlcNAc-terminated glycosphingolipids	NAP	Teneberg <i>et al.</i> (1997)
Lactosylceramide	Not defined	Angstrom <i>et al.</i> (1998)
Galactosylceramide/glucosylceramide	Not defined	Abul-Milh <i>et al.</i> (2001)
Lactotetraosylceramide	Not defined	Teneberg <i>et al.</i> (2002)
Neolactoseries glycosphingolipids	Not defined	Miller-Podraza <i>et al.</i> (2005, 2009)
Ganglioseries gangliosides	HP0721	Bennett and Roberts (2005)
Galabiosaosylceramide	VacA	Roche <i>et al.</i> (2007)

^aAbbreviations: Hsp70, heat-shock protein 70; NAP, neutrophil-activating protein; SabA, sialic acid-binding adhesion; BabA, blood group-binding adhesin; VacA, vacuolating cytotoxin.

H. pylori glycosphingolipid-binding activities and their corresponding bacterial carbohydrate-binding proteins are described.

Sulfatide

While binding of *H. pylori* to both the GM₃ ganglioside and sulfatide has been reported (Saitoh *et al.*, 1991), binding to GM₃ was later disputed when only sulfatide binding was observed upon examination of binding of *H. pylori* to Kato III cells (Kamisago *et al.*, 1996). The heat-shock protein HSP70 has been proposed to be the adhesin involved in binding of *H. pylori* to sulfatide (Huesca *et al.*, 1996, 1998). Another candidate is *H. pylori* NAP, which binds to sulfatide and sulfated gangliotetraosylceramide [(SO₃→3)- β -Gal-(1→3)- β -GalNAc-(1→4)- β -Gal-(1→4)- β -Glc-(1→1)-Cer] (Teneberg *et al.*, 1997). However, NAP also binds to sulfated oligosaccharides, e.g. (SO₃→3)-Gal, (SO₃→3)-GlcNAc and sulfated Le^a, of human high-molecular-mass salivary mucins (Namavar *et al.*, 1998).

Neolactoseries gangliosides

H. pylori was initially demonstrated to bind sialylated glycoconjugates and gangliosides

(Evans *et al.*, 1988; Miller-Podraza *et al.*, 1997). Subsequently, the *H. pylori* adhesin SabA was identified and a novel high-affinity receptor for this adhesin was isolated from a human gallbladder adenocarcinoma and characterized as sialyl-dimeric-Le^x (Mahdavi *et al.*, 2002). The structural requirements for SabA-mediated *H. pylori* ganglioside binding were defined: N-acetyl-lactosamine (LacNAc)-based gangliosides with terminal α -(2→3)-linked Neu5Ac were recognized by wild-type SabA-expressing bacteria, whereas no binding to gangliosides or LacNAc-based gangliosides with terminal α -Neu5Ac-(2→6), α -NeuGc-(2→3) or α -Neu5Ac-(2→8)- α -Neu5Ac-(2→3) occurred (Roche *et al.*, 2004). The minimal epitope required for SabA-mediated *H. pylori* binding to gangliosides is α -Neu5Ac-(2→3)-Gal (Hirno *et al.*, 1996; Johansson and Karlsson, 1998; Roche *et al.*, 2004). Nevertheless, comparative binding studies (Roche *et al.*, 2004) have shown that the binding affinity is increased by: (i) increased length of LacNAc core chain, whereby this effect is most likely caused by an improved accessibility of the α -Neu5Ac-(2→3)-Gal head group when presented on a longer core chain; (ii) branches of the carbohydrate chain, i.e. a divalent presentation of

the binding epitope; and (iii) fucose substitution of the LacNAc core chain, whereby the fucose residues may either interact with the carbohydrate-binding site of the SabA adhesin or affect the conformation of the ganglioside, providing an optimal presentation of the α -Neu5Ac-(2→3)-Gal epitope.

Neutrophil infiltration and activity is believed to play an important role in gastritis severity and disease progression. Several of the *H. pylori*-binding gangliosides, for example α -sialyl-(2→3)-neolactotetraosylceramide, α -sialyl-(2→3)-neolactohexaosylceramide, α -sialyl-(2→3)-neolactooctaosylceramide, the VIM-2 ganglioside and sialyl-dimeric-Le^x glycosphingolipid, are present on human neutrophils (Stroud *et al.*, 1996), suggesting that the ganglioside-binding capacity may be involved in neutrophil activation. Indeed, the *H. pylori*-induced neutrophil oxidative burst reaction can be inhibited by pre-incubation with sialylated oligosaccharides (Teneberg *et al.*, 2000). The relative contributions of SabA and NAP in neutrophil activation have been investigated using a battery of *H. pylori* deletion mutants (Unemo *et al.*, 2005). Mutant and wild-type strains lacking SabA were devoid of neutrophil-activating capacity, while the absence of either NAP or the Le^b-binding BabA had no effect on neutrophil activation. Thus, SabA-mediated binding of *H. pylori* bacterial cells to sialylated neutrophil receptors can play an important initial role in the adherence and phagocytosis of the bacterium, and the induction of the neutrophil oxidative burst reaction.

α -Neu5Ac-(2→3)- β -Gal-(1→4)- β -GlcNAc-(1→3)- β -Gal-(1→4)-GlcNAc-terminated glycosphingolipids

When the potential carbohydrate recognition by NAP was investigated by binding studies using glycosphingolipid fractions from various sources, distinct binding to two compounds (gangliosides) in the acid fraction of human neutrophils (the target cells of NAP) was obtained (Teneberg *et al.*, 1997). These binding-active gangliosides were subsequently identified as α -Neu5Ac-(2→3)-neolactohexaosylceramide [α -Neu5Ac-(1→3)- β -Gal-(1→4)- β -GlcNAc-(1→3)- β -

Gal-(1→4)- β -GlcNAc-(1→3)- β -Gal-(1→4)- β -Glc-(1→1)-Cer] and α -Neu5Ac-(2→3)-neolactooctaosylceramide [α -Neu5Ac-(2→3)- β -Gal-(1→4)- β -GlcNAc-(1→3)- β -Gal-(1→4)- β -GlcNAc-(1→3)- β -Gal-(1→4)- β -GlcNAc-(1→3)- β -Gal-(1→4)- β -Glc-(1→1)-Cer]. A terminal α -NeuGc-(2→3) was also tolerated, i.e. NAP also bound to α -NeuGc-(2→3)-neolactohexaosylceramide and α -NeuGc-(2→3)-neolactooctaosylceramide, in contrast to the SabA adhesin which requires a terminal α -Neu5Ac-(2→3) for binding.

Fucosylated blood-group antigen-carrying glycosphingolipids

In the initial study where the binding of *H. pylori* to the H5-type-1/Le^b determinants was defined, no binding to glycosphingolipids carrying these determinants was observed (Borén *et al.*, 1993). Nevertheless, subsequent studies have shown that BabA-expressing *H. pylori* strains bind to glycosphingolipids with Le^b-related carbohydrate epitopes, with the same recognition patterns as found with neoglycoproteins (Aspholm-Hurtig *et al.*, 2004).

Lactosylceramide

Many *H. pylori* strains, as well as other bacteria including both pathogens and commensals (Karlsson, 1989), bind to lactosylceramide [β -Gal-(1→4)- β -Glc-(1→1)-Cer] and isoglobotriaosylceramide [α -Gal-(1→3)- β -Gal-(1→4)- β -Glc-(1→1)-Cer] with a concomitant binding to gangliotetraosylceramide [β -Gal-(1→3)- β -GalNAc-(1→4)- β -Gal-(1→4)- β -Glc-(1→1)-Cer] (Angstrom *et al.*, 1998). Interestingly, a putative glycosphingolipid-binding motif in *H. pylori* adhesin A (HpaA) was recently identified by structure similarity searches (Fantini *et al.*, 2006). A synthetic peptide corresponding to this motif bound to lactosylceramide, but not to globotriaosylceramide, consistent with the glycosphingolipid-binding pattern of whole *H. pylori* bacterial cells (Angstrom *et al.*, 1998). The identification of this glycosphingolipid-binding motif may allow further dissection of the lactosylceramide-binding properties of

H. pylori, and also other lactosylceramide-binding bacteria, in the future.

*Additional glycolipid structures to which
H. pylori binds*

H. pylori has been demonstrated to bind to a range of additional glycolipid structures, for which the bacterial adhesin has not been identified, such as galactosylceramide with sphingosine and both hydroxy and non-hydroxy fatty acids (Abul-Milh *et al.*, 2001), the neolacto [β -Gal-(1 $\rightarrow$ 4)- β -GlcNAc] core structure (Miller-Podraza *et al.*, 2005, 2009) and lactotetraosylceramide [β -Gal(1 $\rightarrow$ 3)- β -GlcNAc-(1 $\rightarrow$ 3)- β -Gal-(1 $\rightarrow$ 4)- β -Glc-(1 $\rightarrow$ 1)-Cer] (Mahdavi *et al.*, 2002; Teneberg *et al.*, 2002). A novel sialic acid-binding protein of *H. pylori* (HP0721) was recently isolated (Bennett and Roberts, 2005) and bound to the GM₃ ganglioside [α -Neu5Ac-(2 $\rightarrow$ 3)- β -Gal-(1 $\rightarrow$ 4)- β -Glc-(1 $\rightarrow$ 1)-Cer], the ganglio-series gangliosides GM₁ [β -Gal-(1 $\rightarrow$ 3)- β -GalNAc-(1 $\rightarrow$ 4)-[α -Neu5Ac-(2 $\rightarrow$ 3)-]- β -Gal-(1 $\rightarrow$ 4)- β -Glc-(1 $\rightarrow$ 1)-Cer] and GD_{1a} [α -Neu5Ac-(2 $\rightarrow$ 3)- β -Gal-(1 $\rightarrow$ 3)- β -GalNAc-(1 $\rightarrow$ 4)-[α -Neu5Ac-(2 $\rightarrow$ 3)-]- β -Gal-(1 $\rightarrow$ 4)- β -Glc-(1 $\rightarrow$ 1)-Cer], and also to gangliotetraosylceramide [β -Gal-(1 $\rightarrow$ 3)- β -GalNAc-(1 $\rightarrow$ 4)- β -Gal-(1 $\rightarrow$ 4)- β -Glc-(1 $\rightarrow$ 1)-Cer], i.e. a non-sialylated glycolipid. Unfortunately no negative reference glycosphingolipid was included in this assay. Since *H. pylori* cells have previously been shown not to recognize the GM₃ ganglioside, or gangliosides based on the ganglio core structure (Kamisago *et al.*, 1996; Miller-Podraza *et al.*, 1997; Roche *et al.*, 2004), the role of the sialic acid binding of HP0721 needs to be clarified.

*Vacuolating cytotoxin binding to
galabiosaosylceramide*

Human gastric glycosphingolipids recognized by the important *H. pylori* virulence factor vacuolating cytotoxin (VacA) have been characterized as galactosylceramide [β -Gal-(1 $\rightarrow$ 1)-Cer] and galabiosaosylceramide [α -Gal-(1 $\rightarrow$ 4)- β -Gal-(1 $\rightarrow$ 1)-Cer] (Roche *et al.*, 2007). Comparison of the binding preferences of VacA using reference glycosphingolipids from other sources showed the additional

recognition of glucosylceramide [β -Glc-(1 $\rightarrow$ 1)-Cer], lactosylceramide [β -Gal-(1 $\rightarrow$ 4)- β -Glc-(1 $\rightarrow$ 1)-Cer] and globotriaosylceramide [α -Gal-(1 $\rightarrow$ 4)- β -Gal-(1 $\rightarrow$ 4)- β -Glc-(1 $\rightarrow$ 1)-Cer]. No binding to the glycosphingolipids recognized by the VacA holotoxin was obtained with a mutant toxin with a deletion of the 37 kDa fragment of VacA (p58 molecule), indicating that carbohydrate recognition is mediated by the 37 kDa moiety (Roche *et al.*, 2007). Since both toxin domains are required for binding and internalization of VacA, and also for its vacuolating cytotoxic effect (Torres *et al.*, 2005), this suggests a scenario where target cell specificity is obtained by an initial binding of the toxin to high-affinity protein receptors via the p58 molecule, followed by an interaction via the p37 domain to short-chain glycosphingolipids. Binding to mono- and di-glycosylceramides would provide VacA with a membrane-close attachment that may facilitate the internalization of the toxin.

12.4 Conclusions

The presence of multiple binding abilities of *H. pylori* implies that adherence is a multistep process, and that different adhesins mediate adherence to different sites along the colonization axis and during the varying inflammatory status of the host. The personal repertoire of glycans on mucins and glycolipids and transient changes in mucin glycosylation in response to infection or stress (Bosch *et al.*, 2003), as well as circadian variations in acidity in the gastric lumen and *H. pylori* adhesin repertoire, all affect binding. The continuous secretion of mucins which can bind *H. pylori* via BabA, SabA and the low pH-binding modes are likely to have a protective function, interfering with the bacterial aim of binding to the gastric cell surface. Similarly, SP-D interaction with *H. pylori* LPS can also play an important protective role, but this may be subverted by the production of escape variants. Competition between binding to the cell surface-bound structures such as glycolipids and cell-associated mucins, the adherent gastric mucus layer, the mixture of sloughed-off gastric mucins and swallowed saliva in

the gastric juice in the lumen of the stomach is likely to influence the ability of the bacteria to colonize this region.

Acknowledgements

Studies in the authors' laboratories are supported by Vetenskapsrådet (SKL), Cancerfonden (SKL), the Mucosal Immunobiology and Vaccine Center (SKL), Science Foundation Ireland (APM) and the EU Marie Curie Programme (APM).

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13 Helicobacteromics – Genomics of a Highly Variable, Well-adapted and Persistent Pathogen

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13.1 From the Landmarks of *Helicobacter pylori* Research to ‘Helicobacteromics’

Helicobacter pylori-related diseases represent a common problem that many individuals encounter during their lifetime. The discovery of *H. pylori* led to a major breakthrough in gastroenterology. While reports on spiral bacteria in the stomach date back to the 19th century (Bizzozzero, 1893; Konjetzny, 1923), many severe inflammatory diseases of the upper intestinal tract remained mostly incurable before the discovery of *H. pylori* by Marshall and Warren (<http://nobelprize.org>; Marshall and Warren, 1984; Marshall *et al.*, 1985). The corresponding novel and revolutionary insights offered the possibility to treat gastric diseases by antimicrobial therapy and provided a novel understanding of mechanisms underlying gastritis, ulcerations, gastric cancer and lymphoma (Suerbaum and Michetti, 2002; Ahmed and Sechi, 2005; Cover and Blaser, 2009).

In the last decade, scientific investigation of *H. pylori* and infection-related diseases was greatly advanced by the release of genome sequences of several *H. pylori* strains (Tomb *et al.*, 1997; Alm *et al.*, 1999; Baltrus *et al.*, 2009b; <http://www.genomesonline.org>), which opened the way to study and compare complete gene contents of individual strains. The results of such studies demonstrate a high

degree of overall genetic instability (micro-evolution) within individual hosts. The rapid and ongoing process of functional and comparative genomics has revealed novel aspects of microbial biology and evolution, as well as of pathogenicity and gastric adaptation. In this chapter on ‘Helicobacteromics’, we focus on genomic and proteomic aspects of *H. pylori* and highlight the role of this novel information in the study of host-adaptation strategies and pathogen evolution (reviewed in de Reuse and Bereswill, 2007). Population genomics of *H. pylori* strains from different locations worldwide have metamorphosed epidemiology and provided astonishing information on the mechanisms that affect host adaptation and persistence (reviewed by Linz and Schuster, 2007). In addition, Helicobacteromics has also shed new light on the forces that shape the evolutionary history of bacterial pathogenesis and virulence acquisition, in some cases through co-evolution with the host. Even more spectacular is the genome information obtained from *H. pylori* strains worldwide that has been used to successfully retrace the migration of ancient human populations. More generally, multiple genomic sequences provide insights into the evolutionary processes that have shaped the *H. pylori* genome evolution and generated its unusual genetic instability. Analyses of genome plasticity and gene pools have led to

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the definition of the core genome (genes in common to all sequenced strains) and the pan genome (the sum of the core and of dispensable genes shared by all sequenced strains). The overwhelming quantity of novel genetic information could not have resulted in answers to biologically relevant questions without a concomitant revolution in the development of bioinformatic approaches and high-throughput experimental technologies (functional genomics). This chapter also covers these different aspects and novel trends in Helicobacteromics.

By taking individual aspects of different disease manifestations as examples, we discuss the evolutionary forces that accompany the complex host interactions in the light of bacterial ecology. Most important frameworks of host adaptation are illustrated by the specificity for the human host and the lifelong persistence of this infection. Other paragraphs summarize how *H. pylori* has evolved through several mechanisms, with horizontal gene transfer and DNA recombination playing a major role. The resulting genetic drift has equipped *H. pylori* strains with different pathogenic potential and adaptation strategies. Some have acquired the capacity to rapidly adapt to changing environments in order to enhance the spectrum of sites within the stomach that can be infected. In conclusion, the combination of topics dealing with pathogenesis and evolution provides the reader with a global view of current and future trends in Helicobacteromics. To complete insights into this research focus, we discuss possible mechanisms involved in colonization of the restricted and hostile natural reservoir (reviewed by Kusters *et al.*, 2006) including inter-species transmission, genetic diversity and regulatory pathways.

13.2 Comparative Genomics in *H. pylori* Research

13.2.1 Overall genetic variability – a key to host specificity and gastric adaptation

In the gastric mucosa, *H. pylori* is continuously faced with harsh conditions and a vigorous immune response. The need for

adaptation to this extreme and variable micro-environment (van Vliet *et al.*, 2001) is thought to drive the inter-strain genetic variation observed in the *H. pylori* population worldwide. Point mutations, insertions and deletions of genes (Blaser, 1994) and intergenic regions (Bereswill *et al.*, 2000a) present in all *H. pylori* isolates provide flexible mechanisms to subvert host immunity (Cooke *et al.*, 2005) and to adapt to unfavourable conditions (van Vliet *et al.*, 2001). The genome displays a high degree of plasticity (Jiang *et al.*, 1996; reviewed in Baltrus *et al.*, 2009a). The inter-strain diversity of *H. pylori* is extended by plasmids (Hofreuter and Haas, 2002). Genetic recombination is promoted by active DNA import (Karnholz *et al.*, 2006), which is a driving force of environmental adaptation (Baltrus *et al.*, 2008). However, detailed molecular mechanisms mediating genetic variability remain to be elucidated and are currently being intensively investigated (reviewed in Baltrus *et al.*, 2009a). As *H. pylori* encounters multiple forms of stress in the gastric environment that promote DNA damage, genome-wide analysis of the presence or absence of orthologues of DNA recombination and repair enzymes may provide clues towards understanding the microbial diversity related to host and niche. A recent study reported that *H. pylori* carries all biochemical functions that are associated with genome dynamics, namely DNA recombination and horizontal gene transfer, but lacks some of the most important enzymes involved in post-replication and DNA repair (Ambur *et al.*, 2009). However, an understanding of the precise molecular functions of the enzymes participating in DNA metabolism and the possible future role for these enzymes as targets for therapeutic intervention await detailed investigations.

The release of the genome sequences of *H. pylori* strains 26695 and J99 (Tomb *et al.*, 1997; Alm *et al.*, 1999) offered the basis for the first investigation of genomic instability and macro-evolution. At that time, about one-third of the approximately 1600 genes predicted in the 1.6 Mbp genome were considered to be *H. pylori*-specific due to the absence of homologues in other organisms (Marais *et al.*, 1999; Boneca *et al.*, 2003). The fact that even these two genomes displayed a high degree of diversity in relation

to insertions, deletions, overall genome structure and plasticity led to the proposal that the *H. pylori* population represents a 'quasi-species' (Covacci and Rappuoli, 1998) with a panmictic structure due to free recombination (Suerbaum *et al.*, 1998; Suerbaum and Achtman, 2004). The repertoire of mechanisms by which *H. pylori* can generate genetic variability is completed by sequence changes affecting phase-variable genes in which mutations can 'turn on' or 'shut off' gene expression (De Vries *et al.*, 2001). Comprehensive analysis of homopolymeric tracts and dinucleotide repeats in phase-variable gene subsets deduced from strains 26695 and J99 has revealed nucleotide variations that can be present or absent in these strains (Salaun *et al.*, 2004). A recent global genomic investigation of single-sequence repeats (SSR) in pathogenic bacteria revealed that *H. pylori* encodes a number of SSR-associated proteins (Guo and Mrázek, 2008). The SSR-associated genes encode an ATP-binding protein (*mvp*), a rod shape-determining protein (*mreB*), a hypothetical protein (HP0059) and the cytotoxin-associated genes (*cag*) product, CagA. The authors conclude that location of longer SSRs near genes encoding membrane proteins and virulence genes is consistent with their possible role in antigenic variation.

The fact that 6–7% of genes in the first two sequenced *H. pylori* genomes were strain-specific (Alm *et al.*, 1999) provided the first evidence for the presence of a variable gene pool, which is indicative of horizontal gene transfer between *H. pylori* strains. Because these strain-specific genes could be involved in gastric adaptation during co-evolution, this flexible gene pool has been extensively investigated in many strains worldwide. *In silico* analysis of the two *H. pylori* genomes (Garcia-Vallve *et al.*, 2002; Saunders *et al.*, 2005) revealed that both housekeeping genes and virulence genes are transferred among *H. pylori* strains. Divergent codon usage and GC content provided evidence that 69 genes were acquired from other species. Genetic variability and DNA exchange among *H. pylori* strains was further studied by microarray analysis. The results demonstrated that the gene content of *H. pylori* isolates from the same (Israel *et al.*, 2001) and different (Salama *et al.*, 2000) individuals displayed between 3 and 22% varia-

bility, respectively. Based on global gene distribution in 15 unrelated *H. pylori* strains obtained from different geographical origins (Salama *et al.*, 2000), the core genome has been estimated to consist of 1280 genes, including those coding for central (housekeeping), conserved and essential functions (Salama *et al.*, 2000). More than 300 genes are not homogeneously distributed and many of the dispensable genes are located in the so-called 'plasticity zones' and in the *cag* pathogenicity island (PAI). Other variable genes are involved in the synthesis of surface structures and in DNA modification or transposition. It is striking that many genes in the flexible pool encode *H. pylori*-specific proteins or conserved proteins of as yet unknown functions.

The extreme host specificity of *H. pylori* offers the possibility to use genome comparisons of related bacterial species to identify the genes responsible for adaptation to the human host. The availability of genomes of closely related species such as *Helicobacter hepaticus* (Suerbaum *et al.*, 2003), *Campylobacter jejuni* (Parkhill *et al.*, 2000) and *Wolinella succinogenes* (Baar *et al.*, 2003) offered the opportunity to extend our knowledge regarding *H. pylori* evolution (Eppinger *et al.*, 2004). A comprehensive analysis of the genomes available from these four different *Campylobacteriales* species revealed that *H. pylori* and two of these other host-adapted pathogens have lost regulatory and environmental sensing circuits, while in the non-pathogenic commensal *W. succinogenes* (Eppinger *et al.*, 2004) these are still conserved. The finding that *Wolinella* also contains more complete metabolic pathways supports the conclusion that the *Helicobacter* species analysed so far fulfil the paradigm of reductive evolution (as a consequence of host adaptation). In addition, comparison of the *Campylobacteriales* genomes with the genetic information of the sequence databases has allowed the identification of genes and molecular signatures that are unique to members of the epsilon proteobacteria (Gupta, 2006). Whole-genome clustering of *H. pylori* and *C. jejuni* has demonstrated that 648 *H. pylori* genes are species-specific (Janssen *et al.*, 2001). The fact that 95% of the 162 *H. pylori* genes displaying inter-strain variability are absent in other bacteria

indicates that genes of the flexible gene pool are exchanged among *Helicobacter* species, but are not transferred to bacteria of other genera.

Based on recent genome comparisons of *H. pylori* and *Helicobacter acinonychis*, which colonizes felines, it was postulated that a host jump occurred about 200,000 years ago, at which time *H. pylori* was transferred from humans to cats (Eppinger *et al.*, 2006). Extended microarray analysis of the gene distribution and multilocus sequence typing (MLST) of seven core genes of 56 *H. pylori* strains (Gressmann *et al.*, 2005) revealed that 25% of genes that are common to both 26695 and J99 were missing in at least one isolate, and that 21% of genes were absent or variable in *H. acinonychis*. In addition, the authors of that study concluded that there was a core genome of 1111 genes and predicted that the *cagPAI* genes were acquired 'en bloc' after speciation. Variable genes are small, possess unusual GC content and encode mostly proteins of unknown function or outer membrane proteins. Many proteins of unknown function and transposases were predicted to have been acquired prior to speciation (Gressmann *et al.*, 2005). The fascinating insights and novel hypotheses provided by genome-wide analysis of *H. pylori* are continuously further expanded and verified by the ongoing release of novel genome sequences of additional *H. pylori* strains (Baltrus *et al.*, 2009b) and closely related *Helicobacter* species, such as *Helicobacter canadensis* (Loman *et al.*, 2009). A complete and actual listing is available on the Internet (<http://www.genomesonline.org>).

13.2.2 Diversification of *H. pylori* strains in individual hosts

Changes in outer membrane proteins may represent a dynamic response in the *H. pylori* surface structure that facilitates adherence to the gastric epithelium in individual human hosts, as well as promoting chronic infection. This conclusion is supported by the extensive genotypic diversity displayed by individual human *H. pylori* isolates, as well as within strains colonizing individual patients at

different time points, independent of disease development. As gene loss or gain occurring in a single host might play an important role in gastric adaptation, the resulting microevolution is a driving force for genetic diversity of *H. pylori*. The gene content of *H. pylori* strains isolated sequentially from single patients during persistent infection has confirmed that gene loss and acquisition of exogenous DNA occurs (Israel *et al.*, 2001). Furthermore, multilocus sequence analysis of ten genes in paired *H. pylori* isolates from 26 different individuals (Falush *et al.*, 2001) has shown that point mutations occur in the stomach of a single host, and that mostly small mosaic DNA segments with a median size of 417 bp are exchanged. Calculations of mutation and recombination frequencies with respect to insert sizes revealed that genetic diversity displayed by the panmictic population structure is a result of continuous DNA exchange between parental strains (without mutations) and daughter strains, which have accumulated mutations. This finding was supported by gene content analysis of isolates taken from single patients at different time points, which demonstrated that the great majority of genetic changes were caused by homologous recombination, indicating that the adaptation of *H. pylori* to the host is more frequently mediated by sequence changes acquired by recombination events, rather than loss or gain of genes (Kraft *et al.*, 2006).

13.2.3 Worldwide co-evolution and spreading with the human host

The rapid progress in sequencing technologies and related *in silico* software tools for sequence analysis has paved the way for investigations focused upon the gene content and genetic diversity of *H. pylori* populations worldwide. There is strong evidence that *H. pylori* has colonized humans for more than 100,000 years (Covacci *et al.*, 1999) and that there is a significant correlation between *H. pylori* genotypes and prehistoric human migrations (reviewed in Suerbaum and Achtman, 2004; Linz and Schuster, 2007; Suerbaum and Josenhans, 2007; Moodley and Linz, 2009). MLST analysis of selected core

genes in *H. pylori* isolates from ethnic subpopulations of people worldwide has established that the global *H. pylori* population can be subdivided into seven genetically distinct subpopulations, which derived their gene pools from ancestral populations that arose in Africa, Central Asia and East Asia (Falush *et al.*, 2003a). The optimization of the mathematical basis for the use of multilocus genotype data (Falush *et al.*, 2003b) has revealed close associations between *H. pylori* subtypes and human subpopulations in one continent or even ethnic subgroups within small geographic regions (Wirth *et al.*, 2004). Thus, *H. pylori* subtypes can be used to trace human migrations during history, and the spreading of distinct *H. pylori* subtypes could be attributed to prehistoric and modern migratory fluxes (Suerbaum and Achtman, 2004; Linz *et al.*, 2007). Our view of the speciation and evolution biology of *H. pylori* has been further expanded by analysis of isolates from human subpopulations and ethnic subgroups in India (Devi *et al.*, 2007), South-east Asia, Australia and the Pacific (Moodley *et al.*, 2009). The genotyping of *H. pylori* isolates in India revealed that the Indian and European *H. pylori* isolates share a common ancestral origin (Devi *et al.*, 2007). Moreover, the newly discovered *H. pylori* genotypes allowed the differentiation of human migrations to New Guinea, Australia and the Polynesian islands (Moodley *et al.*, 2009). These results verified again that distinct local *H. pylori* subpopulations are shaped by complex human migrations and the mixing or separation of local ethnic subpopulations. In another study, *H. pylori* isolates from Malaysia could be assigned to previously identified *H. pylori* ancestral populations, hpEastAsia, hpAsia2 and hpEurope (Tay *et al.*, 2009). Most interestingly, the results allowed the identification of a novel *H. pylori* subpopulation, hspIndia, within hpAsia2. Sequence comparisons with Chinese and Indian isolates revealed that the majority of the Malay and Indian *H. pylori* isolates share the same origin, while the Malaysian and Chinese *H. pylori* populations are distinctive. This suggests that the Malay population gained the pathogen only recently by ethnological mixing with other subpopulations (Tay *et al.*, 2009).

13.2.4 Horizontal gene transfer – the driving force of strain diversity?

Lifelong persistence and intra-familial transmission of *H. pylori* support the view that genetic material is continuously exchanged among different generations of the bacterium via horizontal gene transfer. The importance of horizontal gene transfer in the variability of *H. pylori* has been underlined by the finding that clonal lineages of *H. pylori* populations coexist in individuals and evolve separately from one another (Ahmed and Sechi, 2005). Continuous horizontal gene transfer between strains of different generations is thought to be causative for: (i) genome plasticity (Baltrus *et al.*, 2009a); (ii) evolution of distinct sets of *H. pylori* genotypes in individual human hosts and populations; (iii) the completely diverse and panmictic genetic population structure; and (iv) the association of defined *H. pylori* genotypes with particular geographic regions. The definitive coexistence of distinct sequence variants, and transfer from one *H. pylori* population in a given local area to another *H. pylori* population in another geographic region via human migration, is clearly driven by the high potential of *H. pylori* for DNA uptake and recombination. However, this makes the association of particular genotypes with specific geographic locations even more difficult. Hence, while polymorphisms within the *H. pylori* genome can serve as useful markers for studying ancient human migrations, these types of analyses are certainly complicated by the mixing of *H. pylori* strains from migrated and native populations (Ahmed and Sechi, 2005).

13.2.5 *H. pylori* microevolution and disease progression

The identification of flexible genome areas, plasticity zones, operons and single genes associated with severe pathologies represents a major and ongoing challenge in *H. pylori* research. Molecular analyses during the pre-genomic era established that the sequences of virulence-associated genes coding for the vacuolating cytotoxin VacA (Atherton *et al.*,

1995) and the CagA protein (Blaser, 1994) varied considerably among strains. In addition, at that time, specific genotypes of VacA were associated with ulcer development (Atherton *et al.*, 1995) and the presence of the *cagPAI* promoting injection of the CagA protein into host cells correlated with pronounced inflammation and more severe pathologies (see Backert *et al.*, Chapter 11, this volume). The rhesus macaque model has greatly contributed to the elucidation of genomic changes in *H. pylori* that occur early during experimental infection (Solnick *et al.*, 2004). Microarray analysis demonstrated that *H. pylori* recovered from infected macaques carried deletions in the locus coding for BabA, an adhesin that mediates attachment of *H. pylori* to gastric epithelia. In some isolates the *babA* gene was not expressed or was replaced by *babB*, which encodes a related protein. The absence of *babA* and duplication of *babB* has also been observed in *H. pylori* isolates derived from human clinical samples, suggesting that this gene conversion is of relevance to the human host and might reflect diverging selective pressures for adhesion either across hosts or within an individual (Colbeck *et al.*, 2006).

Persistent colonization of the human stomach is associated with asymptomatic gastric inflammation (gastritis) and an increased risk of duodenal ulceration, gastric ulceration and non-cardia gastric cancer. Results from genome analysis of *H. pylori* isolates from patients suffering from different diseases (for a review, see Linz and Schuster, 2007) suggest that the genome content accounts for the development of different pathologies (Romo-González *et al.*, 2009). The comparative investigation of genome sequences revealed that *H. pylori* strains isolated from patients with gastric cancer or gastric ulcer disease are most closely related to strains of East Asian or European origin, respectively (McClain *et al.*, 2009). Among the core genes determined, a subset of alleles was found to be highly divergent in the East Asian strain, encoding proteins that exhibited <90% amino acid sequence identity compared with corresponding proteins in the strains of other geographic origin. Unique strain-specific genes were identified in each of the newly sequenced strains, and a set of strain-

specific genes was shared among *H. pylori* strains associated with gastric cancer or malignant processes.

Although *H. pylori* causes an initial acute superficial gastritis which over time develops into a chronic gastritis, in some infected individuals this will progress to chronic atrophic gastritis (ChAG). This pathology is characterized by diminished numbers of acid-producing parietal cells and an increased risk for the development of gastric adenocarcinoma. The complete genome analysis of the first *H. pylori* isolate from a patient with ChAG gave further insights into gene sets that could be involved in causing this specific disease outcome (Oh *et al.*, 2006). Whole-genome analysis of additional *H. pylori* isolates collected from an individual who progressed from ChAG to gastric adenocarcinoma revealed a gene signature shared among ChAG strains, as well as genes that may have been lost or gained during progression to adenocarcinoma. Many of these genes encode components of metal uptake and utilization pathways, outer membrane proteins and virulence factors, indicating that the bacteria adapt effectively to environmental changes during ChAG disease progression. Taken together, these data provide insight into the diversity that exists among *H. pylori* strains from different clinical manifestations. Highly divergent alleles and strain-specific genes identified in this study may represent useful biomarkers for analysing geographic partitioning of *H. pylori* and for identifying strains capable of inducing malignant or premalignant gastric lesions.

13.2.6 Novel proteomics tools identify biomarkers of disease progression

More recently, proteomic tools have been used for the identification of novel diagnostic *H. pylori* biomarkers for predicting disease progression (Wu *et al.*, 2008). The current disease paradigm suggests that the final outcome of *H. pylori* infection is strongly dependent on host genetics and bacterial virulence, both playing important roles in modulating disease development. By using high-throughput platforms, the authors

identified several crucial proteins that have pathogenic and prognostic potential. For example, antibodies to alkaline hydroperoxide reductase and the co-chaperonin GroES of *H. pylori* could be utilized for identifying patients at high risk of disease complications after *H. pylori* infection. In addition, 'immuno-proteomics' has provided global information about antigens relevant in different disease states, and thus might be suitable for identifying novel vaccine candidates or serological markers. For example, the humoral immune response to *H. pylori* was used for diagnostic purposes and as a basis for vaccine development (reviewed by Bernardini *et al.*, 2007). In another study, the analysis of protein patterns of *H. pylori* strains isolated from Korean and Colombian patients suffering from duodenal ulcer or gastric cancer by high-throughput surface-enhanced laser desorption/ionization time-of-flight mass spectrometry identified 18 statistically significant candidate biomarkers discriminating between the two clinical outcomes (Khoder *et al.*, 2009). Three biomarker proteins were purified and identified as the neutrophil-activating protein NapA, an RNA-binding protein and a DNA-binding histone-like protein. These novel biomarkers might be of use for predicting the evolution to gastric cancer in *H. pylori*-infected patients.

Such proteomic technologies are predicted to facilitate biomarker identification that in the future may have the potential to provide antigens for use in vaccines for preventing *H. pylori* infection. Together with appropriate clinical phenotyping and genotyping, this information will clearly enhance our understanding of disease progression/pathogenesis and lead to more precise prediction of variable disease outcomes.

13.3 Functional Genomics to Study the Adaptation of *H. pylori* to the Gastric Environment: Transcriptome Studies

The stomach is the unique niche colonized by *H. pylori*, where it proliferates without competition, as no other microbe is able to permanently inhabit this hostile environment. To

multiply and persist in the gastric environment, *H. pylori* has developed specific molecular strategies and factors to deal with adverse and unstable environmental conditions such as acidity, fluctuating nutrient availability, changes in oxygen tension and the intense immune response of the host. Despite these variable environments, *H. pylori* is a particularly successful pathogen, being able to proliferate in its host over many decades. In addition, there is a link between the *H. pylori* adaptive response and its pathogenicity, as illustrated by the roles of the strongly regulated urease virulence factor. In bacteria, transcriptional regulation is an initial and rapid response to changing environments and stresses. Since two complete genome sequences of *H. pylori* were already available at the end of the 20th century, profiling of gene expression using DNA arrays was the approach of choice to investigate the regulons and the stimulons of this pathogen. A regulon represents the group of genes regulated by a given transcriptional regulator and a stimulon, a set of genes that are transcriptionally regulated in response to a perturbation of the environment. Accordingly, genome-wide transcriptional profiling using a DNA array aims to define the changes in the transcriptome of an organism either exposed to a modification of its environment/growth conditions or that carries a mutation. Numerous transcriptomic studies have been carried out for *H. pylori* (Table 13.1) leading to the conclusion that *H. pylori* has a unique and particularly complex adaptation potential through modulation of gene expression. In the following sections we discuss mainly the currently known functions regulated under these conditions; however, this is not to say that a large number of other as yet unknown genes are not also regulated.

13.3.1 Transcriptomics to characterize the *H. pylori* stimulons *in vitro*

In the case of *H. pylori*, a major effort has concentrated on examining its response to conditions mimicking those encountered during host colonization.

Table 13.1. Studies using whole-genome transcriptional profiling of *Helicobacter pylori*.

Condition or protein factor tested	Experimental procedure	Strain(s) used	Total number of regulated genes	Reference
<i>H. pylori</i> transcriptome in conditions mimicking those encountered in the human host				
In response to acidity	Growth on plates (48 h) at pH 5.5 versus pH 7	26695	84	Ang <i>et al.</i> (2001)
	Exposure to pH 4 versus pH 7 for 30 min	26695	11	Allan <i>et al.</i> (2001)
	Exposure to pH 5 of a liquid culture grown at pH 7. Time course from 0.5 to 2 h	G27	118	Merrell <i>et al.</i> (2003a)
	Exposure to pH 4.5, 5.5, 6.2, 7.4 with and without 5 mM urea for 30 min	26695	300	Wen <i>et al.</i> (2003)
	Exponential growth in liquid medium at pH 5 and pH 7	26695	101	Bury-Moné <i>et al.</i> (2004)
As a function of growth phase	Growth in liquid medium from exponential to stationary phase. Time course from 0 to 50 h	SS1	325	Thompson <i>et al.</i> (2003)
In response to iron starvation	Iron chelation or add-back of bacteria in exponential and stationary phase. Time course from 0 to 100 min	SS1	183	Merrell <i>et al.</i> (2003b)
In response to AGS cell attachment	Bacteria attached to AGS cells over a 4 h period versus in the same conditions without cells	69a	43	Kim <i>et al.</i> (2004)
In the gerbil stomach	Colonization during 10 days versus <i>in vitro</i> growth	69a	Not provided	Scott <i>et al.</i> (2007)
<i>H. pylori</i> transcriptome in mutants				
NikR ^a	WT versus $\Delta nikR$ mutant, in the presence of nickel	SS1	42	Contreras <i>et al.</i> (2003)
Fur ^b	WT versus Δfur mutant in iron-restricted or iron-replete conditions grown for 20 h	26695	97 iron-responsive regulation 43 Fur-dependent regulation	Ernst <i>et al.</i> (2005a)
Fur ^b	WT versus Δfur mutant, exponential and stationary growth phase	G27	29	Gancz <i>et al.</i> (2006)
Fur ^b	WT versus Δfur mutant, exponential and stationary growth phase	G27	26 in exponential phase 90 in stationary phase	Danielli <i>et al.</i> (2006)
ArsRS ^c (HP166–HP165)	WT versus a HP165 mutant	26695 and B128	7	Forsyth <i>et al.</i> (2002)
σ^{28} , FlgM ^d	WT versus mutants	N6	NA	Josenhans <i>et al.</i> (2002)

σ^{54} , FlgRS, FlhF, FlhA ^d	WT versus mutants and mutants versus mutants	N6 and 88-3887 (a motile variant of 26695)	NA	Niehus <i>et al.</i> (2004)
HP1021 ^e	WT versus mutant	26695	79	Pflock <i>et al.</i> (2007a)
HP0958 ^d	WT versus mutant	NTCC 17874	44	Douillard <i>et al.</i> (2008)
FliK ^d (HP0906)	WT versus mutant	NTCC 17874	54	Douillard <i>et al.</i> (2009)
HrcA, HspR and HrcA-HspR ^f	WT versus single and double mutants	G27	NA	Roncarati <i>et al.</i> (2007)
<i>H. pylori</i> transcriptome in mutants in response to acidity				
Fur ^b	WT versus Δfur mutant, exposure of a liquid culture grown at pH 7 to pH 5. Time course from 0.5 to 1.5 h	G27	95	Gancz <i>et al.</i> (2006)
NikR ^a and Fur ^b	$\Delta nikR$ - Δfur mutant during growth at pH 5 versus pH 7	26695	36	Bury-Moné <i>et al.</i> (2004)
ArsRS ^c (HP166–HP165)	WT versus $\Delta arsS$ mutant, exposure to pH 5 for 1 h	G27	109	Pflock <i>et al.</i> (2006a)
ArsRS ^c (HP166–HP165)	WT versus <i>arsS</i> mutant, exposure to pH 5 for 75 min	J99	68 on 101 considered genes	Loh and Cover (2006)
CrdRS ^g (HP1364)	WT versus a <i>crdS</i> mutant, exposure to pH 5 for 75 min	J99	63 on 101 considered genes	Loh and Cover (2006)
FlgS ^d (HP0244)	WT versus a <i>flgS</i> mutant, exposure to pH 7.4, 4.5 and 2.5 with 10 mM urea	26695	NA	Wen <i>et al.</i> (2009)

WT, wild type; NA, not applicable.

^aNikR (HP1338): nickel-responsive regulator.

^bFur (HP1027): iron-responsive regulator.

^cArsR-S (HP166–HP165): two-component system involved in acid-responsive signalling. ArsR: response regulator, ArsS: sensor histidine kinase.

^d σ^{28} (FlhA, HP1032), FlgM (anti- σ^{28} , HP1122), σ^{54} (RpoN, HP0714), FlgRS (HP0703-HP0244), FlhF (HP1035), FlhA (HP1041), HP0958, FliK (HP0906): regulators of the flagellar genes expression or components of the flagellar machinery.

^eHP1021: orphan response regulator.

^fHrcA (HP0111), HspR (HP1025): heat shock transcriptional regulators.

^gCrdRS (HP1365–HP1364): two-component system involved in regulation of copper resistance determinant. CrdR: response regulator, CrdS: sensor histidine kinase.

Response to acidity

One of the most remarkable properties of *H. pylori* is its unusual capacity to persistently colonize an extremely acidic niche. While this gastric pathogen is able to survive acidity in the stomach lumen in the initial phase of infection (median pH of 1.4), persistent colonization mainly establishes within the mucus layer where a less acidic pH prevails (Schreiber *et al.*, 2004). Adhesion to the epithelial cells at a pH close to neutrality only occurs in about 20% of the *H. pylori* population and this process is thought to be dynamic, with bacteria continually attaching to and detaching from the epithelial cell layer. In this environment, *H. pylori* is frequently exposed to a fluctuating pH to which it needs to adapt rapidly in an appropriate and well-coordinated way. As a consequence, it became rapidly clear that the response of *H. pylori* to pH is much more complicated than that of other urease-negative gastrointestinal pathogens, such as *Escherichia coli* or *Salmonella enteritidis*.

Given this, low pH was the first environmental condition to be extensively investigated by transcriptional profiling of *H. pylori* (Allan *et al.*, 2001; Ang *et al.*, 2001; Merrell *et al.*, 2003a; Wen *et al.*, 2003; Bury-Moné *et al.*, 2004; Table 13.1). The poor overlap between the results of these five transcriptomic studies in both the nature and the total number of regulated genes is probably related to the variety of experimental setups with shorter or longer exposure times to different acidic pH values, different culture media supplemented or not with urea, and also different *H. pylori* strains (Table 13.1). Strikingly, two studies (Merrell *et al.*, 2003a; Bury-Moné *et al.*, 2004) – although performed under different conditions – reported a similar number of acid-regulated genes (118 and 101), of which 22% were in common. Despite the differences, some common features can be extracted from these studies. First, these results clearly indicate that *H. pylori* modifies gene expression at low pH in order to induce mechanisms of protection against protons. It was previously established that ammonia production is the major strategy for acid resistance in *H. pylori*, mainly by its highly active and abundant urease enzyme that hydrolyses urea to produce

ammonia and bicarbonate. Accordingly, most studies observed acid-induced expression of *H. pylori* urease operons and of two other ammonia-producing enzymes, the AmiE amidase and AmiF formamidase, that we previously characterized (Skouloubris *et al.*, 1997, 2001). Furthermore, an acid protection strategy of *H. pylori* is suggested by the repeated observation of down-regulation in the expression of membrane proteins including transporters, permeases and outer membrane proteins. A common theme is also the enhanced expression of genes related to motility, including structural proteins of the flagellar apparatus and motor-related proteins. Stimulation of *H. pylori* motility by acid was experimentally demonstrated (Merrell *et al.*, 2003a) and might be indicative of a strategy to escape acidity or of a pH-driven response directing the bacteria through the mucus pH gradient to a suitable site for multiplication or adhesion, as previously suggested (Schreiber *et al.*, 2004). Two studies have indicated reduced expression of the VacA cytotoxin and the SabA adhesin at acidic as compared with neutral pH (Merrell *et al.*, 2003a; Bury-Moné *et al.*, 2004). This regulation, confirmed at the protein level for VacA using comparative two-dimensional gel electrophoresis (2-D GE) (Jungblut *et al.*, 2000), is thought to optimize the production of virulence factors in the neutral pH environment found near the gastric epithelial cells.

Moreover, our investigations of the response of *H. pylori* to exponential growth at pH 5 have unravelled a novel category of acid-regulated genes, encoding proteins related to metal metabolism or regulated by metal-ion availability (Bury-Moné *et al.*, 2004). Solubility of nickel, iron and other metal ions is known to be strongly enhanced by acid, and the role of the Fur iron-responsive regulator in the *H. pylori* acid resistance had already been reported (Bijlsma *et al.*, 2002). In addition, urease activity depends on a nickel cofactor, underlining that acid resistance of *H. pylori* is also tightly linked to nickel metabolism. As a protective mechanism against the toxic side-effects of metal ions, we found enhanced expression of metal storage proteins and diminished expression of metal-ion transport systems under acidic conditions (Bury-Moné *et al.*, 2004).

Finally, these transcriptome studies also revealed three major regulators of the acid response network in *H. pylori* that have been further examined: the two metal regulators Fur and NikR, responsive to iron and to nickel, respectively (van Vliet *et al.*, 2003; Bury-Moné *et al.*, 2004), and the HP165–HP166 genes that encode a two-component regulatory system (TCS) (Bury-Moné *et al.*, 2004; Wen *et al.*, 2003, 2006) designated ArsRS and further analysed by the group of D. Beier (Pflock *et al.*, 2004, 2005) (see below).

Growth phase-dependent regulation

The growth phase-regulated genes of *H. pylori* have been identified in a time-course transcriptome study (Thompson *et al.*, 2003). A major switch in gene expression was observed during the transition between late logarithmic and stationary phase, with the expression of several genes related to virulence being modified (Thompson *et al.*, 2003). During this switch, genes encoding CagA, the NapA bacterioferritin and the FlaA flagellin were induced, while genes encoding proteins involved in iron homeostasis were regulated as if the bacteria were submitted to iron overload. The regulatory mechanisms governing the entry into stationary phase remain a black box in the case of *H. pylori*, particularly since there is no known homologue of the alternative sigma factor RpoS that is central to this switch in *E. coli*. HrcA, a heat-shock regulator found to be up-regulated during the logarithmic–stationary switch, might be a potential first candidate to play a role in the growth phase switch (Thompson *et al.*, 2003).

Response to iron availability

During persistent stomach colonization and as a function of diet, *H. pylori* experiences periods of both iron overload and starvation. In addition, low pH will render iron more soluble. The human host sequesters this iron to prevent oxidative stress and to limit bacterial proliferation. Indeed, while iron is essential for several bacterial functions, it is toxic if its intracellular concentration becomes too high. Thus, iron homeostasis consists of a fine-tuned balance between uptake, efflux,

utilization and storage. A time-course transcriptomic study was performed in response to iron starvation in both the exponential and stationary phase (Merrell *et al.*, 2003b). Of the 183 differentially regulated genes, only 30 were common between the two growth phases. The observation of a phase-dependent expression of the global ferric uptake regulator protein Fur, with enhanced production in the stationary phase (Danielli *et al.*, 2006), might partially explain this observation. Regulated genes comprised those involved in iron sequestration, storage and transport. Among them was *pfr*, which encodes ferritin, previously shown to be repressed by the Fur regulator (Bereswill *et al.*, 1998) in the absence of iron (Bereswill *et al.*, 2000b). This unusual function of Fur has been independently confirmed and the binding site of iron-free Fur on the *pfr* gene promoter has subsequently been identified (Delany *et al.*, 2002). In contrast, in the study of Merrell *et al.* (2003b), the *H. pylori* iron-binding protein neutrophil-activating protein (NAP) and the putative iron outer membrane transporters FrpB1 and FecA1/2/3 were induced in the presence of Fur and iron. In addition, a number of virulence factors were affected as a function of iron. Both VacA and NapA were induced by iron starvation and motility was affected in a complex growth phase-dependent manner. The two amidase genes (*amiE* and *amiF*) were induced by iron starvation in both growth phases, while a weaker induction of the *ureAB* genes was measured only in the stationary phase (Merrell *et al.*, 2003b).

Response to epithelial cell attachment and to colonization in an animal model

To gain insight into the *H. pylori* response to adhesion to gastric epithelial cells, Kim *et al.* (2004) compared the global gene expression of *H. pylori* after attachment to AGS gastric cancer cells with that of bacteria incubated in the cell culture medium. Only 43 genes presented differential expression, including the down-regulation of genes related to motility that was compatible with close adhesion.

In an attempt to directly appraise the response of *H. pylori* to infection, the transcriptome of *H. pylori* after infection of Mongolian

gerbils for 10 days was compared with that of *H. pylori* grown *in vitro* at pH 7.4 (Scott *et al.*, 2007). From a considerable number of genes presenting differential expression, some conclusions could be drawn. As expected for an acidic niche, up-regulation of many acid acclimatization genes was measured, including the urease genes, *rocF* coding for arginase, *hp1186* encoding the α -carbonic anhydrase (Marcus *et al.*, 2005; Bury-Moné *et al.*, 2008) and those of the two amidases *amiE* and *amiF*. Transcription of two groups of motility and chemotaxis genes was also enhanced, as well as nine genes of the *cagPAI* including *CagA*. Strikingly, several of these genes were under the control of the ArsRS acid-responsive regulator system. This type of study is crucial to understand the behaviour of a pathogen in its host, although it remains technically challenging as only small amounts of RNA can be recovered from *in vivo*-infecting bacteria.

13.3.2 Transcriptomics to characterize *H. pylori* regulons using mutant strains

Results from whole-genome expression profiling have led to novel hypotheses on processes or mechanisms that are switched on in response to environmental changes. Generally, such switches are mediated by transcriptional regulators that act on multiple targets in a hierarchically organized way. All the genes controlled by a given regulator constitute its regulon, which can be defined by comparing the transcriptome of a wild-type strain with that of an isogenic mutant deficient in the regulator of interest, with or without an environmental stimulus.

H. pylori has a very limited number of global regulators including sigma factors with the housekeeping σ^{80} , and two alternative sigma factors, σ^{54} (RpoN) and σ^{28} (FliA). It also possesses few transcriptional regulators, including the NikR and Fur metalloregulators that respond to nickel and iron, respectively, the carbon storage regulator CsrA, and two heat-shock regulators HspR and HrcA. In comparison to other bacteria, the number of TCSs is also dramatically reduced in *H. pylori*. Most enterobacteria possess more than 30 such

systems. The TCSs are specialized in translating environmental stimuli into transcriptional regulation and usually comprise a histidine kinase sensor and a response regulator. Apart from two orphan response regulators (HP1043 and HP1021), *H. pylori* possess three complete TCSs that have been well characterized: HP1365–HP1364 (CrdRS; Waidner *et al.*, 2005), HP166–HP165 (AsrRS; Pflock *et al.*, 2004, 2005, 2006a) and HP703–HP244 (FlgRS; Niehus *et al.*, 2004), involved in the response to copper ions, to acidity, and in flagellar gene expression as well as the response to acidity, respectively. A few other annotated transcriptional regulators have not yet been characterized. The low number of TCSs in *H. pylori* is proposed to be related to its unique environmental niche and small genome size, which is perhaps the result of reductive evolution in the course of co-evolution and host adaptation.

Regulation of the flagellar system

Flagellar motility is essential for colonization of the stomach by *H. pylori*. In several bacteria, the flagellar system is controlled by a complex transcriptional hierarchy acting on a large number of genes and by sophisticated feedback regulation mechanisms. In *H. pylori*, about 40 genes are related to motility. The flagellar transcriptional network has been investigated by DNA arrays using different mutants (Niehus *et al.*, 2004). The role of the two sigma factors recruited for the expression of flagellar genes, σ^{54} (RpoN) and σ^{28} (FliA) with its cognate anti-sigma FlgM (Colland *et al.*, 2001; Josenhans *et al.*, 2002), have been studied in particular detail. Transcriptome analysis showed that negative feedback regulation of the FliA regulon was dependent on FlgM, which was, in addition, involved in FlhA- but not FlhF-dependent feedback control of the RpoN regulon (Niehus *et al.*, 2004). Examination of the regulatory function of FhlA, encoding a flagellar basal body protein, revealed that it controls a large number of genes including those of FliA and RpoN regulons. In addition, analysis of the role of the FlgRS TCS supported the function of FlgR as an exclusive RpoN-associated regulator (Niehus *et al.*, 2004). These investigations emphasized the complexity of this

system and revealed more unique features of *H. pylori*, such as the σ^{54} - and σ^{28} -independent expression of chemotaxis and flagellar motor genes, as well as the absence of a true master regulator for flagellar regulation.

A recent transcriptome study (Douillard *et al.*, 2008) evaluated the role of HP0958 (a novel RpoN chaperone) using a mutant of HP0958, previously shown to have impaired motility (Ryan *et al.*, 2005). A total of 44 genes were found to be differentially expressed in the HP0958 mutant, including the majority of the RpoN-dependent flagellar genes. Most other flagellar genes were not significantly differentially expressed in the HP0958 mutant. Interestingly, HP0958 was found to post-transcriptionally regulate the *flaA* gene encoding the major flagellin protein, by a mechanism that remains to be defined (Douillard *et al.*, 2008). In addition, the effect of deleting the *fliK* gene (encoding a protein that controls the hook length during flagellar assembly) on the transcriptome of *H. pylori* was evaluated (Douillard *et al.*, 2009). Again, only transcription of the genes under control of RpoN was differentially regulated in the mutant, suggesting specific and localized FliK-dependent feedback on the RpoN regulon (Douillard *et al.*, 2009).

The Fur and NikR regulons

The role of Fur, the iron-responsive regulator, has been assessed by transcriptional profiling of both a wild-type strain (26695) and a Δfur mutant in iron-restricted and iron-replete conditions (Ernst *et al.*, 2005a). Pair-wise comparisons of these four conditions resulted in identification of complex regulatory patterns, with both genes being positively or negatively regulated by Fur bound to iron or in apo-Fur form, and also in a significant number of Fur-independent iron-regulated genes. Strikingly, out of 97 iron-responsive genes, only 43 were Fur-dependent, pointing to additional regulatory mechanisms responding to iron in *H. pylori*. Fur+iron-repressed genes comprised those involved in iron uptake and cofactor metabolism, as well as the amidases. Similar results have been reported with a Δfur mutant in another genetic background (strain G27; Gancz *et al.*, 2006). In

that study, the iron-free Fur-repressed genes comprised the oxidative stress response protein SodB and hydrogenase subunits. To clarify these complex iron and Fur regulatory patterns, and to distinguish between direct and indirect effects, it is essential to combine both individual gene expression measurements (as for SodB; Ernst *et al.*, 2005b; or for *amiE*; van Vliet *et al.* 2003) and whole-genome DNA-binding assays. One such study used a Fur chromatin immunoprecipitation (ChIP) on chip approach to globally identify the Fur-binding targets (Danielli *et al.*, 2006). Fur-IP-DNA pools were hybridized to *H. pylori* DNA arrays. Two hundred candidate targets were initially identified. Among these regions, only those also identified by transcriptome analysis of wild-type versus Δfur mutant strains were retained. This resulted in a Fur regulon consisting of 59 directly regulated genes, 25 of which were positively regulated (Danielli *et al.*, 2006).

Nickel, like iron, is a double-edged sword since it is both required and potentially toxic, if present at too high an intracellular concentration. In *H. pylori*, nickel is of particular importance as the cofactor of two enzymes essential for colonization, namely urease and hydrogenase. The regulon of the nickel-responsive regulator NikR was investigated under conditions of nickel excess by comparing the response of a wild-type strain to that of a $\Delta nikR$ mutant (Contreras *et al.*, 2003). The study revealed that *H. pylori* NikR is a pleiotropic regulator, in contrast to its homologue in *E. coli*, which only represses the NikABCDE nickel uptake system under nickel excess conditions (De Pina *et al.*, 1999). In *H. pylori*, NikR associated with nickel becomes active as an autoregulator controlling nickel uptake (NixA permease), metabolism (structural subunits of urease) and storage (Hpn, Hpn-like), thereby contributing to nickel homeostasis (Contreras *et al.*, 2003). In addition, NikR also controls the expression of stress response and flagellar genes (Contreras *et al.*, 2003). Although NikR was originally thought to be a repressor, its regulon has been shown to comprise both negatively and positively regulated genes. Direct binding of NikR to promoter regions of activated target genes such as *ureAB* confirmed its role as a nickel-

responsive activator (Delany *et al.*, 2005; Ernst *et al.*, 2005b). Finally, an overlap between the nickel and iron metabolism has been observed in which NikR not only represses the expression of the iron-responsive regulator Fur, but also that of different proteins involved in iron acquisition, including the *exbB/exbD/tonB* operon (*hp1399–1340–1341*) coding for the TonB machinery, a system energizing iron uptake through the outer membrane (Contreras *et al.*, 2003; Delany *et al.*, 2005). The physiological basis for the tight interconnection between the nickel- and iron-responsive gene networks is now better understood as a result of our recent demonstration that the *H. pylori* TonB machinery is, in addition to iron transport, also required for nickel uptake (Schauer *et al.*, 2007). This new finding was substantiated by the identification of the corresponding nickel outer membrane transporter FrpB4, whose expression is exclusively regulated by NikR (Schauer *et al.*, 2007).

Regulons of the heat-shock regulators HspR and HrcA

The global response of two previously identified heat-shock regulators of *H. pylori*, HrcA and HspR, was analysed using mutant strains deficient in one or both regulators (Roncarati *et al.*, 2007). Expression of 14 genes was found to be negatively controlled by one or by the two regulators, while a set of 29 genes was shown to be positively regulated. DNase I footprinting experiments with the promoter regions of two of the newly identified regulated genes *hp0630* and *flaB* did not show binding of HrcA or HspR, suggesting that these genes are indirect targets. Interestingly, half of the regulated genes were related to flagellar motility and were positively controlled by HrcA or HspR. This points to a possible intersection between stress response and flagellar assembly that was confirmed by the loss of motility of the HrcA and the HspR deletion mutants (Roncarati *et al.*, 2007).

Regulons of the two-component system ArsRS and of the orphan regulator HP1021

Among the few TCSs of *H. pylori*, HP165–HP166 has attracted most attention

since it was found to play a central role in the *H. pylori* adaptive response to acidity, therefore being named Ars for acid-responsive signalling (Pflock *et al.*, 2005). The importance of this system in *H. pylori* is illustrated by the fact that a mutant of the ArsS histidine kinase HP165 was unable to colonize mice (Panthel *et al.*, 2003) while the ArsR response regulator HP166 (of the OmpR family) is essential for *in vitro* growth of *H. pylori* (Beier and Frank, 2000). This suggests unusual distinct functions for ArsR in its phosphorylated and unphosphorylated forms, with the latter regulating the expression of an essential, as yet unknown gene.

Two global studies identified targets of the ArsRS system, using either a DNA magnetocapture assay with bound recombinant ArsR (Dietz *et al.*, 2002) or DNA arrays with a strain deficient in ArsS (Forsyth *et al.* 2002). The lack of overlap between the results of these studies emphasizes the difficulties and possible side-effects of these approaches. However, recent analysis of ArsRS-mediated acid regulation by these two groups (Loh and Cover, 2006; Pflock *et al.*, 2006a) confirmed some of the data, including the negative autoregulation of ArsR (Dietz *et al.* 2002) and the regulation of the Hpn nickel storage protein (HP1432)- and arginase (HP1399)-encoding genes (Forsyth *et al.*, 2002). Little information is available for the response regulator HP1021, which apparently misses a cognate sensor. Expression profiling of an *hp1021* deletion mutant only revealed differential expression of ten genes (Pflock *et al.*, 2007a). For the product of five of these genes, the regulation was confirmed by comparative proteomic 2-D GE analysis of the wild-type strain and *hp1021* mutant.

Role of NikR, Fur, ArsRS, CrdRS and FlgS regulators in the acid response

Comparison of the numerous transcriptional profiling studies presented above reveals striking overlaps between the subset of genes differentially regulated under several environmental conditions or in mutant strains. Complex regulatory networks have become apparent, in particular for responses to acidity and metal ions. To date, four transcriptional

regulators have been found to be involved in the response of *H. pylori* to acidity. The precursor work of Bijlsma *et al.* (2002) showed that Fur was required for acid resistance in *H. pylori*, as in other bacteria (e.g. *Salmonella enterica* serovar Typhimurium). The involvement of NikR and ArsR in addition to Fur as effectors of the global response of *H. pylori* to acidity was revealed by comparison of our transcriptome data with that of mutants deficient in these regulators (Contreras *et al.* 2003; Bury-Moné *et al.*, 2004; Loh and Cover, 2006; Pflock *et al.*, 2006a). For the first time, a nickel-responsive regulator, NikR, was found to be involved in the global acid response (reviewed by van Vliet *et al.*, 2004b).

To assess the role of these effectors in the acid response, several gene expression profiling experiments were performed in mutants exposed to low pH (Table 13.1). In our study, we found that, during growth at pH 5, the number of acid-responding genes dropped from 101 in a wild-type strain to only 36 genes in an isogenic $\Delta fur\Delta nikR$ double mutant (Bury-Moné *et al.*, 2004). In a more recent study, time-course transcriptome analysis was used to dissect iron and pH regulation in *H. pylori* and compare acid-regulated genes in a wild-type and Δfur mutant strain (Gancz *et al.*, 2006). A list of 95 genes which were aberrantly regulated at acidic pH in the absence of Fur was obtained with a strong overlap with previously identified acid-regulated genes. Fur-dependent acid-regulated genes comprised those involved in ammonia production (amidase, asparaginase), detoxification (SodB and the KatA catalase), pathogenicity (proteins of the *cagPAI* and *NapA*) and transcriptional regulation (ArsR). In addition, 89% of the Fur/acid-regulated genes were previously identified as being regulated by Fur, iron or acid. ArsR is an OmpR-like response regulator similar to its orthologue in *S. enterica* which is known to be involved in stationary phase-dependent pH-induced acid tolerance via positive autoregulation (Bang *et al.*, 2002). Low pH has been shown to be a signal triggering the autophosphorylation of the *H. pylori* ArsS histidine kinase and subsequent phosphorylation of its cognate response regulator ArsR (Pflock *et al.*, 2004). Comparison of the transcriptome of a $\Delta arsS$ mutant versus

a wild-type strain exposed to pH 5 revealed differential expression of as many as 109 genes including several already identified acid-regulated genes such as those encoding urease subunits and the *AmiE* and *AmiF* amidases (Pflock *et al.*, 2005, 2006a). For the three corresponding promoter regions, direct binding of phosphorylated ArsR protein was demonstrated. Several genes differentially regulated in the ArsS-deficient mutant were also under the control of NikR and Fur. In another study, global gene regulation in response to acidity of mutants deficient in ArsS, CrdS and FlgS was compared to that of the wild-type strain J99. While no difference was observed with the FlgS mutant of 101 analysed acid-regulated genes, 68 and 63 were no longer responsive to acidity in the ArsS and CrdS mutants, respectively, with about 70% common genes (Loh and Cover, 2006). Surprisingly, no difference in the global genetic response to acidity was observed in *crdS* mutants of other *H. pylori* strains (Pflock *et al.*, 2007b). Also in contradiction with previous work was the recent study of Wen *et al.* (2009), who found that FlgS in strain 26695, in addition to its function as a flagellar genes regulator, controls about 100 genes in the response to acidity through a pathway independent of its FlgR cognate regulator. The discrepancies between these different studies might be attributable to different experiments setups and/or to different genetic backgrounds of the *H. pylori* strains analysed. However, it underlines the care required when interpreting transcriptomic results, and the importance of validation of the results by other more direct techniques.

Finally, to make the picture even more complex, one should mention negative autoregulation of each of the three best-characterized regulators, Fur, NikR and ArsRS, as well as autorepression being stimulated by acidity for Fur and ArsRS. An additional level of complexity is given by cross-regulation whereby NikR negatively controls the expression of *fur* (Bury-Moné *et al.*, 2004; van Vliet *et al.*, 2004a) and vice versa (Delany *et al.*, 2005). Furthermore, ArsR does not regulate the expression of *nikR* and *fur* (Pflock *et al.*, 2006a,b) but Fur represses the expression of *arsR* in response to iron (Merrell

et al., 2003b; Gancz *et al.*, 2006), suggesting that the ArsR acid response is part of a complex regulatory network including acid- and metal-dependent regulation.

13.4 Conclusions from Global Gene Expression Studies in *H. pylori*

Understanding the mechanisms underlying *H. pylori*'s persistent colonization, and its unique adaptive capacity in the gastric environment, has represented a fascinating challenge for the research community almost since its discovery. A plethora of gene expression profiling studies has been published (see Table 13.1) generating a considerable amount of data, which are often hard to merge. The multiple transcriptomic studies are difficult to compare since they were performed with different reference strains and diverse experimental test conditions, in particular those used to mimic the environment encountered in the host (Table 13.1). While regulators and adaptive mechanisms have started to be identified and transcriptional networks to be unravelled, a closer look at the data produces a rather confusing picture with the expression of a significant number of genes varying in every condition or mutant strain tested.

Despite this, a common theme of these transcriptome analyses is the convincing connection between metal metabolism, acid response and virulence. Accordingly, *H. pylori* strains deficient in each of the transcriptional regulators involved in these responses (NikR, Fur, ArsRS) results in attenuated or abolished colonization capacities in animal models (Pantheil *et al.* 2003; Bury-Moné *et al.* 2004; Gancz *et al.* 2006). Because metal ions become more soluble at low pH, a tempting hypothesis is that higher metal bioavailability is a signal sensed by *H. pylori* during acid stress (Bury-Moné *et al.*, 2004; van Vliet *et al.*, 2004a,b). It therefore makes biological sense that metal-ion regulators of *H. pylori* are involved in the response to acidity. We previously proposed a model (Bury-Moné *et al.*, 2004) in which acidity is a 'spatial-temporal' signal for *H. pylori* that indicates its location in the stomach, either in the acidic mucus or close to the neutral epithelial cells, and

accordingly regulates several virulence factors. The work of Schreiber *et al.* (2004) on the spatial orientation of *H. pylori* in the gastric mucus fits well with such a model.

In addition, a major concern with transcriptomic studies is that they uncover both directly regulated genes and genes differentially expressed as a consequence of indirect effects. Growth phase strongly influences global gene expression in *H. pylori* (Merrell *et al.* 2003b; Thompson *et al.* 2003) including Fur production itself (Danielli *et al.*, 2006), and it might well be that the conditions tested in mutants or upon stress have influenced this parameter. Direct transcriptional regulation analysis has been used to validate the genomic data. Using EMSA (electrophoresis mobility shift assays) or DNase I protection assays, direct binding of Fur, NikR, phosphorylated ArsR or CrdR to the promoter region of a number of genes has been demonstrated (Delany *et al.*, 2002, 2005; Contreras *et al.*, 2003; van Vliet *et al.*, 2003, 2004a,b; Pflock *et al.*, 2004, 2005, 2006a,b; Ernst *et al.*, 2005c; Waidner *et al.*, 2005; Wen *et al.*, 2006). Also, to identify every genomic target of Fur, a ChIP on chip strategy has been used (Danielli *et al.*, 2006). This powerful strategy will be interesting to apply to the study of NikR, phosphorylated ArsR or FlgR in *H. pylori* cells incubated under different conditions. Second, despite numerous analyses, the DNA-binding site consensus that has been proposed for the Fur, NikR or ArsR regulators is weakly specific (short and AT-rich), and therefore poorly convincing, in an AT-rich organism such as *H. pylori*. This strongly suggests that additional parameters on the target DNA sequences or other cofactors are still to be identified. Such additional effectors could be small regulatory RNAs, such as identified for the Fur-mediated iron response in *E. coli* (Massé *et al.*, 2007). In addition, based on currently available data, no hierarchy in the regulatory networks of *H. pylori* can be established. Nevertheless, the overlapping regulatory pathways (acidity, flagella, metal and heat-shock stresses) are most intriguing and are evocative of the existence of a master regulator such as, for example, ComK, which controls more than 100 genes involved in competence in *Bacillus subtilis* (Maamar and Dubnau, 2005).

Importantly, such a 'conductor' could function through a novel global regulatory mechanism.

13.5 Summary and Outlook

At the end of the 20th century, *H. pylori* was the first bacterial pathogen for which two complete genome sequences were available. Genomics has rapidly become the approach of choice to study this 'young' pathogen. Several complete genome sequences are available today and many more will certainly be published in the coming years. Tools such as massive sequencing, comparative genomics, proteomics and transcriptomics have generated new insights into *H. pylori*'s spectacular diversity, pathogenicity and gastric adaptation. Thus, Helicobacteromics has provided a better understanding of the genetic drift, genomic instability, host specificity, environmental adaptation and evolutionary biology of this human pathogen. *H. pylori* has a remarkably variable genome that indicates a high rate of genetic change. It is therefore surprising that recent studies have failed to experimentally capture *de novo* genetic/genomic changes. The sensitivity of these analyses could be enhanced by novel technologies (i.e. tiling arrays, deep sequencing) to detect single-nucleotide polymorphisms at a genomic level. In addition, ongoing studies aim to track genomic changes occurring during infection by completely sequencing the chromosomes of *H. pylori* strains isolated from a single patient at different time points during disease progression.

Numerous transcriptome studies have helped to gain a better insight into the regulatory response of *H. pylori* to environmental and/or stress conditions that this pathogen must deal with in the stomach. Some mechanisms underlying the survival strategies of *H. pylori* in the gastric environment are now better understood, such as the response to acidity or to metal ions. Transcriptional regulators involved have been identified and some regulatory networks unravelled. However, the regulation of the expression of a number of genes does not depend on any established

regulators and many of the networks overlap to a surprisingly high degree. This points to complex regulatory cascades and the involvement of additional regulatory factors, such as unknown transcriptional regulators or small regulatory RNAs. With new high-throughput sequencing technologies such as Illumina SOLEXA or 454-ROCHE, deep sequencing approaches are now available for exploring, for instance, the RNome of a given organism (every RNA expressed in a cell). The groups of J. Vogel (MPIIB Berlin, Germany) and F. Darfeuille (INSERM, Bordeaux, France) recently performed such a global study defining the RNome of *H. pylori* strain 26695 and identified a number of small non-coding RNAs, some of which could play regulatory roles (Sharma *et al.*, 2010). The future directions will be to follow the changes of the *H. pylori* RNome under varying conditions or in different mutants and in parallel to test experimentally the role of the candidate regulatory RNAs.

In conclusion, this chapter illustrates the adaptive strategies of *H. pylori*. A short-term strategy involves coordinated control of gene expression which is an immediate and reversible response affecting the entire bacterial population. A long-term strategy implies genome plasticity, not homogeneous among a population but stabilized under external selective pressure. These characteristics of *H. pylori* have certainly been determinant in making this bacterium a successful pathogen that has coexisted with humans for hundreds of thousands of years, that once established persistently colonizes the host stomach for decades and that still infects half of all people in the world.

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Index

Note: page numbers in *italics* refer to figures and tables

- Abl kinase 231
- Abl oncoproteins 230–231, 232–233
- acid response 278–279, 284
- acid response network regulators 279, 282–284
- acquired immune response 94
- activator protein 1 (AP-1) 147
- acyltransferase 195
- adherence properties, regulatory
 - mechanisms 249–252
- adherence-associated lipoproteins (Alp) 249
- adhesins 248–254
 - phase variation 250–252
 - saliva-specific 256
- adhesion of *H. pylori* to gastric surface 248–262
 - host structure interactions 254–261
 - lipopolysaccharide-mediated 252–254
 - mode 248–254
- adipokines, vaccine-induced immunity
 - mechanisms 177–178
- African enigma 31
- age
 - prevalence of infection 3, 7–8
 - see also* children
- AID DNA-editing enzyme 213
- alkaline hydroperoxide reductase antibodies 275
- allergic disease 78–79
 - H. pylori*-mediated 104
- AmiE amidase 278
- AmiF formamidase 278
- ammonia 84
 - production for acid resistance 278
- amoxicillin 46, 47
 - antibiotic resistance 51, 52–53
 - mutation detection 59
 - PPI-based triple therapy 19–20, 48
- amoxicillin–metronidazole combination 49
- amoxicillin–rifabutin combination 49
- anaemia, iron-deficiency 71–74
- antibiotic(s)
 - absorbable 46–47
 - combination use 47
 - diffusion 46–47
 - eradication of *H. pylori* 18–19, 36
 - minimal inhibitory concentrations 46, 54
- antibiotic resistance 36, 45–51, 52–53, 54–60
 - clinical impact 51, 54
 - cut-off values 54
 - detection 54–60
 - genotypic methods of detection 55–60
 - multiple 51
 - mutations 47
 - detection 55–60
 - phenotypic methods of detection 54–55
 - prevalence 50–51, 52–53
 - risk 19
 - see also* named antibiotics
- antibodies
 - agglutinating 176
 - vaccine-induced immunity
 - mechanisms 173–175
- antigen-presenting cells 94
- antigens
 - candidate vaccines 167–168
 - multiple 168
- antimicrobial susceptibility testing 48–49
- antisecretory drugs 45–46
- apoptosis, VacA protein induction 225
- apoptosis-associated speck-like protein (ASC) 150
- arginase-encoding gene 282

- ArsRS acid-responsive regulator system 280, 283
 promoter region binding 284
 regulons 282
- Asia
 epidemiology of infection 13–18
 eradication of *H. pylori* 18–19
 gastric cancer
 epidemiology 16
 incidence 14, 15
 mortality 15
 prevention 18–19
 risk 14
 IL-8 polymorphisms 123
 infection in 13–20
 seroprevalence rate 13–15
 treatment regimens for *H. pylori* 19–20
- asthma 78–82
 CagA antibodies 80
H. pylori-mediated 104
 mechanisms of *H. pylori* infection
 association 80–82
- atherosclerosis 75
 CagA antibodies 77–78
 carotid artery 76–77
H. pylori direct role 77
 heat-shock proteins 78
 risk factors 77
- atopy, *H. pylori* infection association 81
- atrophic gastritis 2
 chronic 35, 36, 274
 gastric cancer risk 29
 IL-1 β polymorphisms 121
 IL-8 polymorphisms 123
 TLR4 association 151
 TLR4 polymorphisms 125
- autoimmune pancreatitis 84–85
- autoimmunity, *H. pylori*-mediated 104–105
- babA* gene 248–249
see also blood group-binding adhesin (BabA)
- bacterial danger signals 152–153
- Barrett's oesophagus, eradication of *H. pylori*
 complication 36
- B-cell lymphoma
 gastric 1
 mucosa-associated lymphoid tissue
 (MALT) 69
- B-cell response 94–95
- biomarkers 275
 disease progression 274–275
- bismuth-based quadruple therapy 20, 49
- blood group-binding adhesin (BabA) 248–249
 deletions in locus coding for 274
 ligands 256
 mucin binding 258
 phase variation 250–251
- cag* pathogenicity island (*cagPAI*) 17, 32, 213, 216,
 217, 227–237
 crystal structures of proteins 228–229, 230
 discovery 227
 dispensable gene location 271
 gene acquisition 272
 type IV secretion system 227, 228–229,
 229–230
- CagA antibodies
 asthma association 80
 atherosclerosis association 77–78
 lymphocyte cell cycle arrest 95
 multiunit vaccine 181–182
- cagA* gene 17, 32, 213
- CagA protein 17, 33, 213
 carotid atherosclerosis progression 76–77
 discovery 227
 EPIYA motifs 17, 33
 EPIYA region 230–231
 gene sequences 273–274
H. pylori-induced gastric damage 213, 214–215,
 215, 216
 idiopathic thrombocytopenic purpura 71
 immunoblot 2
 injection into epithelial cells 229, 230
 innate immune response initiation 152–153
 intracellular functions 231, 234–235, 235–236
 ischaemic heart disease 75–76
 oncogenesis role 215
 phosphorylation by Src and Abl
 oncoproteins 230–231, 232–233
 phosphorylation-dependent signalling 231,
 234–235, 235
 phosphorylation-independent
 signalling 234–235, 235–236
 salt levels 25
 signalling to nucleus 236
- CagD protein 230
- cagE* gene 17
- CagF protein 230
- CagL protein 228–229, 229
 crystal structure 230
 integrin signalling 237
- CagS protein 230
- CagZ protein 230
- carcinogenesis mechanisms 34–35
- cardiovascular disease 75–78
 CagA protein association 75–76
 carotid artery intimal-medial thickness 76–77
- catalase 216
- β -catenin, activation by CagA 236
- cathelicidin LL-37 155–156, 197
- cationic antimicrobial peptides (CAMPs),
 resistance 197–198
- CD4⁺ T cells 95, 96
 VacA suppressive effects 225, 226
- CD8⁺ cytotoxic T cells 100

-
- CD14
 lipopolysaccharide low immunoactivity 198
 precancerous lesion association 151
 CD18 224, 225
 host cell motility/elongation 230
 CD80 152
 CD86 152
CDH1 gene mutations 31
 cell-mediated immunity, vaccine-induced
 immunity mechanisms 175–177
 chemokines
 neutrophil-activating 176
 proinflammatory 156
 chemotaxis genes 255
 children, prevalence of infection 4–5, 7–8
 China, IL-1 β polymorphisms 121–122
 cholera toxin (CT) 169, 170–171, 172, 173, 180
 cholesterol- α -glycosyltransferase 216
 cigarette smoking, gastric cancer 26
 clarithromycin 46–47
 antibiotic resistance 47–48, 50–51, 52–53
 mutation detection 55–58
 chemical stability 45
 PPI-based triple therapy 19–20
 colonization
 animal model 279–280
 flagellar motility 280–281
 persistent of stomach 274, 284
 ComK regulator 284
 coronary heart disease (CHD) 75–76
 coronin-1 225
 Correa's hypothesis 16, 26, 27
 CpG oligonucleotides 170, 171–172, 173
 cranberry juice 50
 CrdRS acid response regulator 283
 promoter region binding 284
 Crohn's disease 82–83
 C-type lectin 203
 CXCL8 147, 148, 149, 150, 151–152
 induction by CagA 153
 cyclo-oxygenase 1 (COX-1) inhibition 1–2
 cyclo-oxygenase 2 (COX-2), VacA effects on
 expression 227
 cytokines 117, 118
 adipokines 177–178
 inflammation-modulating 120–124
 proinflammatory 147, 198
 T-helper cell 117, 119–120
 cytotoxic T lymphocyte-associated antigen 4
 (CTLA-4) 102–103
 cytotoxin-associated gene A protein (CagA) *see*
 CagA protein
 decay-accelerating factor (DAF) 237
DEFB3 gene expression 155
 defensins 154–156
 α -defensins 154
 dendritic cells 152, 156
 host immune response 179
 dendritic cell-specific intercellular adhesion
 molecule 3-grabbing non-integrin
 (DC-SIGN) 203
 developing countries, epidemiology of
 infection 2–3
 dextran sulfate 249
 dietary factors 117
 gastric cancer 25–26, 32
 disease progression 273–274
 biomarkers 274–275
 DNA arrays 275
 DNA recombination 270
 DNA sequences 284
 DNA vaccines 171, 172, 173
 DNA-strip test, clarithromycin resistance mutation
 detection 57
 dual-priming oligonucleotide-based multiplex
 polymerase chain reaction (PCR),
 clarithromycin resistance mutation
 detection 56–57
 duodenal ulcers 1
 NOD1 polymorphisms 125
 non-atrophic gastritis 33
 TGF β polymorphisms 124
 dysplasia 25, 29–30
 high-grade 29, 30
 identification 36
 low-grade 29–30
 Padova classification 30
 E-cadherin 31
 efflux pumps 48
 endosomal maturation, VacA effects 222–223, 224,
 226
 environmental factors 117
 gastric cancer 26
 gender bias 129
 prevalence of infection 7–8
 epidemiology of infection 1–9
 Asia 13–18
 prevalence 2–5
 changing 4–5
 environmental changes 7–8
 gastric cancer rate 14, 16, 31–32
 infection-induced ulcers 8–9
 transmission routes 5–6
 epidermal growth factor (EGF) 226
 epidermal growth factor receptor (EGFR)
 signalling 226
 epithelial cells
 attachment 279–280
 CagA protein injection 229, 230
 sialylation of infected gastric cells 257

- epithelial cells *continued*
 VacA
 effects on monolayer permeability 224–225
 effects on vacuolation 224
 targeting of multiple host surface molecules 220, 221, 222–223, 223–224
 EPIYA motifs 17, 33, 230–231
 Epstein–Barr virus (EBV), gastric cancer risk 27
 eradication of *H. pylori* 36
 complications 36
 gastric cancer prevention strategy 18–19
 mouth 255
 treatment regimens 19–20
 ethnic groups
 IL-1 β polymorphisms 121–122
 prevalence of infection 4
 subpopulations 273
 evolution
 microevolution of *H. pylori* 273–274
 reductive 271
 extragastric manifestations of infection 69–85
 asthma 78–82
 cardiovascular disease 75–78
 idiopathic thrombocytopenic purpura 70–71
 inflammatory bowel disease 82–83
- faecal–oral transmission of infection 5, 6
 family transmission of infection 5–6
 Fc γ receptors, idiopathic thrombocytopenic purpura 71
 fimbriae 248
 FlaA flagellin 279
 flagellar motility 280–281
 flagellar proteins 255
 flagellar system regulation 280–281
 flagellin 146–148, 198, 279
 FlgR cognate regulator 283
 fluorescent *in situ* hybridization (FISH),
 clarithromycin resistance mutation
 detection 57–58
 fluoroquinolone resistance 52–53, 54
 mutation detection 58
 focal adhesion kinase (FAK) 228–229, 229,
 230, 235
 FOXP3 transcription factor 100–101, 103, 104, 130
 fruit intake 117
 gastric carcinoma protection 25–26
 fucosylated blood-group antigen-carrying
 glycosphingolipids 260
 fucosyltransferase (FucT) gene 200
 functional genomics, gastric adaptation 275,
 276–277, 278–284
 Fur metalloregulator 279, 280, 281–282
 acid response network regulator 283
 promoter region binding 284
 furazolidone 47
- galabiasylceramide, VacA binding 261
 galectin-3 253
 gastric adaptation 270–272, 284
 functional genomics 275, 276–277, 278–284
 Lewis antigens 200–203
 gastric atrophy 32
 iron-deficiency anaemia 74
 gastric cancer (adenocarcinoma) 1, 24–37, 69
 aetiology 25–27
 carcinogenesis mechanisms 34–35
 causation model 26, 27
 cellular immune response variability 103
 classification 28–29
 control 35–37
 diagnosis 24
 dietary factors 25–26, 32
 diffuse-type 31
 early 28
 epidemiology in Asia 16
 of gastric cardia 28
 gastric inflammation site 116
 gender effects 128–129
 genomic analysis 274
 hazard ratio 35, 36
 hereditary diffuse 31
 histopathology 28–31
 human leucocyte antigens 120
 host factors 18
 IL-1 β polymorphisms 121
 IL-6 polymorphisms 123
 IL-8 polymorphisms 123
 IL-10 polymorphisms 124
 incidence 24
 in Asia 14, 15
 rate 35, 36
 intestinal type 29–31
 invasive 28
 Laurén classification 28–29
 morbidity/mortality 212
 mortality in Asia 15
 MUC1 short allele polymorphisms 126, 127
 pathology 28–31
 poorly differentiated 31
 precancerous process 29–31
 precursor lesions 2
 prevalence 2
 prevention 36–37
 strategy 18–19
 prognosis 35–36
 risk 14
 H. pylori infection rate 31–32
 smoking association 128
 TGF β polymorphisms 124
 TLR2 polymorphisms 125, 151
 TLR4 polymorphisms 125
 Tregs 103, 104
 well differentiated 31
 WHO classification 28

- gastric cardia, gastric adenocarcinoma 28
gastric erosion, blood loss 71–72
gastric mucosa 270
 iron scavenging 72–73
gastric precancerous multistep model 24, 25
gastric ulcers 1
 gastric inflammation site 116
 IL-8 polymorphisms 123
gastritis
 chronic active 25, 29, 30
 colonization density 253
 gender effects 128–129
 IL-12 122–123
 MUC1 short allele polymorphisms 126, 127
 non-atrophic 25, 29, 30, 32–33
 persistent colonization of stomach 274
 post-immunization 175–176, 178
 site 116
 Treg response 100–101
 see also atrophic gastritis
gatifloxacin resistance 54
gender effects 126–130
 animal models 128–129
 environmental factors 129
gene(s) 16–18
 horizontal transfer 270, 273
 motility 278
 phase-variable 271
 plasticity zones 271
 point mutations 272
 strain-specific 271
 worldwide content 272–273
 see also housekeeping genes; virulence genes
gene expression
 profiling 275, 276–277
 switch 279
gene pools 273
genetic diversity, worldwide 272–273
genetic factors
 bacterial 117
 gastric cancer 26
 human gene polymorphisms 117, 118, 119–130
 prevalence of infection 8
genetic variability 270–272
genome
 different pathologies development 274
 plasticity 270
 sequence 16, 270–271
genomics of *H. pylori* 269–285
 comparative 270–275
 functional 275, 276–277, 278–284
genotypic diversity 272
geographical distribution 2–4
 γ -glutamyl transpeptidase 95, 216
glycans 255
glycocalyx 258
glycosphingolipids, *H. pylori* binding 258–261
GM₃ ganglioside, *H. pylori* binding 258, 259
GroES co-chaperonin 275
growth phase-regulated genes 279

H. pylori neutrophil-activating protein
 (HP-NAP) 152
heat-labile toxin of *Escherichia coli* (LT) 169,
 170–171, 172, 173, 179–181
heat-shock protein(s) (HSP)
 atherosclerosis association 78
 vaccine antigen 168
heat-shock protein 60 152
heat-shock regulators 279, 282
Helicobacter acinonychis 272
helicobacteromics 269–285
helminths, gastric cancer risk 32
heparan sulfate 249
hepatic encephalopathy 84
hepatitis A 79
hepatitis C 84
hepatobiliary disease 83–84
hepatocellular carcinoma 84
HLA-DP antigens 120
HLA-DQ antigens 120
HLA-DR antigens 120
HOP genes 250
host adaptation 271
host defence, lactoferrin 153
host factors 18
 variability impact on pathogen recognition
 receptors 124–126
host genetic factors 116–130
host immune response
 bypassing in protective immunity
 induction 178–179
 dendritic cells 179
 evasion 250
 innate immune initiators 142–152
 Tregs 178–179
host–pathogen interactions 212–213
housekeeping genes 16, 271
 multilocus sequence typing 33–34
Hp(2-20) peptide 197
HP1021 orphan regulator, regulon 282
hpAsia2 273
Hpn nickel storage protein 282
HrcA heat-shock regulator 279
 regulon 282
hspIndia 273
HspR heat-shock regulator, regulons 282
human β -defensins 154–155, 197
human cationic antibacterial protein
 (hCAP18) 155
human gene polymorphisms 117, 118, 119–130
human leucocyte antigen(s) (HLA) 120
human migration 273

-
- human neutrophil peptides (HNPs) 154
 - humoral response 95
 - diagnostic use 275
 - vaccine development 275
 - hygiene hypothesis 36, 78–79
 - idiopathic thrombocytopenic purpura (ITP) 70–71
 - iron-deficiency anaemia 71–74
 - IFNGR 119
 - IL-1 receptor-associated kinase-1 (IRAK1) 143
 - immune cells
 - VacA suppressive effects 225–226
 - VacA targeting of multiple host surface molecules 220, 221, 222–223, 223–224
 - immune response 94
 - acquired 94
 - bypassing of host regulatory response 178–179
 - components 119
 - gastric mucosa 270
 - innate 95–97, 96, 120, 142–157
 - vaccines 168, 175–177, 275
 - immunization
 - antibody requirement 175–177
 - candidate antigens 167–168
 - dual antigen 168
 - gastritis 175–176
 - mucosal 172–173
 - parenteral 173
 - routes 172–173
 - Th1 immune response 176
 - immunoglobulin A (IgA) antibodies 94–95
 - immunoglobulin E (IgE) antibodies 79
 - immunoglobulin G (IgG) antibodies 79, 94–95
 - immunomodulation, *H. pylori*-mediated 104
 - Indian dilemma 14, 16
 - indoleamine 2,3-dioxygenase (IDO) activity 103
 - inflammasomes 151
 - inflammation
 - severity 116–117
 - site 116
 - see also* gastritis
 - inflammatory bowel disease 82–83
 - inflammatory cells 156
 - inflammatory response, modulation by Lewis
 - antigens 203
 - innate immune effector responses 153–156
 - innate immune initiators 142–152
 - bacterial danger signals 152–153
 - innate immune system, inflammation-modulating cytokines 120–124
 - β_2 -integrin *see* CD18
 - intercellular adhesion molecule 1 (ICAM-1) 216
 - interferon γ (IFN γ) 96, 119
 - vaccine-induced immunity 177
 - interleukin 1 β (IL-1 β) 120–122
 - gene cluster polymorphisms 18, 26, 27
 - interleukin 4 (IL-4) 119–120
 - interleukin 6 (IL-6) 123
 - interleukin 8 (IL-8) 123
 - salt levels 25
 - interleukin 10 (IL-10) 97, 102, 123–124
 - immunity down-regulation 178–179
 - interleukin 12 (IL-12) 122–123
 - interleukin 12p40 (IL-12p40) 175–177
 - interleukin 17 (IL-17) 97, 98–99
 - vaccine-induced immunity 176–177
 - interleukin 23 (IL-23) 99
 - vaccine-induced immunity 176, 177
 - intestinal metaplasia 2, 25, 29, 30
 - IL-10 polymorphisms 124
 - TLR4 association 151
 - TLR4 polymorphisms 125
 - intestinal parasites, gastric cancer risk 32
 - intimal-medial thickness 76–77
 - iron
 - absorption 72, 202–203
 - availability 279
 - homeostasis 279
 - metabolism 282
 - overload 279
 - regulation 281
 - scavenging in gastric mucosa 72–73
 - starvation 279
 - virulence factor effects 279
 - iron-deficiency anaemia 71–74
 - H. pylori* role 73–74
 - iron-repressible outer membrane proteins (IROMP) 73
 - ischaemic heart disease
 - CagA protein association 75–76
 - risk factors 77
 - ISCOMATRIX™ 170, 172
 - ISCOMS™ (immune-stimulating complexes) 170, 172
 - Japanese populations
 - HLA molecule impact 120
 - IL-1 β polymorphisms 121
 - junctional adhesion molecule (JAM) 235
 - Kdo sugars 195, 196
 - lactoferrin 50, 153–154
 - antimicrobial function 153–154
 - lactosylceramide 260–261
 - laminin 253–254
 - Laurén gastric adenocarcinoma
 - classification 28–29
 - leptin 178
 - levofloxacin 47
 - antibiotic resistance 50–51, 54

- levofloxacin-based triple therapy 20, 48
- Lewis antigen expression 198, 199, 200
- BabA phase variation 250–251
 - colonization density in gastritis 253
 - gastric adaptation 200–203
 - inflammatory response modulation 203
 - lipopolysaccharide-mediated
 - adhesion 252–253
 - mucus 255, 257–258
- Lewis* gene 255
- lipid A 190, 191
- acyl chains 193, 194, 194–195
 - acylation pattern 192
 - biosynthetic pathway 194–196
 - CAMP resistance promotion 197
 - excretion by exocytosis 198
 - Kdo residues 195, 196
 - low immunoactivity 192, 197, 198
 - H. pylori* pathogenesis 197–198
 - PEtN group 196, 197
 - structural analysis 192, 193, 194
 - tetra-acylated 196
- lipid A kinase 196
- lipopolysaccharide(s) (LPS) 153, 190–204
- adhesin function 248
 - adhesion function 252–254
 - excretion by exocytosis 198
 - H. pylori* adhesion 252
 - Lewis antigen expression 198, 199, 200
 - gastric adaptation 200–203
 - low immunoactivity 191–192, 213
 - H. pylori* pathogenesis 197–198
 - mucin synthesis reduction 256
 - O-polysaccharide chain 190, 191, 198, 200, 201, 202
 - H. pylori* adhesion 252–253
 - surfactant protein D binding 253
 - saccharide component structures 199
 - see also lipid A
- LL-37 155–156
- LPS-binding protein 198
- LpxK lipid A kinase 196
- lymphocyte function-associated molecule 1 (LFA-1) 216, 224
- lysosomes, VacA effects 226
- macrophages 156
- activation in idiopathic thrombocytopenic purpura 71
- major histocompatibility complex (MHC)
- antigens 120
- mast cells, vaccine-induced immunity
- mechanisms 177
- matrix metalloproteinase 1 (MMP-1), activation by CagA 236
- megasomes 237
- metal metabolism 284
- metal storage proteins 278
- metal-ion regulators 284
- metal-ion transport systems 278
- metronidazole 46
- antibiotic resistance 50–51, 52–53
 - mutation detection 59–60
 - PPI-based triple therapy 19, 20, 48
 - sequential therapy 48
- microevolution of *H. pylori* 273–274
- mitogen-activated protein (MAP) kinase 226, 230
- molecular epidemiology 16–18
- molecular mimicry 77–78
- motility genes 278
- mouth, eradication of *H. pylori* 255
- MUC1 mucin 126, 258
- MUC5AC mucin 255
- BabA ligands 257
 - glycoforms 258
 - SabA ligands 257
- MUC6 mucin 255
- mucins 117, 118, 126
- adhesion modes 257
 - glycosylation 254–255
 - H. pylori* binding 256–258
 - membrane-associated 258
 - polymorphism 254–255
 - salivary 256–258
- mucosa-associated lymphoid tissue (MALT)
- lymphoma 69, 103
- HLAs 120
- morbidity/mortality 212
- NOD2 association 151
- TLR4 polymorphisms 125
- mucus barrier 254–258
- multilocus sequence typing (MLST) 33–34
- multiple sclerosis, *H. pylori*-mediated 104
- mutations 272
- MyD88 adapter-like protein (MAL) 143
- myeloid cell leukaemia sequence 1 (MCL-1) 236
- myosin regulatory light chain (MLC) 225
- NACHT-LRR-PYD-containing proteins (NALPs) 150–151
- NapA bacterioferritin 279
- natural killer (NK) cells 95
- neolactoseries gangliosides 259–260
- α -Neu5Ac-(2-3)- β -Gal-(1-4)- β -GlcNAc(1-3)- β -Gal-(1-4)-GlcNAc-terminated glycosphingolipids 260
- neutrophil-activating protein (NAP) 249
- biomarker protein 275
 - neolactoseries gangliosides 259, 260
- neutrophils
- priming by LPS 191
 - vaccine-mediated protection 176–177

- nickel 281–282
- NikR metalloregulator 280, 281–282, 283
 promoter region binding 284
- nitric oxide (NO) 214–215, 216
- nitric oxide synthase (NOS) 198
- nitric oxide synthase, inducible (iNOS) 35
- nitrosative stress 34–35
- NLR protein family 150
- non-autoimmune chronic pancreatitis 84
- non-steroidal anti-inflammatory drugs (NSAIDs),
 peptic ulcers 1–2
- nuclear factor- κ B (NF κ B) 122, 144, 147, 149
 activation 150, 236
- nucleosome assembly protein (NAP) 181–182
- nucleotide-binding oligomerization domain
 (NOD) 124, 125–126, 148–150
- nucleotide-binding oligomerization domain 1
 (NOD1) 148–150, 156, 198
 proinflammatory signalling 237
- nucleotide-binding oligomerization domain 2
 (NOD2) 148–149
 mucosa-associated lymphoid tissue lymphoma
 association 151
- occludin 225
- oesophageal adenocarcinoma, eradication of
 H. pylori complication 36
- oipA* gene 18, 230
- O-polysaccharide chain 190, 191, 198, 200,
 201, 202
 H. pylori adhesion 252–253
 surfactant protein D binding 253
- oral cavity colonization 255–256
- oral–oral transmission 5, 6
- outer inflammatory protein A (OipA) 213
- oxidative stress 34–35
- p38 MAP kinase 226
- pancreatic disease 84–85
- PAR1 235, 236
- pathogen-associated molecular patterns
 (PAMPs) 143, 144, 147
- pathogenicity factors, virulence factor
 interplay 212–213
- pathogen-recognition molecules (PRMs) 142, 143,
 144, 147, 149
 gene polymorphisms 151
- pathogen-recognition receptors 124–126
- paxillin 230
- pepsinogen, serum levels 35, 37
- pepsinogen I/pepsinogen II (PGI/PGII)
 ratio 35, 37
- peptic ulcers/peptic ulcer disease 1–2, 69
 gender factors 128
 morbidity/mortality 212
 prevalence 8–9
 Treg protection 101–102
- peptidoglycan 213
- persistence of *H. pylori*, lifelong 273
- PEtN group 196, 197
- pfr* gene 279
- phase shift variation 250, 271
- polio virus replicons 170, 172
- probiotics 50
- proliferation-inhibiting protein 95
- proteomics, novel tools 274–275
- proton pump inhibitor(s) (PPIs) 45–46
- proton pump inhibitor (PPI)-based quadruple
 therapy 49
- proton pump inhibitor (PPI)-based triple
 therapy 19–20, 47–48
 antibiotic resistance clinical impact 51, 54
- racial groups, prevalence of infection 4, 7
- real-time polymerase chain reaction (RT-PCR),
 clarithromycin resistance mutation
 detection 55–56
- receptor protein-tyrosine phosphatase (RPTP) 220,
 221, 222–223, 223, 226
- reductive evolution 271
- reflux oesophagitis 36
- regulatory T-cells (Tregs) 95, 96, 97, 100–104
 gastric adenocarcinoma 103, 104
 H. pylori disease associations 103–104
 host response 178–179
 IL-10-secreting 102
 inducible 100
 natural 100
 peptic ulcer disease protection 101–102
 response 94
 gastritis 100–101
 TGF β -secreting 102
- regulons of *H. pylori* 280–284
- rifabutin 47
 antibiotic resistance 59
- rifabutin-based triple therapy 20
- rifampicins 47
- rifamycins, antibiotic resistance 59
- RIP-like interacting CLARP kinase (RICK) 149
- RNA profiling, vaccine-induced immunity
 mechanisms 177–178
- RpoN regulon 281
- SabA adhesin 249, 251
 acidity effects 278
 binding 258
 ligands 256
 neolactoseries gangliosides 259, 260
 pH effects on expression 251–252
- sabA* gene, phase variation 251

- saliva 256
- Salmonella* recombinant vaccines 171, 172
- salt intake 25, 117
- salvage therapy 20
- screen-and-treat strategy 19
- secretors 255
- sequence variants, coexistence 273
- sequential therapy 20, 48
- seroprevalence rate, Asia 13–15
- sex chromosomes 129–130
- sexual dimorphism 127–128
- Shp tyrosine phosphatase 231, 235
- sialic acid-binding adhesin (SabA) *see* SabA adhesin
- sialyl-dimeric-Le^x glycosphingolipid 260
- single-sequence repeats (SSR) 271
- Sjögren's syndrome 256
- slipped-strand mispairing (SSM) 250
- BabA phase variation 251
- small regulatory RNAs 284
- smoking 117
- gastric cancer development 128
- socioeconomic status
- gastric cancer 26
- prevalence of infection 3–4, 5
- sphingomyelin 223–224
- Src family kinases (SFKs) 231
- Src oncoproteins
- CagA phosphorylation 230–231, 232–233
- inhibition 235
- Sry* gene 130
- stimulons of *H. pylori* 275, 278–280
- stomach, corpus region 116
- strains of *H. pylori* 16–17
- subpopulations, genetically distinct 273
- subtypes of *H. pylori* 273
- sulfatide, *H. pylori* binding 259
- superoxide dismutase 216
- surfactant protein D (SP-D) 253
- systemic lupus erythematosus (SLE), *H. pylori*-mediated 104
- T cells
- CD8⁺ cytotoxic 100
- response suppression 95
- VacA suppression 224, 225
- see also* CD4⁺ T cells; regulatory T-cells (Tregs); T-helper (Th) cells
- T4SS transmembrane transporters 227, 228–229, 229–230
- CagA-independent signalling 236–237
- tetracycline, antibiotic resistance 51, 52–53, 59
- Thai dilemma 14, 16
- T-helper (Th) cells
- cytokines 117, 119–120
- development 96
- recruitment to gastric mucosa 156
- subset responses 97–99
- T-helper 1 (Th1) cells 97–98, 156
- immune response effector phase 203
- T-helper 1 (Th1) response
- asthma 82
- vaccination 176
- T-helper 2 (Th2) cells 96, 97, 98
- immune response effector phase 203
- T-helper 2 (Th2) response
- asthma 82
- gastric cancer risk 32
- T-helper 9 (Th9) cells 98
- T-helper 17 (Th17) cells 96–97, 98–99
- Toll IL-1 receptor (TIR) 143
- Toll-like receptor(s) (TLRs) 124–125, 143–148
- expression in epithelial cells 145–148
- LPS low immunoactivity 198
- Toll-like receptor 2 (TLR 2) 125
- expression in epithelial cells 145–146
- gastric cancer association 151
- immune response in infection 198
- Toll-like receptor 4 (TLR4) 125
- expression in epithelial cells 145–146
- induction by lipopolysaccharide 252
- precancerous lesion association 151
- Toll-like receptor 5 (TLR5) 198
- expression in epithelial cells 146–148
- Toll-like receptor 7 (TLR 7) 130
- TonB machinery of *H. pylori* 282
- Toxoplasma gondii* 79
- transcriptome studies 275, 276–277, 278–284
- regulons of *H. pylori* 280–284
- stimulons of *H. pylori* 275, 278–280
- transepithelial resistance (TER) 224
- transforming growth factor β (TGF β) 96, 124
- Tregs 102
- transmission
- intra-familial 273
- routes of infection 5–6
- treatment for *H. pylori*
- adjuvant therapy 50
- basis 45–47
- currently recommended 47–50
- regimens 19–20
- salvage 20
- second-line therapy 49
- Treg *see* regulatory T-cells (Tregs)
- triggering receptor expressed on myeloid cells (TREM) 151–152
- tryptophan aspartate-containing coat protein (TACO) 225
- tumour necrosis factor α (TNF α) 121–122, 152
- tumour necrosis factor α (TNF α)-inducing protein (Tip α) 33
- two-component regulatory system (TCS) in *H. pylori* 279, 280, 282

- ulcerative colitis 82–83
- urea degradation 84
- urease 179–181, 255, 278
 - vaccine antigen 168
- VacA protein 17, 18, 33, 213
 - acidity effects 278
 - activity 220, 222–223, 223–224
 - allelic variation 218–220
 - apoptotic cell death induction 225
 - binding to galabiosaosylceramide 261
 - discovery 216–217
 - endosomal maturation effects 222–223, 224
 - epithelial cells
 - monolayer permeability 224–225
 - vacuolation 224
 - expression 218–220
 - gene sequences 273–274
 - genotypes 274
 - H. pylori*-induced gastric damage 213, 214–215, 215, 216
 - immune cell suppression 225–226
 - interaction partners 220, 221
 - iron starvation 279
 - lipid interactions 223
 - maturation 218–220
 - molecular structure 218–219, 220
 - multiple host surface molecule targeting 220, 222–223, 223–224
 - multiunit vaccine 181–182
 - secretion 218–220
 - signal transduction pathway effects 226–227
 - sphingomyelin receptor role 223–224
 - T-cell response inhibition 95
 - transepithelial resistance 224
- vaccines 167–182
 - adjuvants 169, 170–171, 171–173, 176
 - clinical trials 180–181
 - cell-mediated immunity 175–177
 - clinical trials 180–182
 - DNA 171, 172, 173
 - immune response 168, 175–177, 275
 - immunity induction mechanisms 173–178
 - inactivated whole-cell 180
 - mucosal 172–173, 179–181
 - multivalent subunit 168
 - non-human primate trials 179–182
 - parenteral 173, 181–182
 - recombinant bacterial vector 181
 - vector 171, 172
 - see also* immunization
- vacuolating cytotoxin (VacA) protein *see* VacA protein
- vegetable intake 117
 - gastric carcinoma protection 25–26
- vegetable products 50
- VIM-2 ganglioside 260
- VirB9 protein 228–229, 230
- VirB10 protein 227, 228–229, 229, 230
- VirB11 protein 228–229, 230
- virulence 284
- virulence factors 32–33, 212–237
 - animal models 213, 214–215, 215
 - cell surface interactions 256
 - in vitro* models 214–215, 215–216, 217
 - iron effects 279
 - pathogenicity factor interplay 212–213
 - see also* CagA protein; VacA protein
- virulence genes 17, 271
- virulence potential, *H. pylori* strains 32–33
- water quality, transmission of infection 6
- World Health Organization (WHO), gastric adenocarcinoma classification 28
- zona occludens 1 (ZO-1) 235