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CHAPTER 1

Evolution of the Probiotic Concept: From Conception to Validation and Acceptance in Medical Science

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Contents

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Abstract Two pioneering achievements by Ilya Ilyich Metchnikoff were recorded in 1908. Most notable was his Nobel Prize in Medicine for discovering the innate cellular immune response to an infectious challenge. Of lesser note was his recommendation, "...to absorb large quantities of microbes, as a general belief is that microbes are harmful. This belief is erroneous. There are many useful microbes, amongst which the lactic bacilli have an honorable place." While his discovery of the inflammatory response was rapidly incorporated into our understanding of cellular immunity, his recommendation "to absorb large quantities of microbes," on the other hand, languished for decades in limbos of indifference, skepticism, and disbelief. The present chapter is a synopsis of salient discoveries made during the past 100 years, which gradually displaced these skepticisms, validated his concept of "useful microbes," and propelled his "lactic bacilli" into the mainstream of modern medical science, practice, and therapy.

I. INTRODUCTION

Conception and evolution of the probiotic concept

A reader who has little knowledge of such matters may be surprised by my recommendation to absorb large quantities of microbes, as a general belief is that microbes are harmful. This belief is erroneous. There are many useful microbes, amongst which the lactic bacilli have an honorable place (Metchnikoff, 1907).

This proclamation reported more than a century ago by Nobel Laureate Ilya Ilyich Metchnikoff is widely accepted as the origin of the ''probiotic concept'' (Bibel, 1988; Fuller, 1992a; Parker, 1974), a concept generally defined today as the ability of ''live microorganisms which, when administered in adequate amounts confer a health benefit on the host'' (Reid *et al.*, 2003). During this past century, our perceptions, definitions, scientific understandings, and practical expectations concerning this concept have undergone significant changes. It has encountered periods of indifference, skepticism, rejection, and commercial exploitation, but also, periods of serious scientific scrutiny and eventual discoveries that led to its validation. In addition, it now appears to have evolved into what Nobel Laureate Joshua Lederberg has termed the ''microbiome concept'' (Lederberg, 2004). It is interesting to note Metchnikoff's prescience concerning the microbiome in his additional musing that, ''Systematic investigations should be made on the relation of intestinal microbes to precocious old age, and on the influence of diets which prevent intestinal putrefaction in prolonging life and maintaining the forces of the body'' (Metchnikoff, 1907).

The present review attempts to record seminal events and varied phases that took place during the century-long journey from Metchnikoff to the Microbiome: a journey that realized identification of Metchnikoff's ''lactic bacilli,'' validated the probiotic concept, and led to today's systematic metagenomic and metabolomic investigations of human and animal microbiomes. The reader is referred to additional perspectives and reviews concerning both the historical aspects and the present status of probiotic affairs. Included among these are the following recent works by De Vrese and Schrezenmier (2008), Lebeer *et al.* (2008), Walter (2008), Michail (2008), Lesser and Molbak (2009), Preidis and Versalovic (2009) and Fuller (1989, 1992b). These resources notwithstanding, most researchers interested in probiotics, microbiomics, and related disciplines are aware of the exponential growth that occurred in these fields during the past decade: growth that has begun to overwhelm attempts to review all relevant studies and the increasing number of prospective probiotic strains under consideration. The authors of the present review have chosen to address this dilemma in the following manner. We have abstracted information derived from a plethora of published studies on a well-established probiotic species with which we are most familiar. We believe that the extensive body of information obtained on this unique species during the past 25 years will provide the reader with a sound *conceptual template* from which to overview the evolution of the probiotic concept—from conception to validation to acceptance in the mainstream of biomedicine.

II. EARLY STAGES IN DEVELOPMENT OF THE PROBIOTIC CONCEPT

As noted by Fuller (1992a) and Casas and Dobrogosz (2000), the *first stage* in the evolution of the probiosis was its conceptual birth by Metchnikoff' (1907). Following his death in 1916, Rettger and Chaplin (1921), Kopeloff (1926), and Rettger *et al.* (1935) initiated a *second stage* using *Lactobacillus acidophilus* as a dietary supplement. They conducted systematic investigations and even initiated human clinical trials in which intestinal isolates of *L. acidophilus* were used. But it was not until after World War II, particularly during the 1950s–1980s era, when research concerning interactions between gut microbes and their host entered into a *third stage* of development. Both negative and positive interactions, as predicted by Metchnikoff, were investigated during this stage using animal hosts. A negative role for gastrointestinal (GI) microorganisms was clearly established during this period. This was shown, for example, when certain peroral antibiotic treatments resulted in an increased growth of chickens. Antibiotics continue to be added to animal feeds today as growth promoters. During the 1950s, germ-free technologies also became available, particularly in the United States, Sweden, Japan, and the United Kingdom. Again, a negative effect of gut microbes was shown. Germ-free animals not only maintained good health but in many instances outlived their non-germ-free counterparts, further confirming Metchnikoff's contention that certain gut microbes can adversely affect a host's health and life span.

However, positive, beneficial roles for gut microbes were also revealed during this period. It was proven that indigenous microbes provided their hosts with a mucosa-associated shield, which protected them from a variety of infectious diseases. Seminal findings along these lines by Bonhoff *et al.* (1954), Freter (1956, 1992), and others showed that oral antibiotic administrations rendered experimental mice more susceptible to infections with *Salmonella*, *Shigella*, and *Vibrio* spp. Others found that antibiotic treatments induced pseudomembraneous colitis caused by *Clostridium difficile* (Wilson, 1993), and that administration of fecal suspensions could successfully treat such antibiotic-associated diarrheas. Nurmi and Rantala (1973) demonstrated the protective role of the GI microbes, showing that competitive exclusion (CE) of *Salmonella* from the gut of chickens could be achieved by early oral administrations of cecal extracts obtained from healthy adults. CE attributable to the gut microbes was confirmed for other *Salmonella* spp. (Snoeyenbos *et al.*, 1978,1983) and other enteropathogens such as *Escherichia coli* (Weinack *et al.*, 1981), *Campylobacter* (Soejadi *et al.*, 1982; Soejadi-Liem *et al.*, 1984a), and *Yersinia enterocolitica* (Soejadi-Liem *et al.*, 1984b). Impey *et al.* (1982) showed a similar protective effect following administration of 48 selected gut microbes. Collins and Carter (1978) used

germ-free animals to provide one of the most convincing studies showing the protective effect of gastrointestinal microbes. They showed that while a germfree guinea pig was killed by as few as 10 *Salmonella enteritidis* cells inoculated *per os*, 10^9 cells were required to kill a guinea pig possessing its conventional microbiota. As attested by Havenaar and Huis in't Veld (1992), these animal studies using cecal extracts as probiotic formulations tended to affirm the probiotic concept.

Such positive results notwithstanding, the probiotic concept continued to languish throughout this period. Skepticism persisted, based largely on the poor quality and statistical ineffectiveness of many of the cultured probiotics that were marketed for the animal production industries. Barrow (1992) addressed this matter forthrightly in a critical resume of the flawed and unscientific practices that perpetuated negative impressions and served ''only to create a mystique of probiosis without an adequate, rational assessment of its true value.'' Along these same lines, Freter (1992) noted that ''although we've already passed the hundredth anniversary of the probiotic concept, it is in many ways still the probiotic concept.'' He argued further, that without future critical studies of basic mechanisms and successful field trials, the practice of probiotics would continue to be viewed by many as ''an exploitation of oversimplified ecological theories.'' Perhaps most damaging to the ''probiotic concept'' during this period was the lamentable degree of commercial exploitation of the concept in the marketplace. Gilliland and Speck (1977) reported, among numerous other examples of misuse, that both human and animal probiotic products labeled as containing *L. acidophilus* often do not contain any viable *Lactobacillus* species, let alone the advertised *L. acidophilus*. Furthermore, the study of probiotics had languished too long under the burden of shallowness supported more by anecdotes, abstracts of unpublished findings, and commercial testimonies than by sound scientific analyses. Little wonder that articles appeared in the literature during this period with titles such as: ''Probiotics, Prebiotics or 'Conbiotics'?'' (Berg, 1998), ''Probiotic bacteria: myth or reality'' (Sullivan *et al.*, 1992), and ''Probiotics—snake oil for the new millennium?'' (Atlas, 1999). Freter (1992) expressed a healthy skepticism along these lines by stating that probiotic ''preparations containing a single or few types of bacteria are limited by ecological necessity.'' Impey *et al.* (1982) expressed a similar view based on their finding that as many as 48 strains of bacteria were required to produce a protective effect against colonization of the avian gut by salmonellae. Along these same lines, Hentges (1992) stated that: ''In the restoration of colonization resistance in the gastrointestinal tract, it is improbable—that probiotic measures designed to alter the ecology of the intestinal luminal contents will be successful.''

III. THE VALIDATION STAGE IN DEVELOPMENT OF THE PROBIOTIC CONCEPT

During the mid-1980s and through the 1990s, skeptics notwithstanding, it became apparent that probiotic concept had in fact entered into the *fourth stage* of development—a stage in which researchers, practitioners, and the marketplace rejected uncritical appraisals of probiotics and accepted only sound scientific studies and statistically significant evidence of their efficacy. Clearly, the probiotic concept had evolved during this period into the science of probiotics (Fuller, 1997; Wood, 1992; Salminen and von Wright, 1993, 1998). Attesting to this transition, Havenaar and Huis in't Veld (1992) proposed that: ''If we want to get rid of the mysticism surrounding probiotics, fundamental research is necessary to collect information on how probiotics act. This involves development of adequate methods to quantify, localize, and identify the changes in the intestinal microflora, to establish basic criteria for the selection of bacterial strains, and to perform well-controlled animal experiments, field trials, and studies in humans. The mechanism by which probiotics exert their action must be the subject of future research.''

Tannock (1992) described characteristics expected of an ''ideal probiotic'' strain as follows: It should (a) persist for a long time in the GI tract, (b) produce a substance inhibitory to GI pathogens or stimulate host immunity so as to increase resistance of the host to intestinal infection, (c) contribute to the host's nutrition by synthesizing essential nutrients that thus become more readily available to the host and/or by digesting dietary substances (e.g., lactose) that the host may be physiologically ill-equipped to utilize, (d) be suitable for cultivation on a large commercial scale, (e) be safe and devoid of characteristics that could compromise the host's health, and (f) exhibit stability in all the above characteristics. However, he also theorized that attempts to isolate such an ideal probiotic strain would most likely fail, and that an alternative stratagem would be to derive such a strain by genetic manipulation.

Havenaar and Huis in't Veld (1992) argued that it may be possible to isolate such ideal strains, but only if proper screening methods were used to identify their ''ideal'' traits. They suggested that *in vivo* efficacy testing be conducted only on strains thus selected to possess these ''ideal'' traits. Barrow (1992), on the other hand, was concerned about the high degree of variability observed when *in vivo* efficacy tests were conducted. He attributed this variability, among other factors, to poor characterization of the strains used in the past and/or poor understanding of the microecology of the GI tract, positing that efficacy evaluations conducted using strains lacking either ''ideal'' traits or host specificity are not likely to succeed in any event and, furthermore, that in too many instances, the

occasional ''positive'' results obtained were often overoptimistically and/or uncritically interpreted, serving ''only to create a mystique of probiosis without an adequate rational assessment of its true value.''

Casas and Dobrogosz (2000) summarized a 15-year study that focused on the probiotic potential of previously unheralded heterofermentative *Lactobacillus* species found to inhabit the GI tract in all animals examined to date, including humans (Fujisawa *et al.*, 1996; Mitsuoka, 1992). This species, *L. reuteri*, is reported to be one of only three or four *Lactobacillus* species reported to be truly indigenous inhabitants in the human gut (Reuter, 2001). The Casas and Dobrogosz (2000) report concluded that host-specific strains of *L. reuteri* possessed (indeed exceeded) all characteristics required to be Tannock's (1992) ''ideal probiotic,'' noting that no genetic manipulation was required. The importance of host specificity as an ''ideal trait'' cited by Havenaar and Huis in't Veld (1992) was also deemed an important factor in demonstrating probiotic efficacy. The discovery that *L. reuteri* strains are able to produce an antimicrobial metabolite (termed reuterin) when grown in the presence of glycerol provided a method to selectively isolate, identify, and quantify host-specific strains of *L. reuteri* from gut of all animals used in these studies. Strains thus isolated from varied hosts were grown and administered orally as viable preparations to their respective hosts in order to ascertain their safety and probiotic efficacy. *L. reuteri* cultures isolated from various hosts were shown to exhibit statistically significant probiotic efficacy when administered to their respective challenged hosts. To date, no other ''lactic bacillus'' has accomplished this task or so convincingly validated Metchnikoff's probiotic concept (Casas and Dobrogosz, 2000; Dobrogosz, 2005; Dobrogosz and Roos, 2008).

Most probiotic microbes studied to date belong to the genera *Lactobacillus* and *Bifidobacterium*, although some species of fungi (e.g., *Saccharomyces cerevisiae*) and enterococci (e.g., *Enterococcus faecalis*) are also considered *bona fide* probiotics. Among the lactobacilli, a variety of species have been studied including *L. acidophilus* NCFM, *L. gasseri*, *L. johnsonii*, *L. casei* Shirota, and *L. plantarum* 299v, and among the bifidobacteria *B. longum*, *B. bifidum*, *B. infantis*, *B. adolescentis*, and *B. breve* (De Vrese and Schrezenmier, 2008), and also a special coliform *E. coli* Nissle 1917. Given the near-exponential growth in probiotic, prebiotic, and symbiotic studies, particularly during the past decade, and the numerous recent reviews currently available on these studies, the present review will focus largely, but not exclusively, on the science and technology underlying the safety, probiotic efficacy, clinical availability, and mode(s) of action of *L. reuteri*. It is the probiotic species that is most familiar to the present authors, and may be seen by the reader as the ''conceptual template'' from which to view the evolution of the probiotic concept. In this connection, the entire species has been shown to exhibit probiotic efficacy. *L. reuteri* cultures

isolated from various hosts, ranging phylogenetically from avians to humans, have been shown to exhibit probiotic efficacy when administered to their challenged hosts. To date, no other ''lactic bacillus'' has accomplished this task or so convincingly validated Metchnikoff's probiotic concept (Casas and Dobrogosz, 2000).

IV. EVOLUTION IN PROBIOTIC PERSPECTIVES AND DEFINITIONS

A. Emergence of the microbiome concept

Lederberg (2004) has termed the cohabitation of the myriad microbial genomes within our bodies as a ''microbiome'' and considered additional information on the nature and functions of this microbiome as ''central to understanding the dynamics of health and disease.'' Gill *et al.* (2006) and Ley *et al.* (2006) have undertaken metagenomic and ecological analyses of the human distal gut microbiome, proposing humans to be ''superorganisms whose metabolism represents an amalgamation of microbial and human attributes.'' O'Hara and Shanahan (2006, 2007) view the microbiome and its collective metabolic activity ''equal to a virtual organ within an organ,'' and that ''an improved understanding of this hidden organ will reveal secrets relevant to human health and to several infectious, inflammatory, and neoplastic disease processes.'' They posit that ''studies to elucidate the molecular mechanisms of host–microbiota interactions are needed to lend credence to the use of pharmabiotic strategies in clinical medicine.'' It is evident that host–microbiota interactions have been too long underestimated given (a) the vast cellular, genomic, and functional dimensions of the microbiomic organ; (b) that there are at least 500–1000 microbial species residing within the adult intestine and other mucosal tissues biofilms, harboring at least 100 times as many genes as the human genome; and (c) that these microbiomic components outnumber their human hosts on a cell-to-cell basis by at least 10–1, granting us the status of having evolved as a 90% microbial–10% human superorganism (Ley *et al.*, 2006).

B. Alternative definitions proposed for probiotics

These new perspectives on host–microbe relationships also prompted attempts to redefine the term 'probiotics' and to better understand their specific metabolic and immunological functions as microbiomic components. Clancy (2003) recently suggested that ''the term 'probiotic' had served its useful generic function of drawing attention to 'health promoting' bacteria at mucosal surfaces,'' but to continue to use ''the

term 'probiotic' for those bacteria which promote health by immunomodultory means would appear outmoded, and potentially confusing when communicating about immune-regulating bacterial species.'' He suggested another term—immunobiotics—to identify ''those bacteria that promote health through activation of the mucosal immune apparatus,'' and that ''recognition of bacteria that promote mucosal T cell function as immunobiotics moves probiotic biology forward by focusing on a mechanism of outcome, that is, immunomodulation at distant mucosal sites. It does not, however, diminish the need to attend longstanding concerns regarding the biology and accreditation of these bacteria as health enhancing agents.'' O'Hara and Shanahan (2006, 2007) suggested a more generic term—pharmabiotics—''to encompass any form of therapeutic exploitation of the commensal flora, including the use of live probiotic bacteria, probiotic-derived biologically active metabolites, prebiotics, synbiotics, or genetically modified commensal bacteria.'' Perhaps at a future date, another more generic term such as immunoprobiotic may be deemed more appropriate given that some probiotic strains produce a number of metabolic products, which may contribute to a host's health and well-being by nonimmunological means. *L. reuteri* strains, for example, have been shown to produce active metabolic products, including vitamin B_{12}, conjugated linoleic acid (CLA), bile salts hydrolase activity conferring an adaptive response to bile acid stress and tonicity, and novel glucan and fructan prebiotics, the antimicrobials reuterin and reutericyclin, and various adhesions (Dobrogosz and Roos, 2008). Future studies are needed to determine whether or not any of these metabolic products play a role in *L. reuteri*'s probiotic effectiveness. Future studies may also address yet another terminological issue. Andersson *et al.* (2001) defined the term ''synbiotics'' as ''mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract of the host.'' What term then describes a probiotic that synthesizes its own prebiotics?

C. Multiple roles played by specific microbiomic components

Schiffrin and Blum (2002) offered a synoptic overview of the multiple roles that microbiomic components have been shown to play, namely: ''Specific components of the intestinal microflora, including lactobacilli and bifidobacteria, have been associated with beneficial effects on the host, such as promotion of gut maturation and integrity, antagonisms against pathogens and immune modulation. In addition, the microflora seem to play a significant role in the maintenance of intestinal immune homeostasis and prevention of inflammation.''

As mentioned earlier, *L. reuteri* may be unique among the above-cited specific components of the intestinal microbiota (Casas and Dobrogosz,

2000). It is among few *Lactobacillus* species (along with *L. gasseri*, *L. ruminis*, and to some degree *L. salivarius*) (a) known to be indigenous components of the mucosal microbiome in both humans and animals (Fujisawa *et al.*, 1996; Mitsuoka, 1992; Reuter, 2001), (b) shown to provide all of the above-noted ''beneficial effects'' when viable, host-specific strains are administered to their respective hosts, and (c) shown to confer broad-spectrum protection from an assortment of diseases in a wide range of hosts as evidenced by numerous animal field trials and human clinical trials. These broad-spectrum effects were observed in controlled studies in which subjects lacking detectable *L. reuteri* were compared to those administered host-specific strains of *L. reuteri* (Casas and Dobrogosz, 2000). In each case, administrations of viable *L. reuteri* cells conferred significant protection irrespective of whether the disease was attributable to biological agents (i.e., bacteria, viruses, fungi, or protozoa), certain chemical agents (e.g., methotrexate-associated enteritis, acetic acid-induced colitis), or even environmental stressors (e.g., cold-stress). Furthermore, histological and microbiological analyses of intestinal tissues obtained during these studies showed that *L. reuteri* (a) effectively protected the integrity of the gut mucosal barrier, (b) prevented translocation of luminal microbes to extra-intestinal sites, and (c) effectively downregulated inflammation associated with these biological, chemical, or environmental insults.

D. New perspectives on host–microbiome interrelationships

Rakoff-Naholm *et al.* (2004) published a conceptual breakthrough in understanding the molecular communication networks that exist between certain microbiome components and associated host tissues. New information was provided in which host cell Toll-like receptors (TLRs) are shown to play key roles in creating homeostatic forces that balance immunobiotic protection on the one hand versus pathogen infection/tissue damage on the other.

These studies employed mice deficient in MyD88, an adaptor molecule essential for TLR-mediated induction of inflammatory cytokines (Takeda *et al.*, 2003), as well as mice deficient in TLR2 and TLR4. Wild-type (wt) mice were used as comparative controls. It was hypothesized that the MyD88$^{-/-}$ mice would be unable to mount TRL-dependent inflammatory responses to their commensal bacteria and, therefore, would not be susceptible to intestinal pathology following dextran sodium sulfate (DSS, a sulfated polysaccharide toxic to colonic epithelium) administration. Surprisingly, and in sharp contrast to the wt mice which had 100% survival, the My D88$^{-/-}$ mice showed severe mortality and morbidity from the DSS treatment. Although mortalities were less pronounced, the TLR4$^{-/-}$ and TLR2$^{-/-}$ mice were similarly affected by the DSS treatment, indicating ''the severe mortality seen in the MyD88$^{-/-}$

animals was the result of defective signaling of multiple TLRs induced by various commensally-derived products.'' Severe and extensive colonic erosion, ulceration, and epithelial injury were observed post-DSS administration compared to the wt controls. It was also determined that the mortality of the DSS-treated MyD88 $^{/}$ mice was not caused by commensal overgrowth or by hyperinfiltrating leukocytes.

All additional evidence reported in this rigorous study (Rakoff-Naholm *et al.*, 2004) and other studies (Takeda *et al.*, 2003), leads to the conclusion that ''TLR-mediated recognition of commensals in the colon regulates production of tissue protective factors,'' thereby conferring ''protection from mortality, morbidity, colonic bleeding, and intestinal epithelial damage caused by administration of the injurious DSS.'' Also, TLR-mediated recognition of commensal bacteria occurred both under normal conditions with an intact epithelium as well as during intestinal injury. In further confirmation of these conclusions, Rakoff-Naholm *et al.* (2004) also showed that removal of the commensal microbiota in the wt mice by means of 4-week oral administration of broad-spectrum antibiotics—vancomycin, neomycin, metronidazole, and ampicillin—resulted in severe mortality and morbidity when subsequently administered DSS. Although these data suggested that commensal bacterial products engage TLRs and thus protect from DSS-induced epithelial injury, it remained to be determined whether this commensal-associated protection was caused by recognition of commensal products by TLRs or by metabolic activities of the commensals. The former proved to be the case when either purified lipopolysaccharide (LPS), a TLR4 ligand, or lipoteichoic acid (LTA), a TLR2 ligand, was administered in the drinking water of microbiota-depleted animals 1 week prior to and during administration of DSS. Both ligands completely protected the animals from the DSS-induced mortality, morbidity, and severe colonic bleeding seen in mice with colons depleted of commensal microbiota. They concluded that these findings reveal a new role of commensal microflora and the innate immune system in mammalian physiology and that ''the interaction of commensal bacterial products with host microbial pattern recognition receptors plays a critical role in resistance to epithelial injury and presumably in other aspects of epithelial homeostasis.''

Madara (2004) provided a graphic perspective on the importance of such ongoing microbe–TLR signaling in maintenance of intestinal immune homeostasis, the consequences of which are illustrated in Fig. 1.1. Intact signaling with appropriate health-enhancing microbial ligands results in homeostasis and a robust response to injuries (2nd Panel). On the other hand, disrupted signaling attributable either to faulty TLR receptor functioning (3rd Panel) or to the absence of appropriate microbial ligands (4th Panel) results in enhanced sensitivity to injury and a diminished capacity to induce repair functions.

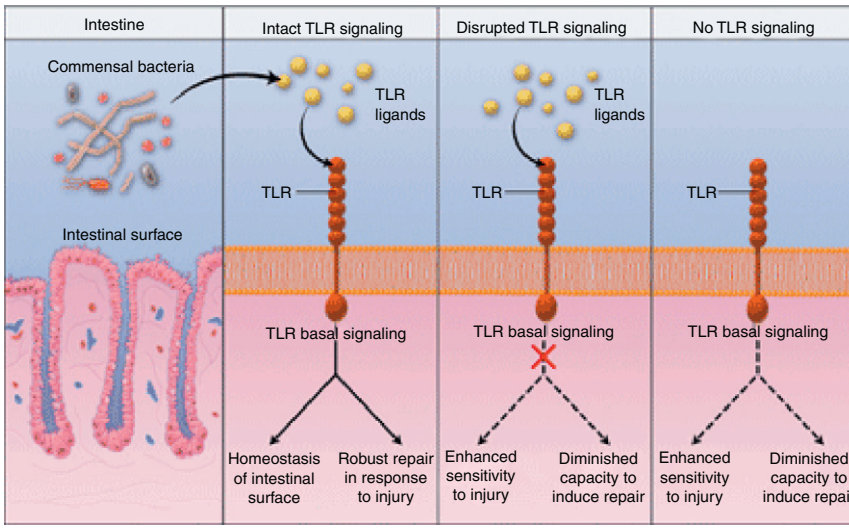

FIGURE 1.1 Role of toll-like receptors (TLRs)—commensal microbe interactions in maintenance of intestinal immune homeostasis. Commensal bacteria secrete TLR ligands such as lipopolysaccharide and lipoteichoic acid, which interact in the normal intestine with a population of surface TLRs. The resultant basal signaling, which is normally ongoing, enhances the ability of the epithelial surface to withstand injury while also priming the surface for enhanced repair responses. Because many types of cells on the surface of the intestine—epithelial cells, lymphocytes between the epithelial cells, subepithelial mesenchymal cells, macrophages, and dendritic cells—express TLRs, it is not known whether this critical homeostatic mechanism is maintained by the cell populations, which also respond by means of TLRs when the surface is breached. Either the disruption of TLR signaling or the removal of TLR ligands compromises the ability of the intestinal surface to withstand insult and effect repair. (Reprinted from Madara, 2004—with permission from NEJM.)

V. PROBIOTIC EFFICACY—CLINICAL TRIALS

As described elsewhere (Casas and Dobrogosz, 2000), numerous controlled safety and gut colonization studies were conducted using a variety of agriculturally important and laboratory animals administered host-specific strains of *L. reuteri*. Following their successful outcomes, human clinical trials were initiated employing *L. reuteri* strain ATCC 55730/SD2112 (known commercially as *Lactobacillus reuteri* Protectis). This strain, isolated from human breast milk, was also tested for safety and is available commercially in a variety of formulations. *L. reuteri* is a natural component of human milk (Sinkiewicz and Ljunggren, 2008), and viable *L. reuteri* has been isolated from every part of the digestive tract: the oral

cavity, the stomach, the small intestine, the colon and feces as well as from the vagina (Reuter, 2001). Connolly (2004), Weizman and Alsheik (2006), and Wolf *et al.* (1995, 1998) reported on the clinical safety of administering viable *L. reuteri* ATCC 55730 to over 600 subjects including premature and full-term infants, preschool children and adults, including HIV positive adults. Connolly *et al.* (2005) (a) reported on the safety of administering viable DL-lactic acid-producing strains to infants (*L. reuteri* is a DL-lactic acid-producing heterofermentative *Lactobacillus* species) and (b) described a new type of probiotic product: an oil suspension of viable *L. reuteri* ATCC 55700 cells able to deliver in five drops (~0.17 ml), a daily dose of 10^8 cfu (colony forming units) to premature and very young infants (Connolly, 2005).

To date, 55 human clinical studies on more than 4100 individuals have been conducted, and the results are published in 28 articles in scientific journals. Another 13 studies are published as abstracts of presentation at scientific conferences. Following are summaries concerning clinical efficacy of this human *L. reuteri* probiotic strain.

A. Efficacy for rotavirus-induced diarrhea in children

Diarrheal diseases are one of the most common health problems encountered during childhood worldwide. During periods of acute diarrhea, the normal GI microbiota is radically changed, including decreases in *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* species (Salminen and Deighton, 1992; Tazume *et al.*, 1993). Several studies have indicated that *Lactobacillus* administrations accelerate normalization of the host's microbial balance and thereby moderate acute episodes of diarrhea. Among these, *L. rhamnosus* GG has been shown to promote clinical recovery from rotavirus gastroenteritis in children and enhance intestinal immune responses (Isolauri *et al.*, 1991; Kaila *et al.*, 1992; Majamaa *et al.*, 1995; Salminen *et al.*, 1995).

Shornikova *et al.* (1997a,b) conducted placebo-controlled, double-blinded clinical studies using *L. reuteri* ATCC 55730, confirming both a bioprophylactic and a biotherapeutic efficacy in treating 6- to 36-month-old Finnish children hospitalized due to acute rotavirus-associated diarrhoea. Eom *et al.* (2005) reported similar results on 50 hospitalized Korean children, aged 6–36 months, 40% having been diagnosed with a rotavirus infection. The children received 10^8 cfu *L. reuteri* twice a day for a 5-day period or a matching placebo. It was also concluded that *L. reuteri* ATCC 55730 was an effective therapeutic agent in children hospitalized with acute diarrhea.

Oil formulations of *L. reuteri* ATCC 55730 were administered for 28 days to premature infants in a prospective, placebo-controlled clinical investigation conducted by Romeo *et al.* (2009). These infants were also administered

liquid suspensions of *L. rhamnosus* GG for comparative purposes. The results showed that both administrations significantly reduced the number of bacterial and *Candida* infections compared to the placebo-controlled infants. Both probiotic strains improved their gastrointestinal symptoms, although greater efficacy was exhibited by *L. reuteri* in this regard. Only the *L. reuteri* administrations, however, reduced the hospital time to only 3 weeks in comparison with the placebo- and *L. rhamnosus* GG-treated subjects who had the usual 6 weeks hospital time.

B. Treatment of infantile colic, feeding tolerance, bowel habits, and gastrointestinal motility in preterm newborns

A prospective, randomized study was conducted by Savino *et al.* (2007) to determine whether *L. reuteri* ATCC 55730 oil administrations would improve confirmed infantile colic in 90 breastfed infants 11–80 days old. The infants were randomly assigned to two treatment groups: one group received *L. reuteri* at a dose of 10^8 cfu/day via the oil formulation, and the other received 60 mg/day of Simethicone. No side effects were observed, and it was concluded that administrations of *L. reuteri* significantly ($p = 0.005$) reduced the crying time within 1 week compared to standard Simethicone therapy in these infants. This positive effect was even more pronounced ($p < 0.001$) at the end of the 4-week study. Indrio *et al.* (2008) reported that this *L. reuteri* supplementation to preterm formula-fed newborns improved their tolerance to the formula, showed a significant decrease in regurgitation and mean daily crying time, and increased the number of stools compared to those given placebo.

C. Protection of infants from infections in child care centers

Weizman *et al.* (2005) also conducted a randomized, double-blind, and placebo-controlled study to evaluate the effects of two probiotics during a 12-week period on the occurrence of infections in 194 infants aged 4–10 months in day care. Three different groups were given formula containing *L. reuteri* ATCC 55730 and *Bifidobacterium lactis* Bb-12 or formula without probiotics. No safety issues were noted during the 3-day study. Both probiotic formulas resulted in fewer infections in these infants compared to the placebo group as measured by the number of febrile episodes and episodes of GI infection. It was also found that *L. reuteri* ATCC 55730 had a significantly superior effect compared to *Bif. lactis* Bb-12 and the control group. There were significantly fewer doctor's visits, less antibiotics, and fewer absent days from day care in the *L. reuteri* group.

D. Effects during anti-*Helicobacter pylori* treatment

1. In children

A double-blind, randomized, and placebo-controlled study investigating the effect of *L. reuteri* ATCC 55730 on GI side effects during and after anti-*Helicobacter pylori* treatment was conducted by Lionetti *et al.* (2006). Forty dyspeptic children, aged 3–18 years, with confirmed *H. pylori* infection were enrolled. They were treated with 10-day sequential antibiotic treatment and randomized to receive either 10^8 cfu/day *L. reuteri* ATCC 55730 or placebo for 20 days, starting from the first day of treatment. The severity of side effects was measured using a validated Gastrointestinal Symptom-Rating Scale (GSRS) scoring system, with both groups having similar GSRS scores at entry of 6.9 and 7.1, respectively. But during eradication therapy and the follow-up, the *L. reuteri*-supplemented children significantly improved their GI health compared to the placebo group: on day 10, the scores were 4.1 in the *L. reuteri* group versus 6.2 in the placebo group ($p < 0.01$) and, on day 20, the scores were 3.2 versus 5.8 ($p < 0.009$), respectively. The rates of *H. pylori* eradication were the same in both groups, but the *L. reuteri* supplementation during and after eradication therapy significantly reduced the frequency and intensity the antibiotic-associated side effects.

2. In adults

A small pilot study to determine whether 6 months' supplementation with *L. reuteri* had any effect on *H. pylori* colonization and gastric mucosal inflammation in seven dyspeptic adult patients showed no effect on either *H. pylori* colonization or degree of stomach inflammation. *L. reuteri* was well tolerated and able to colonize the stomach in these *H. pylori*-infected patients (Glintborg *et al.*, 2006). Three other clinical studies showed positive effects from *L. reuteri* supplementation. Saggioro (2004) conducted a prospective, randomized, double-blind, and placebo-controlled study on eradication of *H. pylori* by *L. reuteri* ATCC 55730. Thirty patients were enrolled, aged 25–56 and suffering from dyspepsia caused by confirmed infection with *H. pylori.* Fifteen patients were given omeprazole (20 mg/day) plus 10^8 cfu *L. reuteri* twice daily, and 15 received omeprazole plus placebo, both for 30 days. The extent of the infection was determined 4 weeks after the end of the therapy. In 60% (9/15) of the patients supplemented with *L. reuteri*, *H. pylori* was totally eradicated, while no eradication occurred in the group that received omeprazole plus placebo ($p < 0.0001$). It was concluded that the *L. reuteri* supplementation had a beneficial effect in treating *H. pylori* infection in humans. Imase *et al.* (2007) investigated whether the urea breath test (UBT) could be used as a marker for burden of *H. pylori* infection, and to determine whether administration of *L. reuteri* chewable tablets could suppress the infection.

From the results of this study, it was concluded that *L. reuteri* administrations significantly decreased UBT values in *H. pylori* positive subjects, demonstrating that *L. reuteri* suppresses *H. pylori* density. Francavilla *et al.* (2008) conducted a prospective, double-blind, and placebo-controlled study in 40 dyspeptic adults infected with *H. pylori*. The test was to determine whether *L. reuteri* reduces the *H. pylori* levels in the stomach, decreases dyspeptic symptoms, and affects the results of conventional treatments with antibiotics. The subjects were randomly assigned to take *L. reuteri* (1×10^8 cfu/day) once daily for 28 days, or placebo. It was concluded that *L. reuteri* effectively suppressed the *H. pylori* infection and reduced the overall occurrence of dyspeptic symptoms, particularly abdominal distension, abnormal defecation, and gas. *L. reuteri* did not affect or interfere with the antibiotic treatment of the infection.

E. Increase work-place healthiness in adults

Tubelius *et al.* (2005) obtained evidence for a significant prophylactic effect of daily ingestion of 10^8 cfu/day of *L. reuteri* ATCC 55730. It enhanced work-place healthiness among Swedish working adults. Day and shift workers healthy at the start of the trial were randomized in double-blind fashion to receive the *L. reuteri* or placebo for 80 days. Of the subjects that complied with the study protocol, 94 received *L. reuteri* and 87 received placebo. Sick leave for defined respiratory or GI infections in the placebo group during this period was 26.4% compared to 10.6% in the *L. reuteri* group ($p < 0.01$). Among the 53 shift workers, 33% in the placebo group reported sick compared to none in the *L. reuteri* group ($p < 0.005$).

F. Improvements on oral health

A series of studies conducted by Nikawa *et al.* (2004) led to the development of *L. reuteri* formulations able to significantly inhibit growth of *Streptococcus mutans*—a bacterium correlated with the risk of dental caries and gingivitis. They screened 18 different fermented dairy products available in Japan, showing that *L. reuteri* was the only strain isolated from these products that inhibited the growth of *S. mutans* and had no harmful effect on dental enamel. This finding was followed by a clinical study with subjects having healthy mouths. Half of them ingested 95 g/day of yoghurt containing *L. reuteri*, while half ingested the same amount of placebo yoghurt. After 2 weeks, the groups changed the study product and the subjects thus served as their own controls. Both groups showed a significant decrease in the number of *S. mutans* during the 2-week period when ingesting *L. reuteri*. A similar clinical outcome was reported in another randomized, placebo-controlled study aimed at investigating the effect on mutans streptococci and lactobacilli of *L. reuteri* delivered

by two nondairy delivery systems, namely, *L. reuteri* tablets and *L. reuteri*-containing straws (Caglar *et al.*, 2006). The study included 120 young adults (21–24 years) divided into four equally sized groups ($n = 30$). Salivary mutan streptococci and lactobacilli were enumerated at baseline and 1 day after the final ingestions. A statistically significant reduction of *S. mutans* was recorded after ingestion of the *L. reuteri* via tablets or straws. A similar but not significant trend was seen for other lactobacilli. Another prospective, randomized, placebo-controlled trial was conducted to study the effect of two strains of *L. reuteri* (Lr-1, Lr-2) versus placebo on gingivitis and dental plaque on subjects suffering from moderate to severe gingivitis (Krasse *et al.*, 2006). The subjects were randomly assigned to one of these three different chewing gum groups. On day 0, the dentist cleaned all surfaces, and the patients were instructed regarding daily oral hygiene and the use of their respective chewing gum thrice daily after brushing. After 2 weeks, the patients visited the dentist for outcome evaluations. Gingival index fell significantly in all groups. The Lr-1 group, but not the Lr-2 group, improved more significantly than placebo ($p < 0.0001$). Plaque index fell significantly in both *L. reuteri* groups between day 0 and 14, whereas there was no significant change in the placebo group. Both strains colonized the saliva—Lr-1 in 65% of patients and Lr-2 in 95% of patients. It was concluded that *L. reuteri* was efficacious in reducing both gingivitis and dental plaque in patients with moderate to severe gingivitis.

VI. PROBIOTICS AS IMMUNOMODULATORY AGENTS

The protective effects of *L. reuteri* administrations described above and elsewhere (Casas and Dobrogosz, 2000) could be attributed to various modes of interactions or combinations thereof, including, for example, (a) competitive exclusion of gut pathogens, (b) inhibition of pathogen persistence by production of antimicrobial agents such as reuterin, reutericyclin, bacteriocins, and/or hydrogen peroxide, or (c) adsorption and/or neutralization of toxic substances. While such nonimmunological activities may play important roles under certain circumstances, beneficial immunomodulatory effects were judged to play the more predominant role. It was shown, for example, that (a) healthy *L. reuteri*-colonized chicks developed significantly deeper ileal crypts and longer villi than their placebo-controlled, healthy counterparts, and (b) healthy *L. reuteri*-treated birds had a significantly higher $CD4^+/CD8^+$ ratio specifically in the ileal regions of their gut than in the ileum of their nontreated counterparts (Casas and Dobrogosz, 2000). A similar effect was observed when the human strain, *L. reuteri* ATCC 55730, was administered to human adult subjects. In a first report of its kind, Valeur *et al.* (2004) demonstrated a

similar effect in human subjects. They obtained *in situ* evidence showing (a) distribution of individual human-specific *L. reuteri* ATCC 55730 cells throughout the human gut, and (b) involvement of this strain in recruitment and/or proliferation of $CD4^+$ T cells specifically in the ileal regions of the gut, concluding that ''*L. reuteri* administrations elicited a recruitment of $CD4^+$ T-helper cells to the human epithelium, and that this recruitment may be one factor in explaining the probiotic effect of this *L. reuteri* strain in man.'' It remains to be determined which subset or subsets of $CD4^+$ T cells were in fact recruited/produced in response to the *L. reuteri* ATCC 55730 administration. A recent study to be addressed later in this review revealed the ability of another human strain, *L. reuteri* ASM 20016, to prime monocyte-derived dendritic cells (DCs) to induce development of regulatory T cells (Tregs). It remains to be determined whether Tregs are prominent among those elicited by *L. reuteri* ATCC 55730 in the Valeur *et al.* (2004) study.

A. *In vitro* and *ex vivo* immunomodulatory activities

Maassen *et al.* (2000a) summarized a number of *in vitro* and *ex vivo* studies showing that certain gut lactobacilli are capable of modulating a host's immune responses to exogenous antigens by either inducing or repressing production and/or release of cytokines and other immunoactive moieties by gut-associated immunocompetent cells. The first of these studies to include *L. reuteri* was published by Marin *et al.* (1997). They introduced heat-killed *L. reuteri in vitro* to macrophage (RAW 264.7 cells) and T-helper cell (EL4.IL-2 thymoma cells) models with and without stimulation by LPS or phorbol 12-myristate-13-acetate (PMA), respectively. *L. reuteri* strain ATCC 23272 induced TNF-α production in both the LPS-stimulated and unstimulated macrophage cultures, and induced IL-6 production in the un-stimulated macrophage but less so in the stimulated macrophage. The T-cell cultures showed enhanced interleukin-2 (IL-2) and interleukin-5 (IL-5) production after *L. reuteri* exposure but only when stimulated by PMA. It was concluded that direct interaction of *L. reuteri* with the model macrophages enhanced cytokine production and that the effects on the T-cell activities were smaller. In a similar study, the inductive effects of the heat-killed whole cells were compared to their respective cell wall and cytoplasmic fractions (Tejada and Pestka, 1999). It was shown that whole *L. reuteri* ATCC 23272 cells as well as their cell wall and cytoplasmic fractions stimulated macrophages to produce significant amounts of inflammatory mediators: TNF-α, IL-6, and nitric oxide (NO). These effects were not seen using splenic or Peyer's patch cell cultures.

Tejada *et al.* (1999) reported studies describing the *ex vivo* production of immunological mediators by primary leukocytes from mice that were

orally administered lactic acid bacteria. Eight different potential probiotic and dairy starter strains were tested including *L. reuteri* ATCC 23272. Eight-week-old female B6C3F$_1$ mice were gavaged with 10^9 cfu of each strain. Eight hours later, peritoneal cells, Peyer's patch, and splenocytes were isolated, cultured for 2 or 5 days in the presence or absence of mitogens, and analyzed for production of IL-6, IL-12, interferon-γ (IFN-γ), TNF-α, and NO. The results showed that prior oral exposure to *L. reuteri* had no effect on *ex vivo* production of these cytokines and NO by Peyer's patch or splenocyte cultures. However, this exposure differentially potentiated or attenuated cytokine and NO production by peritoneal cells. For example, *L. acidophilus* La-2 and *L. casei* ATCC 39539 potentiated production of IL-6 and IL-12, and *L. acidophilus* upregulated IFN-γ and NO. In contrast, *L. reuteri* ATCC 23272 was among a group of lactic acid bacteria (including *L. helveticus* Lr-92, *L. gasseri* NCK101, and *Bifidobacterium* Bf-1) that significantly attenuated production of IL-6, IFN-γ, and NO by peritoneal cells.

Maassen *et al.* (2000b) investigated the ability of orally administered *L. reuteri* ML1 to induce specific mucosal cytokines in BALB/c mice. The mice were immunized i.p. with Chikungunya virus, fed *L. reuteri* for 4 days, and gut sections analyzed immunohistochemically for *in vivo* cytokine production. It was shown that *L. reuteri* ML1 induced several proinflammatory Th1 cytokines within the gut villi, including IL-1b, IL-2, and TNF-α, but not anti-inflammatory Th2 cytokines such as IL-10 and IL-4. TNF-α was produced by cells in the submucosa after oral administration of *L. reuteri*, but not after administration of the other lactobacilli tested. These *in vitro* and *ex vivo* studies revealed the ability of the strain *L. reuteri* ML1 to stimulate pro-inflammatory cytokines, demonstrating the diverging effects of specific strains of *L. reuteri*. It should be kept in mind that most *in vivo* studies conducted to date have provided clear evidence of potent anti-inflammatory activity of *L. reuteri* in the gut (Casas and Dobrogosz, 2000), and that different *L. reuteri* strains may have different effects on the cellular components of the innate immune system (Pena *et al.*, 2004).

B. Adjuvanticity for vaccine effectiveness

Maassen *et al.* (2000b) also reported on the ability of *L. reuteri* ML1 to function as an adjuvant by measuring systemic antibody responses after the mice were immunized i.p. with trinitrophenyl conjugated chicken gamma globulin (TNP-CGG). It was determined that *L. reuteri* ML1 significantly enhanced the systemic IgG antibody response against the parentally administered TNP-CGG antigen, leading them to the conclude that: ''The *Lactobacillus* strains *L. reuteri* (and *L. brevis*) are good candidates for expression of immunogenic antigens for oral vaccination purposes since they may stimulate innate, cellular, and humoral immune responses.''

These findings confirmed earlier observations on *L. reuteri* adjuvant effects on *S. typhimurium* antibody production in young turkey poults (Casas and Dobrogosz, 2000). Hoffmann *et al.* (2008) and Livingston *et al.* (2009) further confirmed this effect using a BALB/c RLF (reconstituted *Lactobacillus*-free) male and female mouse model to show that *L. reuteri* exhibited adjuvanticity for production of antibodies to subunit B of cholera toxin (CTB). The RLF mice were exposed from 3 weeks of age to each of five strains of lactobacilli: two mouse strains of *L. reuteri* (strains 100-23 and L4000), *L. johnsonii* NCC533, *L. paracasei* NCC 2461, or *L. rhamnosus* ATCC 53103. The mice were inoculated by intragastric intubation with 40 mg CTB at 6 weeks of age, and blood collected at 7 weeks of age. The sera were assayed by standard enzyme linked immunosorbant assay (ELISA) procedures for anti-CTB IgG, IgG1, and IgG2a. A differential adjuvant effect was shown: the two *L. reuteri* strains, *L. johnsonii*, and *L. paracasei* produced a statistically significant adjuvant effect; *L. rhamnosus* had no adjuvant effect. The ratio of IgG isotypes in the sera favored IgG1—the isotype characteristic of a Th2-mediated immune response. In the Maassen *et al.* (2000b) study cited earlier, *L. reuteri* ML1 induced relatively high IgG2a levels against CGG, a reflection of adjuvanticity for Th1 activity.

Researchers in Tannock's laboratory (Livingston *et al.*, 2009) also isolated bone marrow dendritic cells (DCs) from their RLF mice and measured the effect these *Lactobacillus* strains had on activation of DCs by assaying expression of cell surface markers CD80, CD86, and MHC class II antigens. Whereas CD86 and MHC class II antigens were unaffected, the CD80 was upregulated by all *Lactobacillus* strains when compared to the nonstimulated controls. Supernatants collected from these DCs stimulated with *L. reuteri* L4000 (marked adjuvant effect), *L. reuteri* 100-23 (small adjuvant effect), or *L. rhamnosus* (no adjuvant effect) were assayed for IL-10 and IL-12 (p70). *L. reuteri* L4000, which had the greatest adjuvant effect, stimulated more IL-10 production than the other two strains. IL-12 was not detected in these studies. Additional studies on *L. reuteri*-DC interactions are addressed below.

C. Modulation of delayed-type hypersensitivity responses

De Waard *et al.* (2002) used a *Listeria monocytogenes*-infected Wistar rat model to show that short-term ingestion of a viable, host-specific *L. reuteri* strain Utr 3 significantly decreased the 24-h delayed-type hypersensitivity (DTH) response to an intradermal challenge with heat-killed *L. monocytogenes*. Two other indigenous *Lactobacillus* strains, *L. johnsonii* Utr-1 and *L. murinus* Utr-2, did not diminish the DTH response, although all three *Lactobacillus* species, including *L. reuteri*, stimulated acquired cellular resistance as measured by significantly reduced viable *L. monocytogenes* in the spleen 36 h post re-infection. This report recalled earlier observations

(Casas and Dobrogosz, unpublished data) showing that, in comparison to untreated chicks, *L. reuteri*-administered chicks (at 5 and 20 days of age) exhibited significantly reduced phytohemaglutinin-P nonspecific DTH responses.

Based on cumulative studies cited above, it became evident that *L. reuteri* strains are well equipped to provoke varied immunomodulatory responses from various components of a host's gut-associated lymphoid tissues. These early finding provided first steps toward understanding *L. reuteri*'s probiotic mode(s) of action. The following four seminal studies transformed these first steps into pathways of new insights concerning microbe–host synergistic relationships in general, and *L. reuteri*'s immunobiotic mode(s) of action in particular.

1. Probiotic immunomodulatory modes of action: Four seminal studies

a. Pathological consequences of L. reuteri *depletion in the gut of IL 10 gene deficient mice*

Madsen *et al.* (1999, 2000) conducted the first of these seminal studies using IL-10-deficient mice that were known to develop a spontaneous, post-weaning colitis by 4 weeks of age. Both deficient mice and their wt counterpart controls were (a) analyzed for pattern changes in luminal and adherent bacteria before and during development of colitis and (b) scored for accompanying gut histological changes. Interesting and hitherto rarely explored relationships were revealed between a host's genetic constitution, the composition of its gut microbiota, and its susceptibility to inflammatory disease. These gene-deficient mice were shown to have a normal colon at 2 weeks of age, but by 4 weeks develop a mild spontaneous colitis that worsens progressively until it plateaus at 8 weeks. The age-matched control mice show no such colonic inflammation. Furthermore, at 2 weeks of age, the deficient mice show a significant increase both in colonic mucosal adherent and translocated aerobic bacteria when compared with their age-matched controls, indicating that a primary alteration had occurred in bacterial colonization of the gut. Also, at 2 weeks of age and prior to development of colonic injury, the deficient mice showed a significant decrease in the total population of protective colonic *Lactobacillus* species. This decrease persisted between 2 and 8 weeks of age—the interval during which colitis develops, implying ''that a critical period may exist when luminal bacteria play a pivotal role in the initiation of the mucosal injury.'' Furthermore, concerning *L. reuteri*, ''Speciation experiments showed that at 2 weeks of age, the control mice were colonized predominantly by *L. reuteri*, whereas the IL-10-gene-deficient mice were predominately colonized by *L. johnsonii*.''

Given these profound microbial alterations, Madsen *et al.* (1999, 2000) asked whether inoculating the deficient mice with an *L. reuteri* strain isolated from healthy control mice at 1 week of age could possibly prevent

development of colitis. The answer was yes. Following an *L. reuteri* inoculation, (a) the total *Lactobacillus* population increased, (b) the colonic mucosal adherent and translocated aerobic bacteria were normalized, and (c) the development of the colitis was attenuated. Other probiotic *Lactobacillus* strains also have been shown to alleviate the spontaneous colitis associated with an IL-10-deficiency. Schultz *et al.* (2002) used *L. plantarum* 299v, and McCarthy *et al.* (2003) used *L. salivarius* 433118 and *B. infantis* 35624, respectively and successfully, in their studies with these mice.

Pena *et al.* (2004) discovered similar differences in gut ecology when *Lactobacillus* populations in colitis-prone IL-10-deficient C57BL/6 mice were compared with those in mice without colitis, namely Swiss Webster and inducible nitric oxide synthetase-deficient C57BL/6 mice. From 20 mice without colitis, six *Lactobacillus* species were recovered; 72% of them being either *L. reuteri* or *L. murinus*. In contrast, only *L. johnsonii* was isolated from 14 IL-10-deficient mice, a finding similar to that reported above by Madsen *et al.* (1999, 2000). In addition, they showed that when representative lactobacilli from both sets of mice were tested for their ability to inhibit TNF-α production by LPS-activated murine RAW 264.7 macrophages, the *Lactobacillus* strains recovered from mice without colitis displayed TNF-α inhibitory properties, whereas none of the *L. johnsonii* isolates from the IL-10-deficient mice exhibited this effect.

Pena *et al.* (2005) selected two mouse-derived *Lactobacillus* strains, *L. reuteri* 6798 and *L. paracasei* 1602, which exhibited potent *in vitro* TNF-α inhibitory activity using this same macrophage cell line. A coculture of these two strains was evaluated for their ability *in vivo* to (a) ameliorate *Helicobacter hepaticus*-induced inflammatory bowel disease-like typhocolitis in IL-10-deficient C57BL/6 mice, and (b) simultaneously modulate their mucosal pro-inflammatory cytokine responses. The results from this study showed that coadministrations of these TNF-α-inhibitory lactobacilli lowered levels of the pro-inflammatory colonic cytokines, TNF-α, and IL-12, and reduced the intestinal inflammation in female *H. hepaticus*-challenged IL-10 deficient mice.

b. Probiotic regulation of dendritic cell functions The second seminal findings was reported by Christensen *et al.* (2002) who noted that prior investigations on effects of probiotic lactobacilli on immune functions tended to be ''inclusive and even conflicting in the absence of clear mechanistic data.'' To this point, they directed attention to dendritic cells (DCs) as primary targets for immunomodulation by probiotic *Lactobacillus* species known to be important members of the commensal gut microbiota. DCs are distributed throughout the GI tract where they reside in the Peyer's patches, lamina propria, and draining lymph nodes (Kelsall and Strober, 1997). They are believed to be gatekeepers of an immune

response, to be principal stimulators of naïve Th cells, and to play a pivotal immunoregulatory role in maintaining the Th1, Th2, and Treg/Tr1/Th3 cell balance (Banchereau and Steinman, 1998). Furthermore, it has been reported that DCs ''open the tight junctions between epithelial cells, send dendrites outside the epithelium, and directly sample bacteria. DCs express tight-junction proteins such as occludins, claudin 1, and zonula occludens 1, thus preserving the integrity of the epithelial barrier'' (Rescigno *et al.*, 2001). It is also known that DCs and other antigen-presenting cells (APCs) recognize the molecular composition of gut microbes via a family of pattern-recognition receptors (PRRs) designated Toll-like receptors (TLRs) (Medzhitov and Janeway, 2000; Rutells and Lemoli, 2004). The studies by Rakoff-Naholm *et al.* (2004), described earlier in this review, provided evidence that protection from infection, response to injuries, and control of gut epithelial homeostasis is largely dependent on TLR recognition of gut microbial components.

Christensen *et al.* (2002) hypothesized that species of *Lactobacillus*, including ingested probiotic strains, exert their effects by modulating the Th1/Th2/Th3/Tr1/Treg-promoting capacity of DCs in the gut. To test this hypothesis, six lethally gamma-irradiated strains of lactobacilli—*L. reuteri* DSM 12246, *L. plantarum* Lb1, *L. fermentum* Lb20, *L. casei* subsp. *lactic* CHCC3137, *L. plantarum* 299v, and *L. johnsonii* La1—were exposed *in vitro* to naïve DCs isolated and cultured from the bone marrow of female C57BL/6 mice. The resultant culture supernatant fractions were analyzed for cytokines IL-6, IL-10, IL-12, and TNF-α and for upregulation of MHC class II and B7-2 (CD86) surface markers. Substantial differences were found among these lactobacilli to induce IL-12 and TNF-α production by the DCs. Similar but less pronounced differences were observed in their abilities to induce production of IL-6 and IL-10, and all strains upregulated surface MHC class II and CD86 markers—indicative of DC maturation, although species with the greatest capacity to induce IL-12 were most effective in this regard.

Particularly interesting results were obtained when the DC cultures were exposed to mixtures of varying concentrations of *L. reuteri* and *L. casei*. It was shown that *L. reuteri*, a poor IL-12 inducer, differentially inhibited IL-12, IL-6, and TNF-α induction during coculture with *L. casei*, while IL-10 production remained unaltered. Similar effects by *L. reuteri* were observed when cocultured with *L. johnsonii* rather than *L. casei*. These findings showed that different strains of *Lactobacillus* signal different DC activation patterns. Furthermore, they showed that *L. reuteri* regulated the DC's responses to other lactobacilli, leading the authors to conclude that ''*L. reuteri* may contribute to an environmental modulation of the intestinal dendritic cell generation favoring tolerance toward antigens bearing no 'danger signal' while at the same time keeping intact the capacity to respond against pathogens recognized via a danger signal like LPS.''

Unlike macrophages, the DCs may be interacting directly with the intestinal microbiota, and ''*L. reuteri* might be a potential fine-targeted treatment effective for downregulating production of IL-12 and TNF-α (and IL-6) while inducing the anti-inflammatory IL-10, thus representing an alternative therapeutic approach to counterbalance the pro-inflammatory intestinal cytokine milieu.'' And thus, ''the potential exists for Th1/Th2/Th3-driving capacities of the gut to be modulated according to composition of gut microflora, including ingested probiotics.''

c. L. reuteri *regulation of dendritic cell activities and Treg cell production*

In the third of these seminal studies, Smits *et al.* (2005) extended the Christensen *et al.* (2002) observations on DC modulations by showing that *L. reuteri* has the ability to prime DCs to stimulate production of Tregs. Rather than a murine model, they employed an *in vitro* human model system, including the human type-strain, *L. reuteri* (ATCC 53609/DSM 20016), immature human DCs generated from monocytes, and naïve human $CD4^+$ T cells. Maturation of the immature DCs was induced (along with specified maturation factors) by different concentrations of *L. reuteri* or two other lactobacilli: *L. casei* (NIZO B255) or *L. plantarum* (NIZO B253). Purified naïve $CD4^+$ T cells were stimulated during coculture with the mature DCs in the presence of superantigen *Staphylococcus aureus* enterotoxin B and rIL-2 plus rIL-15 and expanded for 7 days. Culture supernatants were then harvested and IL-10 content and T-suppressor activity (among other analyses) determined.

The *L. reuteri* and *L. casei* strains (but not the *L. plantarum* strain) were shown to prime the DCs to promote the development of Tregs. The Tregs thus developed were shown to (a) produce increased levels of IL-10, (b) inhibit proliferation of bystander T cells in an IL-10-dependent fashion, and (c) exhibit suppressor activity. Furthermore, they showed that the *L. reuteri* and *L. casei* strains bound to the DCs via a C-type lectin, specifically the DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN). This was established by showing that blocking antibodies to DC-SIGN inhibited the ability *L. reuteri* or *L. casei* to produce mature DCs capable of inducing the conversion of naïve T_H cells into Treg cells. It was noted (a) that UV-killed *L. reuteri* yielded identical results as viable cells, indicating that the regulatory DC priming does not depend on the viability of the bacteria, and (b) that the optimal priming for these regulatory DCs occurred at a bacterial/DC ratio of 1:1, with a lower priming effect occurring when the ratio was 10:1 or 100:1. In this connection, the authors noted that ''This is an intriguing finding, suggesting that these bacteria might activate additional pathways in DCs at high doses. These pathways might interfere with the cross-talk of DC-SIGN signaling, which in the end might (partially) prevent Treg cell development. It can be speculated that this pathway, triggered at high bacterial (even

probiotic bacterial) doses, represents an escape to clearance of bacterial overload, overruling the generalized immune suppression prevailing at low bacterial load. Whether different dosages of probiotic bacteria will also affect the clinical efficacy of probiotic treatment in a number of inflammatory diseases such as allergy remains to be established.''

In a similar series of experiments testing the effects of lactobacilli on DC and T-cell activities, Mohamadzadeh *et al.* (2005) investigated the effects of three human probiotic *Lactobacillus* strains—*L. reuteri* ATCC 23272/DSM 20016), *L. gasseri* ATCC 19992, and *L. johnsonii* ATCC 33200—on proliferation and activation of human myeloid DCs. These lactobacilli (both viable and UV-killed) were shown to modulate the phenotype and functions of the DCs. The lactobacilli-activated DCs cocultured with $CD4^+$ and $CD8^+$ T cells skewed the latter cells to T helper and Tc1 polarization respectively, as evidenced by secretion of IFN-γ, but not IL-4 or IL-13. It is particularly noteworthy, however, that the lactobacilli used in this study were applied in bacterial/DC ratios of 10:1, 100:1, and 1000:1, ratios that exceed the 1:1 ratio determined optimal for DC priming for Treg development as reported by Smits *et al.* (2005). Nevertheless, Mohamadzadeh *et al.* (2005) concluded that their findings: ''... add to the complexity of current evidence indicating the intestinal bacteria and probiotics, including lactobacilli, help maintain gut homeostasis by balancing pro- and anti-inflammatory mucosal responses.''

d. Lactobacillus *associated Immunobiotic DC interactions not involving* L. reuteri In another study on modulation of DCs, Drakes *et al.* (2004) tested the effect of the VSL#3 probiotic bacterial cocktail which contains eight different bacterial strains: *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, *L. plantarum*, *B. longum*, *B. infantis*, *B. brevis*, and *Streptococcus salivarius* subsp. *thermophilus*. They also concluded: ''... that probiotics possess the ability to modulate the dendritic cell surface phenotype and cytokine release in granulocyte-macrophage colony-stimulating factor-stimulated bone marrow-derived dendritic cells. Regulation of dendritic cell cytokines by probiotics may contribute to the benefit of these molecules in treatment of intestinal diseases.''

Hart *et al.* (2004) conducted a similar study on the ability of the VSL#3 cocktail to modulate DC phenotype and function. However, whereas Drakes *et al.* (2004) used bone-marrow-derived murine DCs and studied the effect of the entire cocktail *per se*, Hart *et al.* (2004) used human intestinal and blood DCs and tested the modulatory effects of the cocktail *per se* as well as the effect of each of the eight cocktail components. The cocktail was shown to be a potent inducer of IL-10 by both sets of DCs and inhibited generation of Th1 cells. It diminished pro-inflammatory effects of LPS by decreasing LPS-induced production of IL-12 while maintaining IL-10 production. Consistent with the Christensen *et al.* (2002) findings,

individual strains within the VSL#3 cocktail displayed differential immunomodulatory effects on these DCs. However, ''the most marked anti-inflammatory effects were produced by bifidobacteria strains, which upregulated IL-10 production by dendritic cells, decreased expression of the costimulatory molecule CD80, and decreased interferon-γ production by T cells.''

The ability of DCs to sample enteric antigens and present this information to the gut immune system is well documented. DCs have been referred to as ''the conductor of the immune orchestra,'' creating a harmonic balance within the gut between tolerance and inflammation. Dysregulation or imbalances in immune responses may result in inflammatory GI conditions, including Crohn's disease and ulcerative colitis (Hart *et al.*, 2004). One can extend this orchestral analogy to envision gut microbes and their myriad products as providing the instrumental components. Whether the score produces harmonic balance and immune homeostasis on the one hand, or inflammatory disharmonies on the other, may well depend on a proper balance of microbial components, including dietary immunobiotics, present in the gut at any particular time. Along these lines, Stagg *et al.* (2004) suggest that ''Understanding the interaction between specialized populations of gut DCs and the commensal flora may provide a key to understanding immune regulation in the gut and open the way for new therapeutic approaches for inflammatory bowel diseases.'' Also, that the ''differential modulation of DCs by microbial products suggests that treatment with probiotic bacteria may function in part by modulating the function of gut DCs.''

e. Proposed Model for L. reuteri *DC Interactions* The above-noted studies on *L. reuteri*–DC interactions have been integrated with the groundbreaking studies by Hori *et al.* (2003) and Pasare and Medzhitov (2003) as summarized by Powrie and Maloy (2003). Viewed together, they provide insights as to one of *L. reuteri*'s immunobiotic modes of action. Hori *et al.* (2003) identified the Foxp3 transcription factor as a master regulator promoting Treg cell differentiation. Pasare and Medzhitov (2003) showed that pathogen activation of TLR signaling in DCs also acts in a cell-extrinsic way to enhance T_E (T-effector cell) responses by overcoming Treg cell suppression. Furthermore, this blockade of Treg cell activity was shown to require interleukin-6 (IL-6) secretion by activated DCs (although IL-6 alone is not sufficient because another TLR-induced factor is also needed). Thus, Treg cells ''do not act in isolation, but are themselves influenced by cells of the innate immune system. An equilibrium is thereby established allowing effective responses against dangerous microbes while minimizing immune pathology'' (Pasare and Medzhitov, 2003). Sentinel DCs are believed to regulate both T_E and Treg cell development and activation. Recognition of pathogens by TLRs expressed on DCs (and perhaps macrophages and other APCs) triggers their activation

resulting in rapid and efficient generation of T_E cell activity against pathogens and production of IL-6 (and additional factors), thereby blocking the suppressive effects of the Treg cells on development of T_E activities. On the other hand, *L. reuteri*-TLR signaling shifts the DC equilibrium toward its nonactivated resting state. The resting DCs induce Foxp3 function in naïve T cells, resulting in development of Tregs, stimulation of IL-10 production, and suppression of the T_E cell-associated inflammatory immunopathology. These findings are consistent with those reported by Christensen *et al.* (2002) and Smits *et al.* (2005) who showed that strains of *L. reuteri* provide immunobiotic signals which are processed by sentinel DCs resulting in production and activation of Tregs, thus minimizing immune inflammatory pathologies and restoring a homeostatic state following challenges by pathogens or other noxious substances.

Immunobiotic modulations similar to those described above were foretold in the concepts of ''old friends'' and ''bystander suppression'' described by Rooks *et al.* (2004). They postulated that indigenous and environmental microbiota stimulated expansion of Treg cells and APC clones either directly or via other cell types such as DCs, and that ''old friends'' such as *Lactobacillus* strains may activate regulatory T cells or APCs toward regulatory functions carried out by T_E and Treg cells. Several studies have been published supporting these concepts. In a model of allergic contact dermatitis, for example, oral administration of *L. casei* reduced skin inflammation through expansion of $CD4^+$ IL-10 secreting T cells (Chapat *et al.*, 2004). Also, *L. paracasei*-T cell coculture in mixed lymphocyte reactions inhibited both Th1 and Th2 effector cytokines, resulting in the expansion of IL-10 producing Tr1 cells and TGF-b-secreting Th3 cells (von der Weid *et al.*, 2001). The results reported by Smits *et al.* (2005) provide direct evidence for these mode(s) of action.

f. L. reuteri *regulation of intestinal epithelial cell (IEC) activities* *L. reuteri*'s immunobiotic regulatory effects are not limited to the interactions with DCs/APCs as described above. A fourth seminal discovery by Ma *et al.* (2004) showed that *L. reuteri*–IEC interactions also underlie *L. reuteri*'s anti-inflammatory effectiveness. Human IEC lines T84 and HT29 were cultured in the presence and absence of live, heat-killed, and gamma-irradiated *L. reuteri* ATCC 23272. The cocultures were analyzed for production of nerve growth factor (NGF), IL-10, and TNF-α-induced IL-8. An ELISA assay was used to quantify intracellular and secreted IL-8. Western blotting and confocal microscopy were used to determine the effects of *L. reuteri* ATCC 23272 on IkB and NF-kB formation. The results showed that only live *L. reuteri* cells upregulated NGF activity in both cell lines while inhibiting, in a dose-dependent manner, the constitutive synthesis of IL-8 as well as IL-8 synthesis induced by NF-kB as measured in terms of mRNA and as both intracellular and secreted protein. The importance of

viability on immunobiotic persistence in the gut and in mucosal stimulation was also noted by Maldonado and Predigon (2004). Ma *et al.* (2004) showed that only live *L. reuteri* ATCC 23272 also inhibited IL-8 synthesis induced by *S. enterica* serovar Typhimurium. *L. reuteri* required preincubation and adherence of live cells for these effects. In addition, they showed that only live *L. reuteri* also inhibited translocation of NF-kB to the nuclei of HeLa cells by preventing degradation of IkB, and, '' . . . that *L. reuteri* has potent direct anti-inflammatory activity on human epithelial cells, which is likely to be related to the activity of ingested probiotics. *L. reuteri* also upgrades an unusual anti-inflammatory molecule, NGF, and inhibits NF-kB translocation to the nucleus.''

A model illustrating *L. reuteri* ATCC 23272's ability to prevent degradation of IkB and thereby NF-kB translocation to the IEC nucleus is presented in Fig. 1.2, adapted from an illustration by Beg (2004). The molecular processes employed by viable *L. reuteri* ATCC 23272 cells to inhibit translocation of NF-kB to the IEC nucleus requires further study. Neish *et al.* (2000) have shown that a nonvirulent strain of *Salmonella* does so by inhibiting the ubiquitination modification of the IkB complex. It is interesting to note that another commensal microbe, the strict anaerobic species *Bacteroides thetaiotamicron*, uses yet another means to antagonize the pro-inflammatory transcription factor, NF-kB, in pathogen-challenged Caco-2 cells. It does so by triggering association of the Rel A subunit of NF-kB with the nuclear hormone receptor and transcription factor PPAR-g (peroxisome proliferator activated receptor-g). They proposed that ''This newly formed complex is rapidly exported from the nucleus, thus attenuating expression of NF-kB-regulated inflammatory genes'' (Kelly *et al.*, 2004).

VII. GENETICALLY MODIFIED PROBIOTICS (I.E., DESIGNER PROBIOTICS)

Although the safety and health benefit of probiotic bacteria is well documented, most of the cultures in use today were empirically selected. However, with the current advances in genetics and molecular biology, it is possible to improve the quality of the different probiotic organisms by genetically engineering the appropriate gene(s) that can make them survive better during industrial production, adapt during transitioning in the host's environment, survive passage through the acidic environment of the stomach, persist in the intestinal tract, and/or deliver specific therapeutic agents into the intestinal tract of the host. The following are a few examples to demonstrate this unlimited potential.

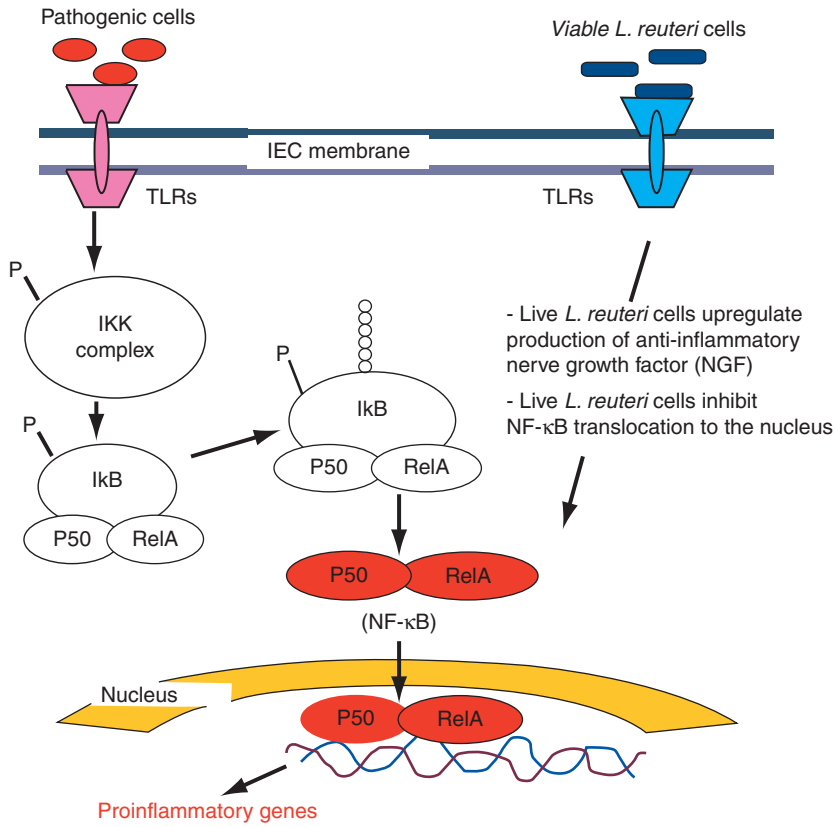

FIGURE 1.2 Regulation of intestinal epithelial cell (IEC) inflammatory responses by viable *Lactobacillus reuteri*–TLR interaction. *Pathogen–IEC–TLR inflammatory function:* The pathogen complex stimulates covalent modifications of IKK resulting in production of NF-κB whose translocation into the nucleus promotes transcription of pro-inflammatory products. *Lactobacillus reuteri–IEC–TLR function:* The *Lactobacillus reuteri* complex stimulates production of the anti-inflammatory agent, NGF, and prevents translocation of NF-κB to the nucleus, thereby minimizing pathogen-induced pathology. (Adapted by permission from Macmillan Publishers Ltd: Beg, 2004.)

A. Genetic enhancement of probiotic efficacy

1. Constructing oxidative stress resistant strains

Most probiotic organisms are obligate fermenters that can accumulate millimolar concentrations of H_2O_2, especially when propagated under oxic conditions, and while some have mechanisms to dispose of metabolic H_2O_2 (i.e. NADH peroxidase), the accumulation is often faster than the disposition (Condon, 1987). Tolerance to reactive oxygen species (ROS), such as O_2^-, H_2O_2, and $HO^\bullet$, is often strain dependent and is one of the

causes that can lead to decreased viability and potency of probiotic organisms found in functional foods, such as yoghurt and mass-produced commercial probiotic formulations (Kullisaar *et al.*, 2002; Talwalkar and Kailasapathy, 2003). Owing to the hazards of ROS, great care and expense must be taken in the preparation of industrial starter cultures and therapeutic formulations of probiotic lactobacilli. One of the defining characteristics of a probiotic organism is its antioxidative activity; therefore, it is worthwhile to identify, characterize, and exploit the antioxidant enzymes found within the probiotic organisms.

Work to engineer probiotic organisms harboring either superoxide dismutases (SODs), catalases, or both has been ongoing in our laboratory for the past 20 years (Roy *et al.*, 1993; Chang and Hassan, 1997; Andrus, *et al.*, 2003; Bruno-Barcena *et al.*, 2004, 2005; Peacock *et al.*, 2008). The manganese-containing superoxide dismutase (MnSOD) gene, *sodA*, present in *Streptococcus thermophilus* was identified (Chang and Hassan, 1997), characterized (Andrus *et al.*, 2003), and heterologously expressed in several probiotic organisms (Bruno-Barcena *et al.*, 2004). Indeed, cells expressing MnSOD were found to be resistant to ROS and to have a longer shelf-life (Bruno-Barcena *et al.*, 2004). Consequently, Bruno-Barcena *et al.* (2005) successfully created a marker-free chromosomal integrant of *sodA* within a probiotic *L. gasseri*. Furthermore, Rochat *et al.* (2006, 2007) and Peacock *et al.* (2008) have ongoing work examining the effect of manganese catalase (MnKAT) expression in heterologous probiotic hosts such as *L. casei* and other probiotic organisms, respectively.

2. Constructing osmo , cryo , and baro stress resistant strains

Osmotic stress is one of several stressors that probiotics must encounter, as a result of either packaging or passage through the intended host. Therefore, a desirable characteristic of a probiotic is its ability to withstand osmotic pressures, brought on either by sugars or by salts. Altermann *et al.* (2004) and Klaenhammer *et al.* (2005) demonstrated the importance of the *cdpA* and *slpA* genes in *L. acidophilus* with regard to its ability to withstand osmotic turgor. Glaasker *et al.* (1996a,b, 1998a,b) characterized mechanisms to combat osmotic stress present in *L. plantarum*, and Piuri *et al.* (2005) demonstrated that in *L. casei*, cell wall modifications involving the penicillin binding protein helped to adapt the cell to high osmolarity conditions. To date, none of the above mentioned genes have been heterologously expressed in other probiotic organisms. However, recently Sheehan *et al.* (2006, 2007) were able to express the *betL* gene (for betain uptake) from the food pathogen *L. monocytogenes* in the probiotic *L. salivarius* and *Bifidobacterium breve* UCC2003 in order to enhance their resistance to osmo-, cryo-, and barostress, resistance to the adverse effects of spray-/freeze-drying as well as GI persistence. The concept of ''using genes from bad bugs to make good

bugs better'' has been termed ''Patho-Biotechnology'' (Sleator and Hill, 2007). This finding clearly demonstrates that the source of the ''good'' gene is irrelevant.

3. Constructing strains for better cell surface adhesion

The ability of probiotics to access mucosal cell surfaces is an important attribute that contributes to competition/inhibition of cell-surface binding by pathogens. Thus, strains that have better adherence to intestinal tissue or mucus are better colonizers. In *Lactobacillus* spp., one mechanism of adhesion is through the production of the S-layer, and, though it is poorly characterized, the S-layer has numerous potential applications that include cell-surface display of antigens and immobilization on solid substrates (Avall-Jääskeläinen and Palva, 2005). Furthermore, Pretzer *et al.* (2005) described the mannose-specific adhesin of probiotic *L. plantarum*, which has the potential for mucosal surface colonization that could improve vaccine delivery and competitive exclusion of pathogens. Recently, Kankainen *et al.* (2009) showed that *L. rhamnosus* possesses a set of genes (*spaCBA*) for pilin proteins and another gene for pilus assemply, which seems to be a plausible mechanism for adherence and gut colonization by some lactobacilli. Clearly, this trait could be a target for cloning and heterologous expression in other probiotic organisms that are not good colonizers.

B. Developing probiotics for vaccine and drug delivery

1. Vaccine delivery

Several studies have investigated the use of both *L. lactis* and *Lactobacillus* spp. for the expression of heterologous antigens to promote the induction of beneficial immune responses. More recently, researchers have utilized *L. lactis* as a delivery vehicle for IL-10 via the bacterial secretory mechanisms. The use of probiotic organisms as transport vehicles for vaccines and other heterologously expressed molecules is not a new concept, but trying to get the vaccine delivered to the right target(s) poses a different challenge. Hols *et al.* (1997) demonstrated that the alanine racemase gene was important for growth of *L. plantarum*, and Palumbo *et al.* (2004) determined that *L. plantarum* knockouts for the alanine racemase gene had septation defects and cell wall perforations. Furthermore, Grangette *et al.* (2004) determined that the alanine racemase knockouts of both *L. lactis* and *L. plantarum*, both of which carried the C subunit of the tetanus toxin, elicited the humoral immune response significantly better than the wt strains. From these reports, it is evident that a modified cell wall results in improved presentation of the antigen by the probiotic organism.

2. Drug delivery

Many etiologies of human diseases such as cancer, emphysema, cirrhosis, atherosclerosis, arthritis, and irritable bowel disorders (IBDs) have been linked to ROS (Halliwell (1984); Kehrer (1993)). Thus, the use of probiotic organisms to deliver antioxidant enzymes and/or anti-inflammatory molecules is of great interest in the treatment of IBDs. Mice deficient in IL-10 develop colitis and serve as a good animal model for studying IBDs. Recently, Carroll *et al.* (2007) examined the anti-inflammatory properties of the probiotic *L. gasseri*, which has been engineered to express the heterologous MnSOD from *S. thermophilus* (Bruno-Barcena *et al.*, 2004, 2005) in the IL-10-deficient mice.

Indeed, the expression of MnSOD in the intestinal tract of this animal model resulted in a significant reduction in the inflammation (Carroll *et al.*, 2007). Furthermore, Rochat *et al.* (2006, 2007) demonstrated that *L. casei* expressing a heterologous manganese catalase (MnKAT) had an anti-inflammatory effect. These findings suggest that ROS may have a role in IBDs and that antioxidant-producing probiotic species could have desirable anti-inflammatory properties. However, ascertaining the correct probiotic strain/the proper antioxidant to be used for the different diseases is a challenge. Clearly, the ability to exploit the antioxidative properties of select lactobacilli or to engineer these enzymes in the strains that lack them for use within pharmaceuticals or nutraceuticals could be extremely valuable.

In another study, Steidler *et al.* (2000) demonstrated that *L. lactis* strains engineered to produce recombinant IL-10 were able to ameliorate colitis in a murine colitis model. Furthermore, a recent phase I study demonstrated some efficacy of *L. lactis* expressing the human *IL-10* gene in treating patients suffering from Crohn's disease (Braat *et al.*, 2006). Indeed, more controlled studies are needed in order to establish the efficacy of these therapeutic approaches for the treatment of IBDs.

VIII. CONCLUSIONS

The seeds Elie Metchnikoff's planted over a hundred years ago—''There are many useful microbes, amongst which the lactic bacilli have an honorable place''—have germinated at long last, and some of his ''lactic bacilli'' are currently being harvested as health-enhancing probiotic products. The disbeliefs and skepticisms concerning their health-enhancing effects have been replaced by insights into the molecular processes underlying their mode(s) of action. Credibility issues concerning Metchnikoff's ''probiotic concept'' have been contravened, and significant contributions to human and animal health are forthcoming. In all likelihood, the future

will witness accelerated scientific research on probiotic agents, and those exhibiting positive probiotic activities will be submitted for clinical scrutiny at an accelerated rate. Some may be genetically modified to enhance their effectiveness. Participants at the second meeting of the International Scientific Association for Probiotics and Prebiotics (ISAPP) joined in calling for this accelerated attention (Rastall *et al.*, 2005), stating that:

> ''Modern molecular, nanotechnology and immunological tools must be directed towards more thorough understanding of microbial community structure and function. In turn, this will generate a new level of understanding of how the human body functions with its microbial constituents, and how such interactions can be modulated for the betterment of the host''.

It is believed that improving existing probiotics through heterologous gene expression will aid in the development of many desirable probiotics for use in the food and pharmaceutical industries. However, acceptance of genetically modified probiotic organisms by the regulatory agents and the consumers remains an important challenge for the full utilization of these organisms. However, these roadblocks will eventually disappear by having in place the proper controls and guidelines plus risk-assessment studies demonstrating the safety and the vast health/economic benefits to be gained from using these organisms in food and medicine.

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The authors dedicate this review to the memory of Dr. Ivan A. Casas who passed away on October 30, 2005. Dr. Casas was a true pioneer microbiologist, whose scientific skills, creativity, and energetic leadership in initiating and coordinating large scale field trials with animals and clinical safety and efficacy trials with human children and adults con tributed immeasurably to validation of the probiotic concept and its recent emergence into the mainstream of medicine.

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CHAPTER 2

Prokaryotic and Eukaryotic Diversity of the Human Gut

Julian R. Marchesi[1]

Contents

Abstract

The human intestinal tract is one of the most densely populated ecosystems studied to date. Recently, the gut microbiota have been implicated as an environmental factor in health and disease; however, as with all ecosystems, a significant proportion of these microbiota are as yet uncultured. Hence culture-independent molecular-based methods have been applied and have started to provide insights into the microbes in this system. This review explores the recent significant findings in the last 5 years in the area of gut microbial ecology. Most significant is the observation that the gut microbiota are dominated by species from the phyla *Firmicutes* and *Bacteroidetes*. Regardless of whether first- or second-generation sequencing technologies are used to explore the microbial diversity, these two phyla are found throughout the intestinal tract, with other microbes such as the viruses and micro-eukayrotes, which, while being present, are either in low

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numbers or have not received much attention. Simply put, the *Firmicutes* and *Bacteroidetes* have made the gut their own, and the next stage in the study of this fascinating system will be to establish the roles they play in the host's health.

I. INTRODUCTION

Human beings are readily colonized by a large variety of microbes. Consequently, many of the microbes have evolved functions that aid their colonization and benefit the host. Additionally, the host has also developed an array of responses to the presence of these microbes. In the last 10 years, the gut and its associated microbiota have received much attention, and the subject area is going through a renaissance as a result of technological advances and the application of culture-independent (CI) approaches developed to study environmental microbiology. The gut is a compartmentalized ecosystem consisting of the oral cavity, esophagus, stomach, small intestine (divided into the duodenum, jejunum, and ileum), and the large intestine (divided into the ascending, transverse, descending and sigmoid colon, and rectum). Microbiological studies in the 1970s showed that these niches were colonized by unique collections of microbes (Savage, 1977), and later it was shown that a significant proportion of the contents of the large intestine were microbial (Stephen and Cummings, 1980). The microbes were considered as both an asset and a liability as they were able to provide the host with functions that were beneficial, for example, supplying the host with fermentation products that are easily metabolized (acetate and butyrate; Cummings *et al.*, 1987), while also generating carcinogens (Hughes *et al.*, 2008; Rumney *et al.*, 1993). Thus, it was evident that we needed to understand the ecology of the microbes in the gut, their functions, and the manner in which they vary and are regulated, in order to understand the contribution that the gut microbiota make to the host's health. To view it in a different way, we should treat the gut microbiota as a virtual organ (O'Hara and Shanahan, 2006), with metabolic activities that rival that of the liver, which are dynamic and can be altered by external factors such as exposure to antibiotics. The gut microbiome is thus an extension of the host's genome and brings an extension to the functions the host is able to perform (Fig. 2.1). The need to understand these functions has been at the forefront of trying to determine what roles the gut microbiome plays in the host's health.

However, the ability to describe functions of the gut microbiome has been impaired by the biases introduced when using traditional microbiological techniques to isolate and enumerate any microbe from its niche (Amann *et al.*, 1995; Savage, 1977; Staley and Konopka, 1985). While the number of uncultivated microbes is not as high as in non-animal systems, it is nonetheless a significant enough proportion to make robust

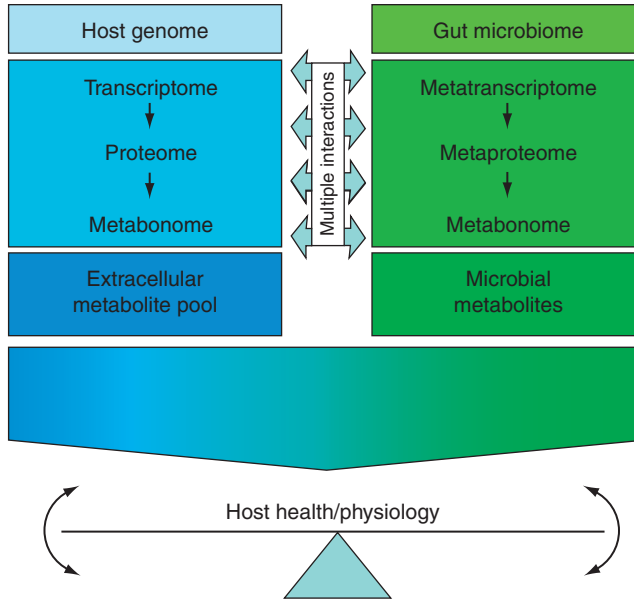

FIGURE 2.1 Schematic representation of the complex interplay between the host and its extended genome, the majority of which resides in the gut. These interactions play a role in the host's health and make significant contributions to where the balance lies in an individual.

conclusions, based on data from cultured isolates, difficult to support. In order to measure the diversity of this uncultivated majority, CI methods have been applied with great success and have helped to fill in the gaps. This review focuses on the recent developments in gut microbiology and how CI methods have provided a new way of looking at this ecosystem.

II. PROKARYOTIC DIVERSITY IN THE GUT

A. Diversity in the large intestine

The majority of the CI studies to date have used Sanger dideoxy chemistry or first-generation sequencing technology to generate inventories of 16S rRNA genes amplified from gut samples and mainly those of the distal gut. Owing to large investment of resources required to generate large datasets of 16S rRNA genes, there are few ''deep'' sequencing projects that have explored the gut microbiota; however, some groups have undertaken such studies. The most comprehensive analysis undertaken was of the guts of three volunteers and published by Eckburg *et al.* (2005). In their study, they isolated 13,355 prokaryotic ribosomal RNA gene

sequences from multiple colonic mucosal sites and feces of healthy subjects. While this was not the first CI analysis of the gut microbiota, being the largest it provided the deepest view of the gut bacteria and surprisingly showed that of the currently described *Bacteria* phyla (24, 34, or 44; EMBL, RDP, or SILVA taxonomies respectively (excluding candidate phyla)), only seven were found in the gut (Fig. 2.2). In addition, the majority of the bacterial sequences (>95%) were from two phyla, the *Bacteroidetes* and *Firmicutes*. The archeal 16S rRNA genes were all matches for the gut archaeon *Methanobrevibacter smithii*. Multivariate analysis of the data led the authors to conclude that while the largest difference in the bacterial community structure was due to inter-subject variation, intra-subject variations were not significant and the similarities in the mucosal community was the same wherever the sample was taken in the large intestine. This latter observation has been verified by Green *et al.* (2006) and Ahmed *et al.* (2007) using CI methods that generate barcode-like data for a bacterial community. The inter-subject median similarity coefficient for the denaturing gradient gel electrophoresis (DGGE) profiles was approximately 34% for 33 subjects, while it was 100% for intra-subject samples (Green *et al.*, 2006). In the study by Ahmed and colleagues, a similar low level of intra-individual variation was reported in mucosal

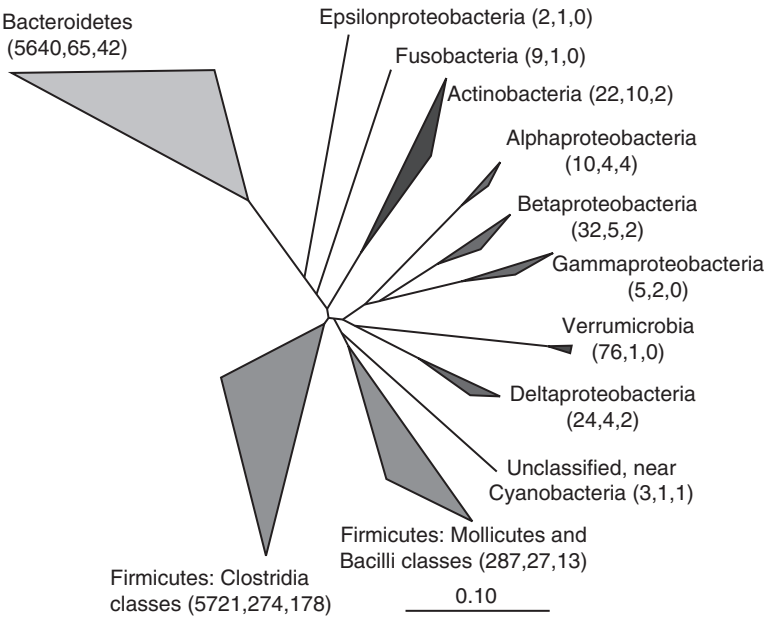

FIGURE 2.2 Phylogenetic tree of the 16S rRNA genes isolated by Eckburg and colleagues. (Reproduced from Eckburg *et al.* (2005).)

samples from subjects who underwent emergency surgery requiring colonic resection and, importantly, were not subject to bowel preparation before surgery, thereby minimizing any potential perturbations of the microbiota. Returning to the inter-subject variability, it seems that all studies point to this being low, and this similarity has been explored recently in an attempt to define the core microbiota of the large intestine (Tap *et al.*, 2009). The gut microbiota of 17 volunteers were analyzed using CI methods, and the authors concluded that the subjects had an estimated richness average of 943 operational taxonomic units (OTUs) per subject (using a cut-off value of 98% similarity). The combined data (10,456 sequences) were all from the phyla described by Eckburg *et al.* (2005), but more striking was the observation that of the 3180 different OTUs, 2500 were not shared and present in only one sample and that none of the OTUs were found in all of the samples. Of the shared OTUs, *Faecalibacterium prausnitzii*, was found in 16/17 samples studied and 66 OTUs (including *F. prausnitzii*) were found more often in the samples and were postulated as a phylogenetic core in the gut. Moreover, these 66 OTUs were also more prevalent in the public datasets of other gut studies (Eckburg *et al.*, 2005; Gill *et al.*, 2006; Li *et al.*, 2008; Manichanh *et al.*, 2006) and were shared between the five datasets investigated (Fig. 2.3). The OTUs were again members of commonly seen phyla, that is, the *Firmicutes* and *Bacteroidetes*. In a similar study, Rajilić-Stojanović and

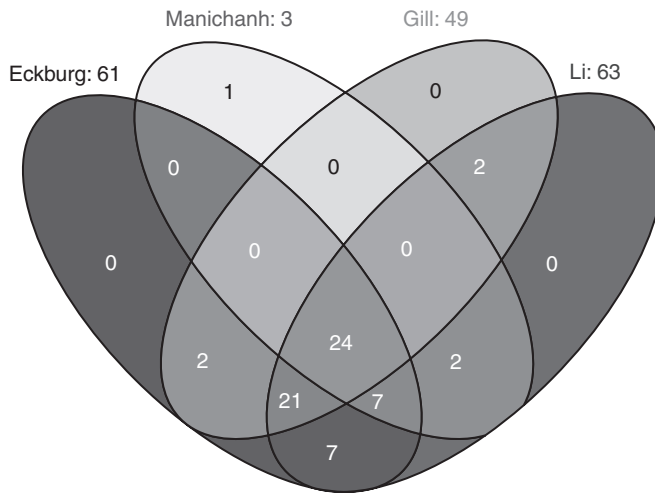

FIGURE 2.3 Venn diagram of the distribution of the 66 putative core OTUs in libraries of 16S rRNA genes isolated from gut microbiota. The four libraries were taken from GenBank and were deposited by Eckburg *et al.* (2005) (2339 sequences), Gill *et al.* (2006) (2062 sequences), Manichanh *et al.* (2006) (539 sequences), Li *et al.* (2008) (5413 sequences). (Reproduced from Tap *et al.* (2009).)

coworkers (Rajilić-Stojanović *et al.*, 2009) used a CI method based on a DNA microarray (containing 1140 phylotypes) to identify the presence of 16S rRNA genes in their samples. Their data again led to the conclusion that the gut microbiota are subject specific and that there exists a core microbiota that does not change significantly with time. Since the microarray approach lends itself much more to analysis of multiple samples, the authors also investigated to what extent age plays a role in shaping the gut microbiota. They concluded that while young subjects share more positive ''hits'' between them than do the elderly subjects, there are shared probes between the two cohorts and this may constitute the phylogenetic or universal core of the distal gut. The groups that were shared, but showed changes with age, were all members of the *Bacteria* that are common to the large intestine, which are shown in Table 2.1. In an interesting corollary of this was the report published by Ley *et al.* (2008), which analyzed the gut microbiota of the mammalian gut *per se*. In this study, 15 phyla were identified from the 60 mammalian species investigated, with the *Firmicutes* (65.7%) and *Bacteroidetes* (16.3%) constituting the majority of the 19,548 classifiable sequences obtained.

As Rajilić-Stojanović and coworkers have shown, creating inventories of 16S rRNA genes are not the only CI method available to determine the composition of the gut microbiota; microarrays have been used by several groups to investigate the gut microbiota (Harrington *et al.*, 2008; Kim *et al.*, 2005; Klaassens *et al.*, 2009; Paliy *et al.*, 2009; Palmer *et al.*, 2006). While they offer a convenient and quick approach to diversity analysis, they are limited by the data from which they were developed. This bias may result in some species not being included on the microarray and thus will not be detected; furthermore, the subject-specific nature of the gut microbiota needs to be addressed and much more work needs to be undertaken to determine, in a much larger cohort, the members of the core gut microbiota. Other approaches have involved quantitative polymerase chain reaction (qPCR) and fluorescent *in situ* hybridization (FISH), which have been used to quantify the types of microbes present, but are only as good as the specificity of the primers or probes designed. Matsuki *et al.* (2004a,b) and Rinttila *et al.* (2004) have developed qPCR primers that amplify the main groups of bacteria found in the colon. In conjunction with FISH analysis undertaken by groups across Europe (Mueller *et al.*, 2006), the same picture emerges of the gut microbiota, with the phyla *Firmicutes* and *Bacteriodetes* as the predominant groups. However, these analyses all suffer because the gut is probably the most densely populated ecosystem on earth, which is composed of predominantly two phyla. First-generation sequencing methods, qPCR, or FISH do not delve deep enough to describe the full range of bacteria present, and therefore we need other options to explore the diversity of this system.

TABLE 2.1 Genus-like groups for which relative abundance was found to be significantly different between younger and elderly adults

Phylum/order	Family/genus	Ratio elderly/younger	*P*-value
Actinobacteria	*Actinomycetaceae*	23	0.0012
	Atopobium	2	0.0135
Bacilli	*Lactobacillus salivarius* et rel.	10	0.0080
	Aerococcus	5	0.0087
	Granulicatella	15	0.0159
	Streptococcus bovis et rel.	5	0.0040
	Streptococcus intermedius et rel.	5	0.0081
Clostridium cluster XIVa	*Eubacterium hallii* et rel.	0.5	0.0416
Clostridium cluster XV	*Eubacterium limosum* et rel.	1.3	0.0144
Bacteroidetes	*Allistipes*	0.20	<0.0001
	Bacteroides ovatus et rel.	0.14	0.0001
	Bacteroides splachnicus et rel.	0.14	<0.0001
	Bacteroides stercoris et rel.	0.09	0.0017
	Parabacteroides	0.14	<0.0001
	Prevotella ruminicola et rel.	0.25	0.0118
	Uncultured *Porphyromondaceae*	0.13	<0.0001
	Uncultured *Bacteroidetes*	0.08	0.0005
Betaproteobacteria	*Aquabacterium*	0.13	0.0004
	Burkholderia	0.33	0.0395
Gammaproteobacteria	*Xanthomonadaceae*	0.06	0.0017

Adapted from Rajilić Stojanović *et al.* (2009).

One approach that does move some way to overcoming the uneven composition of the gut microbiota was developed by Apajalahti and colleagues (Apajalahti *et al.*, 2001; Holben *et al.*, 2004) to investigate the gut of broiler chickens, which was later applied to the human gut to determine the diversity of the gut microbiota in a cohort of irritable bowel syndrome (IBS) sufferers (Kassinen *et al.*, 2007) and overall diversity of healthy subjects (Krogius-Kurikka *et al.*, 2009). The approach fractionates the genomic DNA (based on %G + C) isolated from gut biomass using a cesium chloride gradient and an AT-dependent DNA-binding dye, bis-benzimidazole. The %G + C fractionated DNA is collected and CI analysis undertaken on the individual fractions, thus allowing the user to target less abundant groups. In the comparison of IBS suffers with healthy volunteers, differences in %G + C profiles were investigated by isolating the variable fractions and subjecting them to CI methods. Kassinen *et al.* (2007) reported that the gut microbiota were altered in IBS and that the changes occurred in the genera *Coprococcus*, *Collinsella*, and *Coprobacillus*. Krogius-Kurikka *et al.* (2009) used the %G + C fractionation method to determine to what extent an uneven species composition affects the calculation of diversity. They took the different %G + C fractions and generated 16S rRNA gene clone libraries from the DNA and compared these with a clone library derived from un-fractionated DNA. Their results show that >80% of the clones from the 30–40%G + C fraction were shared with the unfractionated library and that only 32–33% of the clones in the 55–65%G + C fraction were found in the unfractionated library. Upon further inspection of the phylotypes in the %G + C fractions, 26.6% were from the phylum *Actinobacteria* when compared to 3.5% found in the unfractionated sample. While this approach has been used only in some gut-related projects, it does show how biases can easily be introduced when trying to describe a diverse but skewed microbial community.

1. Application of second generation sequencing to explore the gut microbiota

In order to get at the rare biosphere in the gut, second-generation sequencing technologies have been utilized. The rationale for using such high-throughput sequencing (HTS) technology was to overcome the predominance of 16S rRNA gene sequences being obtained from the two main phyla, which may make up over 95% of the colonic microbiota. The main method that has been used was developed by 454 and uses a pyrosequencing approach to generate HTS (Droege and Hill, 2008; Margulies *et al.*, 2005; Marsh, 2007). This technology provides the brute force required to undertake deep sequencing of 16S rRNA gene collections and gut microbiomes, in order to reveal rare or low-abundance species. One of the first forays into the gut microbiota using HTS was an investigation of the role they play in obesity (Turnbaugh *et al.*, 2006). This

application of HTS came on the back of the original observation of an altered ratio of *Bacteroidetes*:*Firmicutes* in obese mice (Margulies *et al.*, 2005); however, the initial applications of HTS to the human gut were published 2 years later (Andersson *et al.*, 2008; Armougom and Raoult, 2008; Dethlefsen *et al.*, 2008; Huse *et al.*, 2008; Keijser *et al.*, 2008). A bioinformatic analysis was performed in 2007 to show that short reads would robustly describe the gut microbiota (Liu *et al.*, 2007). The study conducted by Andersson *et al.* (2008) investigated three niches within the human gut, namely the throat, stomach, and distal gut (using feces as a proxy for this niche), and they used the first-iteration Roche's 454 technology, that is, the GS20 which provided 61,768 reads and after processing left 56,382 reads, with a mean length of 73 nucleotides from their 18 samples. Bioinformatic analysis of the data resulted in each niche clustering with its cognate niche and showing similar taxonomic characteristics (Fig. 2.4). The *Firmicutes* were shown to be the predominant group in the fecal samples, while members of the *Actinobacteria* (previously called high G + C Gram positives) were also a significant proportion of the fecal community. In this study, the *Bacteriodetes* were not as prevalent as previously described, and this result may be due to inter-subject variability (Eckburg *et al.*, 2005) or sample preparation (Claesson *et al.*, 2009; Duncan *et al.*, 2008; Krogius-Kurikka *et al.*, 2009; Ott *et al.*, 2004). The three niches did show site-specific clustering, which is not surprising given the very different selective pressures and environmental conditions that prevail in them. Furthermore, the analysis of the stomach showed a diverse collection of microbiota, which corroborated the data produced by Bik *et al.* (2006) discussed below. The HTS studies of Dethlefsen *et al.* (2008) and Huse *et al.* (2008) both used the second iteration of Roche's 454 HTS, the GS-FLX, which provides longer reads (200–250 bp). Dethlefsen and colleagues investigated the impact that antibiotic administration had on the gut microbiota and reported that in the pre-exposed gut, the number of phyla ranged from 6 to 9 per individual and that in total 10 phyla were found in the distal gut. A similar distribution was reported by Huse and colleagues, who were also investigating the distal gut and fecal microbes using HTS, and the main phyla identified were *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Lentisphaerae*, *Proteobacteria*, candidate division TM7, *Verrucomicrobia*, and *Deinococcus−Thermus*. Huse and coworkers also compared first- and second-generation sequencing technologies and showed that the HTS did indeed extend the range of rare species reported in a sample (Fig. 2.5). Since these first CI HTS studies of the large intestine, several more have been published that looked at human and other animal models of humans disease (Claesson *et al.*, 2009; Dowd *et al.*, 2008a,b; Li *et al.*, 2009; Martínez *et al.*, 2009; McKenna *et al.*, 2008; Roesch *et al.*, 2009; Zhang *et al.*, 2009), thereby attesting to the way in which we will be studying the gut microbiota in

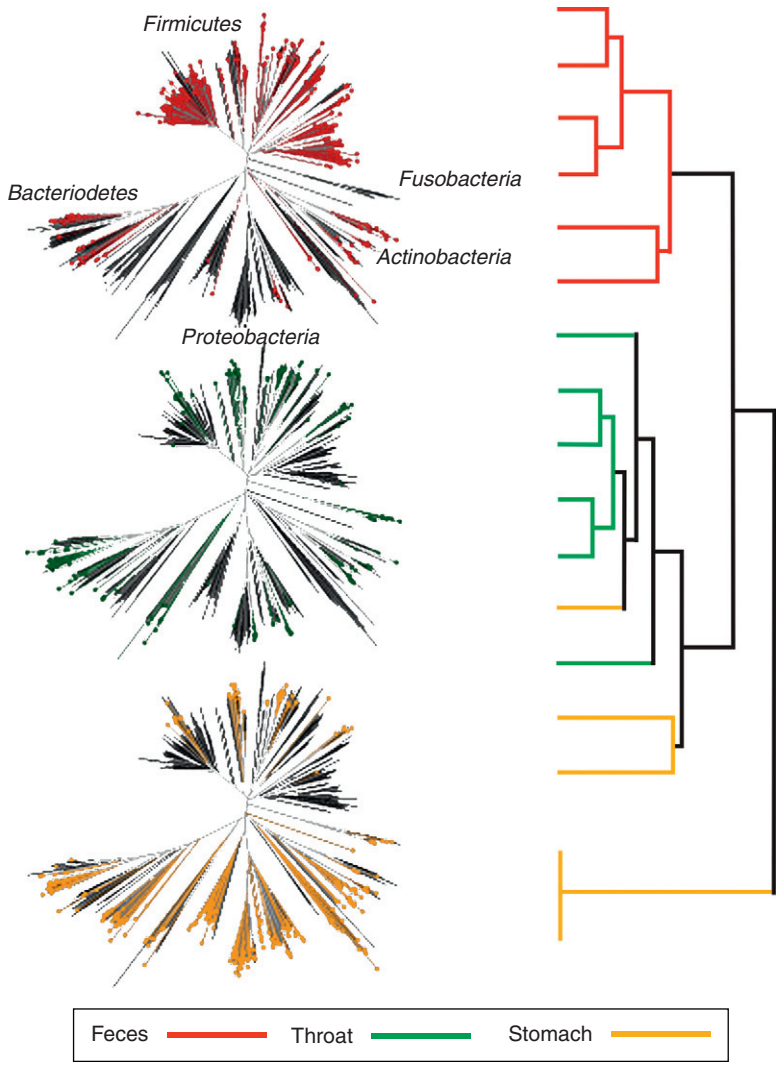

FIGURE 2.4 (A) Phylogenetic trees of the 454 reads from the three niches of the human gut, (B) the way the niches clustered according to the taxonomic data in each sample. (Reproduced from Andersson *et al.* (2008)).

future. The study conducted by Ley *et al.* (2008) of the diversity of the gut microbiota of mammals showed that both diet and host were the key selective drivers of bacterial diversity in the 60 species investigated. Humans, they concluded, were most similar to omnivorous primates and shared the same types of phyla, with the majority of sequences belonging to the *Firmicutes* (65.7%) and *Bacteroidetes* (16.3%), again

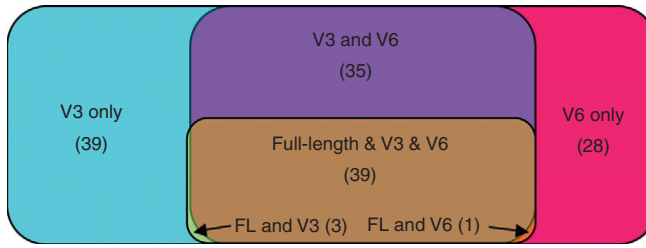

FIGURE 2.5 Venn diagram showing the overlap between the different methods used to determine the diversity of the gut microbiota. The V3 tag sequencing found the most genera (116), V6 found 103 genera, and full-length sequencing found only 43 genera. The V3, V6, and full-length sequencing refer to the V3 and V6 variable regions and near-full-length sequence of the 16S rRNA gene used in the analysis. (Reproduced from Huse *et al.* (2008).)

indicating that members of these phyla have best adapted to colonize the mammalian and human gut. Additionally, the gut microbiota are distinct in nature from the other microbiota associated with the human body (but most closely related to the oral microbiota), which implies a strong selection for the species and functions therein (Costello *et al.*, 2009).

B. Oral, stomach, and small intestine microbiota

Many of the studies undertaken have used fecal or stool material as a proxy for the gut microbiota, the main reason being that it is easily obtained and contains a large proportion of microbial biomass. However, the microbiota of a fecal sample are not fully representative of the intestinal tract and as such several groups have undertaken the investigation of the microbiota in other niches of the gut. One of the easiest sites to access and has been studied in detail is the oral cavity. The most comprehensive analyses to date were recently published (Keijser *et al.*, 2008; Nasidze *et al.*, 2009a,b) and used a combination of 16S rRNA gene clone libraries and HTS of 16S rRNA genes to describe the composition of the saliva and plaque microbiota. In the study of Keijser and coworkers, a repeat of what is seen in the gut was reported, the majority (>99%) of the sequences (73,485 and 124,188 sequences used for analysis for saliva and plaque, respectively) coming from a limited number of phyla: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Spirochaetes*, *Proteobacteria*, and candidate division TM7. Nasidze *et al*. found the same phyla and, in addition, a small number of sequences from *Synergistetes* (genus *Treponema*) and *Tenericutes* (genus *Mycoplasma*). So the oral cavity not only shares similar diversity but also similar phyla with the large intestine. Moving down the gut, we come to the throat and esophagus. Both niches have received

limited attention, but studies that have looked at the microbiota in these areas (Andersson *et al.*, 2008; Pei *et al.*, 2004) used CI analysis of the microbial biomass and concluded that the bacteria in these niches come from six phyla, namely *Firmicutes*, *Bacteroides*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and candidate division TM7.

The stomach, on the other hand, is a strikingly different ecosystem compared to both the oral and large intestine, with a very strong selective pressure due to its low pH. Received wisdom on this organ was that the diversity of its microbiota was very low and devoid of any significant microbiota, excluding *Helicobacter pylori*. However, in 2006, Bik and coworkers (Bik *et al.*, 2006) used a CI approach to look at whether *H. pylori* influenced the gut microbiota and showed that it was far from being the microbial desert depicted in textbooks and the literature. They reported that there were five phyla (recently confirmed by Dicksved *et al.* (2009) using non-HTS methods) represented in the stomach (Fig. 2.6) and that when *H. pylori* was present, it reduced the bacterial diversity and was the predominant sequence recovered. Additionally, using a variety of macroecological algorithms, they estimated that the gastric mucosal ecosystem contained 128 phylotypes. The estimated species richness (Chao 1) was not

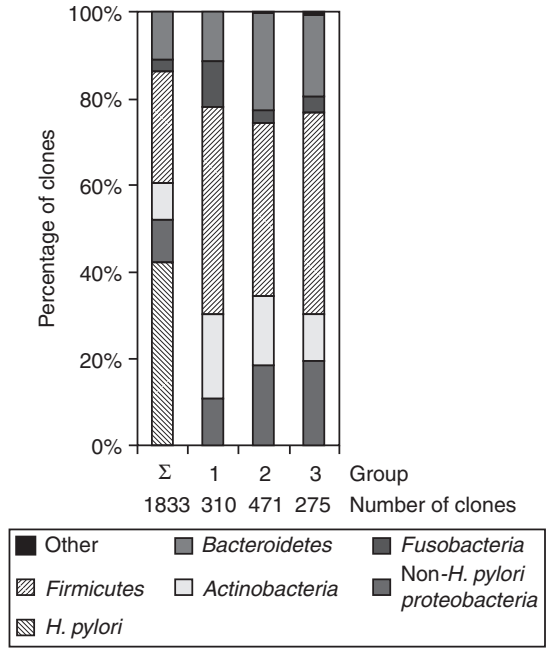

FIGURE 2.6 Phylotype frequencies of 16S rRNA gene clones isolated from gastric specimens. (Reproduced from Bik *et al.* (2006).)

much higher, 193.4, which implies that the majority of the system's diversity was captured in their analysis. When Andersson *et al.* (2008) used HTS to explore the same issue, they also concluded that presence of *H. pylori* correlated with a decrease in the diversity of bacteria observed. However, they found more phyla present in the stomach; an extra eight were added to the list, and these included members of the *Chlamydia* and *Cyanobacteria*. In addition, they measured 276 phylotypes in the uninfected stomach and estimated that there were 375 present. Both these studies led to the conclusion that the stomach is a much more diverse system than previously thought, but does contain low numbers of microbes (Bik *et al.*, 2006).

The final area of the gut that has received very little attention is the small intestine. One reason for the paucity of information on this section of the gut is the difficulty in access and sample collection. However, several groups have strived to shed light on the diversity of this niche, the most notable of which used CI methods to describe the bacterial diversity. The first investigation was reported by Wang *et al.* (2003), in which they took biopsies from the terminal ileum (as well as other parts of the colon) and created inventories of the 16S rRNA gene, which they were able to amplify. Focusing on the ileum, their results led them to report that the bacterial fraction of the mucosal microbiota was dominated by sequences from the *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. A different study several years later (Wang *et al.*, 2005) was undertaken to look at samples from jejunum and ileum, which showed greater diversity (maybe due to it being a more comprehensive analysis), with sequences from six phyla recovered and a significant difference between the two samples sites (Fig. 2.7). The significant difference was the predominance of 16S rRNA gene sequences closely related to the genus *Streptococcus* from the jejunum sample. The Ph.D. thesis of Dr. Carien Booijink (Booijink, 2009) is currently the most comprehensive analysis of the small intestine published. In this thesis, the small intestine microbiota were analyzed using a variety of CI methods including the HITCHIP microarray (Rajilic-Stojanovic *et al.*, 2009), and the core microbiota were described as being from eight phylogenetic groups, with the most abundant genera belonging to *Streptococcus*, *Veillonella*, and *Clostridium*.

III. EUKARYOTIC AND VIRAL DIVERSITY IN THE HUMAN GUT

The gut microbiota, while predominantly composed of members of the *Bacteria*, do contain other classes of microbes. However, there is a relative dearth of information on them compared to that gathered for the *Bacteria*. The majority of work undertaken on the eukaryotic members of the gut has been traditional culture-based approaches looking at fungal diversity and specifically *Candida* and *Saccharomyces* spp. However, in 2006, Scupham and

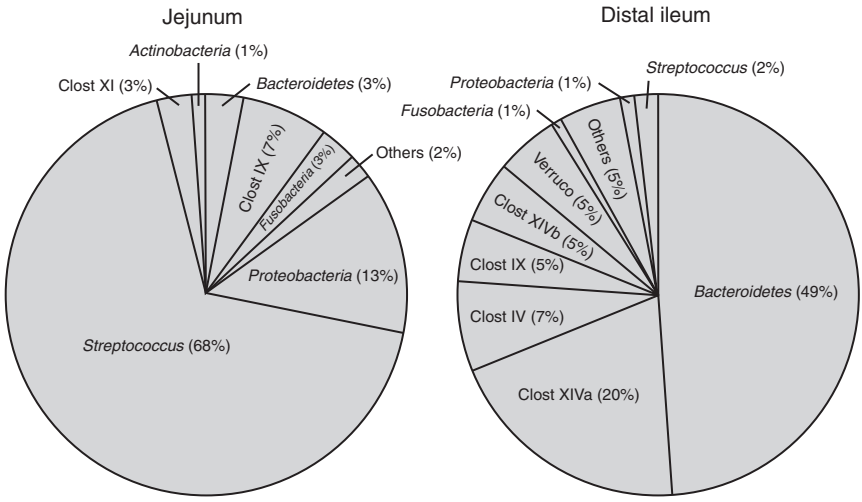

FIGURE 2.7 Phylogenetic distribution of 16S rRNA gene sequences generated from mucosa biopsies of human jejunum and distal ileum. Verruco, *Verrucomicrobia*; Clost, *Clostridium* cluster (Collins *et al.*, 1994). (Adapted and reproduced from Zhu *et al.* (2006).)

colleagues (Scupham *et al.*, 2006) undertook a CI analysis of the fungi in the murine gut. They were able to show, using oligonucleotide fingerprinting of rRNA genes (OFRG), that all four major fungal phyla, *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Zygomycota*, were present. Many genera were represented in the analysis, for example, *Acremonium*, *Monilinia*, *Fusarium*, *Cryptococcus/Filobasidium*, *Scleroderma*, *Catenomyces*, *Spizellomyces*, *Neocallimastix*, *Powellomyces*, *Entophlyctis*, *Mortierella*, and *Smittium*. On the back of this work, Scanlan and Marchesi (2008) undertook a CI and culture-dependent analysis of the distal human gut with a view to establishing whether humans shared such high diversity of micro-eukaryotes. Using the 18S rRNA gene as the surrogate biomarker for micro-eukaryotes, they determined that the human distal gut shows much less diversity than the murine gut. While the cultured isolates were predominantly from the genus *Candida*, the CI analysis showed that the genus *Blastocystis* was the main organism recovered. In the study of Ott *et al.* (2008), in which they investigated the fungal diversity of an IBD versus healthy cohort, a more diverse fungal community was observed; however, it was still much less diverse than the mouse gut. Interestingly, the mucosal and fecal fungal communities were different, with the majority of the mucosal OTUs in the phyla being *Ascomycota* and *Basidiomycota*, while nearly all OTUs from the fecal samples were clustered in *Ascomycota*. This result was in keeping with that of Scanlan and Marchesi (2008), in which the majority of cultured organisms and ribotags were clustered in *Ascomycota*; however, pure culture isolates of

Basidiomycota were obtained (*Rhodotorula mucilaginosa*) when samples were incubated at non-physiological temperatures. These two studies are among the only ones that have used CI approaches to investigate the micro-eukaryotes in the human distal gut, and while they show limited diversity, there is still need to investigate this kingdom further in larger cohorts from geographically different regions and dietary cultures.

One final aspect of the gut microbiota that has received limited attention is the viral component. One of the first CI studies of this diversity was reported in 2003 by Breitbart and colleagues (Breitbart *et al.*, 2003). In their study, they used a metagenomic approach to investigate filter-enriched biomass and concluded that the human gut is replete with viruses and that the majority are members of Siphophage (Fig. 2.8). From the data, they estimated that the community contained approximately 1200 viral genotypes, which makes it the most diverse group of organisms in the gut. Interestingly, in a later study from the same group (Zhang *et al.*,

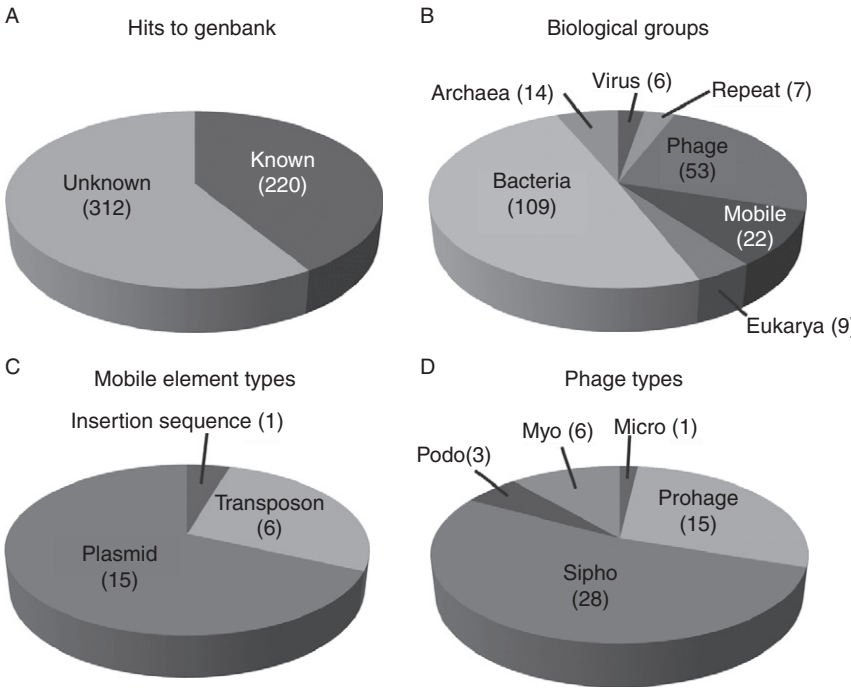

FIGURE 2.8 Genomic overview of the uncultured viral community from human feces based on TBLASTX analysis. (A) Number of sequences with matches in GenBank; (B) Distribution of matches among major classes of biological entities; (C) Types of mobile genetic elements found in the metagenomic library; (D) Families of phages identified in the fecal library. (Reproduced from Breitbart *et al.* (2003).)

2005), the majority of the RNA viruses that they identified were plant parasites, with the pepper mild mottle virus (PMMV) the most abundant (up to 10^9 virions/g of dry weight fecal matter). The authors detected PMMV in 12 (66.7%) of 18 fecal samples collected from two continents and concluded that it is prevalent in the human population. In a small study of the developing infant gut (Breitbart *et al.*, 2008), they also reported that the early colonization of this ecosystem is dominated by bacteriophage, which are derived not from the mother but from the environment. As with their study on adults, the Siphophage were the predominant group obtained; however, only one infant was studied, and further studies would be required in order to develop a more robust view of viral colonization of the human gut.

IV. CONCLUDING REMARKS

The gut microbiota have begun to receive increasing attention in recent years because of its potential role in a variety of functional bowel disorders and other non-intestinal diseases such as diabetes and metabolic syndrome. Thus, it is imperative that we try and define a baseline from which to compare the diseased cohort and to indentify whether members of this community are co-varying with disease biomarkers. In order to achieve this goal, many microbiologists have realized that culture-dependent methods are inadequate and have used CI approaches to describe and capture the diversity in the gut. To this extent, they have been very successful and we now realize that the gut microbiota are composed of mainly members of *Firmicutes* and *Bacteroidetes* with varying levels of the other phyla present. However, we have only just scratched the surface of what we need to do in order to establish this baseline. The number of guts that have been sampled is tiny and the ethnic groups and geographical locations limited. We still lack data on the impact of many lifestyle choices on the gut microbiota and thus on the host. While we are starting to develop a concept of core species in the gut, we know very little on the core functions therein or core metabolites that these functions produce. However, this issue is not intractable, and as new technological developments become available, we will be able to acquire this information and start to develop predictive models of the gut microbiota and their role in health and disease.

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CHAPTER 3

Oxalate-Degrading Bacteria of the Human Gut as Probiotics in the Management of Kidney Stone Disease

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Abstract Humans lack the enzymes needed to metabolize endogenous and dietary oxalate, a toxic compound causing hyperoxaluria and calcium oxalate urolithiasis. Oxalate in humans can be eliminated through (1) excretion in urine, (2) forming insoluble calcium oxalate and elimination in feces, or (3) oxalate degradation by gastrointestinal (GIT) microorganisms. In this article, anaerobic oxalate catabolism in gut bacteria is reviewed, and the possible use of these bacteria as probiotics for treating kidney stone disease is evaluated. *Oxalobacter formigenes* and *Lactobacillus* and *Bifidobacterium* species are the best studied in this regard, with oxalate degradation in the lactic acid bacteria being both species- and strain-specific. The GIT oxalate-degrading bacteria express the catabolic enzymes formyl-CoA transferase (Frc) and oxalyl-CoA decarboxylase (Oxc). The genes encoding these proteins are clustered on the genomes and show strong phylogenetic relationships. Clinical trials investigating reduced hyperoxaluria through administering *O. formigenes* or its enzymes show a promising trend, but the data need confirmation through larger scale, well-controlled trials. Similar studies using *Lactobacillus* and *Bifidobacterium* species also show *in vivo* oxalate reduction, but these data are still controversial. In particular, further investigations need to determine whether there is a direct link between the lack of oxalate-degrading bacteria and hyperoxaluria and whether their absence is a risk factor. Key experiments linking microbial numbers, functional oxalate degradation, molecular analysis of the regulation of the genes involved, and the ability of the bacteria to survive in the gut are crucial elements in identifying suitable probiotics for treating kidney stone disease.

I. INTRODUCTION

A. Oxalate homeostasis in the gut: Sources of oxalate

Oxalic acid is a highly oxidized, strong organic acid that is widely distributed in nature, occurring in both plants and animals. In plants, it generally accumulates as a metabolic end product in the form of free acid. However, it can act as a chelator of cations and is often found as soluble sodium or potassium oxalate, or precipitated as insoluble calcium oxalate

(Lung *et al.*, 1994), which can accumulate as microscopic crystals in the plant tissues, sometimes contributing as much as 80% of the dry weight of the plant (Webb, 1999). Oxalic acid and its oxalate salts occur in the blood (plasma) and urine of animals and humans. Part of the oxalate found in humans comes from ingesting the oxalate-containing plant material in the form of foods such as strawberries, spinach, rhubarb, beets, nuts, wheat bran, chocolate, tea, and coffee (Duncan *et al.*, 2002). A certain amount of oxalate is also formed endogenously in the liver through the metabolism of glycine, glyoxylate, and ascorbic acid (Holmes and Assimos, 1998). However, the relative proportions that exogenous and endogenous sources contribute to the oxalate concentration in the gut are still under debate (Ferraz *et al.*, 2009).

B. Oxalate assimilation in the gut

Humans lack the enzymes that are needed to metabolize oxalate, and this potentially toxic compound is, therefore, dealt with chiefly in three ways. It may be absorbed into the urinary tract and excreted in urine. Alternatively, oxalate in the gut can combine with calcium, forming insoluble calcium oxalate, which is eliminated in feces. Furthermore, it can be degraded by microorganisms present in the gastrointestinal tract (GIT). The relative amounts of calcium and oxalate are important factors affecting the rate of oxalate absorption and urinary excretion (Campieri *et al.*, 2001). It has been shown that, in healthy individuals, an increased intake of dietary oxalate can significantly enhance the excretion of urinary oxalate (Massey *et al.*, 1993). Hatch *et al.* (1993) measured the transport of ^{14}C-oxalate *in vitro* across the various regions of rat or rabbit intestine. They found that in the ileum, jejunum, and proximal colon there was the net movement of oxalate from the serum to the gut mucosa, suggesting that oxalate would be secreted into these regions of the gut *in vivo*. This is different from the situation in the distal colon, which showed a trend towards oxalate absorption (movement from the mucosa to serosa). An implication of this finding would be that the levels of oxalate found in the GIT might vary according to these regions and could influence the local nutrient environment and hence the oxalate-degrading microorganisms found there.

C. Hyperoxaluria and kidney stone disease

High levels of oxalate in humans can have a detrimental, corrosive effect. It can cause a range of medical pathologies including hyperoxaluria and renal failure (Hoppe *et al.*, 2009), calcium oxalate urolithiasis (Campieri *et al.*, 2001), and cardiomyopathy (Van Driessche *et al.*, 2007). Hyperoxaluria is characterized by extremely high levels of urinary oxalate, which

can lead to urolithiasis (stone formation). Although these stones occur most frequently in the kidneys (kidney stones), they can occur anywhere in the urinary tract including the urethra and bladder (Duncan *et al.*, 2002). The process of kidney stone formation involves the formation of crystals that separate from the urine and accumulate on the inner surfaces of the urinary tract. Although up to 80% of kidney stones are comprised of calcium oxalate, the crystals may also contain calcium phosphate and uric acid (Liebman and Costa, 2000).

Kidney stone disease is a worldwide problem affecting between 3% and 20% of people, and many patients have a history of relapse. The development of calcium oxalate stones has been shown to be a multifactorial process, which is influenced by environmental and genetic factors (Knight *et al.*, 2007). Incidences of the disease are associated with a number of risk factors including race (Rodgers and Lewandowski, 2002), diet (Taylor and Curhan, 2004), climate and season (Baker *et al.*, 1993), and gender (Fan *et al.*, 1999), with men having a higher risk of developing kidney stone disease (10%) than women (5%) (Hall, 2009).

Of these risk factors, dietary oxalate plays a major role in influencing levels of oxalate in urine (Hesse *et al.*, 1993; Holmes and Kennedy, 2000) and hyperoxaluria is the most significant risk factor in the formation of kidney stones. Furthermore, it has been shown that the oxalate/calcium molar ratio, normally maintained at 1:10, is critical, and that a change in urinary oxalate concentration is more likely to initiate crystallization and stone formation than changes in the calcium concentration (Siener *et al.*, 2005). The precipitation of calcium oxalate in urine has been found to be dependent on its saturation with both calcium and oxalate ions (Lung *et al.*, 1991)

Certain medical interventions or pathological conditions, which result in increased absorption of oxalate in the colon, can also cause the formation of kidney stones. Examples of these are jejuno-ileal or gastric bypass surgery (Duffey *et al.*, 2008) and pathological conditions such as Crohn's disease (Hanson, 2005) and cystic fibrosis (Sidhu *et al.*, 1998). Urolithiasis and calcium oxalate stone formation have also been identified as risk factors in HIV-infected patients being treated with protease inhibitor antiretroviral therapy (Nadler *et al.*, 2003).

II. OXALATE UTILIZATION BY ANAEROBIC AND FACULTATIVE GUT BACTERIA

The human body is home to a large, dynamic consortium of several hundred species of microorganisms (Macfarlane and Macfarlane, 2004), and the majority of these are found in the GIT. The major components of the adult GIT microbiota are particular to an individual and are generally stably maintained, but can vary to an extent over time within individuals

in response to diet and other factors such as ingesting antibiotics (Iapichino *et al.*, 2008). GIT bacteria carry out a range of biochemical reactions that can affect human health and nutrition (Hooper *et al.*, 2002), and certain bacterial genera, such as *Lactobacillus* and *Bifidobacterium*, in particular, have been used extensively as probiotics in foodstuffs and pharmaceuticals since they are naturally occurring bacteria that contribute positively to human health. GIT bacteria degrade many dietary substances that cannot be digested by humans, and this includes oxalate. Oxalic acid is a simple dicarboxylic acid. It is toxic at higher levels and is generally not the primary source of energy for most bacteria, as it has a low yield of energy during metabolism. However, it appears that two major groups of oxalate-degrading GIT bacteria can be identified (Sahin, 2003). There are the ''generalist oxalotrophs,'' which do not depend entirely on oxalate as an energy source and can ferment many other substrates in addition to it, and the ''specialist oxalotrophs,'' which use oxalate as their sole or major carbon and energy source.

III. MECHANISMS OF OXALATE CATABOLISM IN ANAEROBIC BACTERIA OF THE HUMAN GUT

Oxalate degradation by bacteria occurs via both aerobic and anaerobic respiratory pathways (Sahin, 2003). During aerobic growth, oxalate is metabolized to produce CO_2 and formate, and formate dehydrogenase then oxidizes the latter compound. Anaerobic bacteria, such as those found in the GIT, however, are not able to oxidize the formate, and thus this accumulates as a major end product of the catabolism of oxalate (Cornick and Allison, 1996). The mechanisms, genes, and enzymes involved in anaerobic oxalate catabolism in gut bacteria are reviewed below.

A. *Oxalobacter formigenes*

O. formigenes was the first oxalate-degrading obligate anaerobe to be described in humans (Allison *et al.*, 1985). It is a non-motile, non-spore-forming, Gram-negative bacterium, forming rods that may be curved and occasionally occur in chains of spiral or coiled filaments. The various strains of this species can be differentiated by their cellular fatty acid composition (Allison *et al.*, 1985). *O. formigenes* has served as the paradigm organism in which anaerobic oxalate degradation has been studied, and it has three enzymes involved in the catabolism of oxalic acid (Fig. 3.1). The process begins with the uptake of extracellular oxalate by the membrane-associated oxalate–formate antiporter, OxlT, encoded by the *oxlT* gene (Abe *et al.*, 1996; Anantharam *et al.*, 1989; Ruan *et al.*, 1992). The *frc* gene encodes formyl-CoA transferase, Frc, which activates the intracellular oxalate to

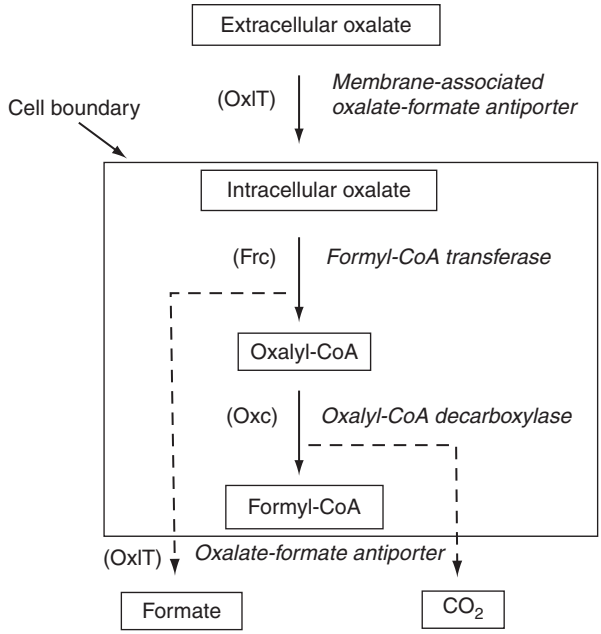

FIGURE 3.1 Oxalate degradation pathway in *O. formigenes*. Substrates and products are shown in boxes; enzyme names are italicized with their protein abbreviations in brackets; dotted lines show secreted product.

form oxalyl-CoA (Baetz and Allison, 1990; Sidhu *et al.*, 1997b). This is decarboxylated in a thiamine PP_i-dependent reaction by the oxalyl-CoA decarboxylase, Oxc, enzyme, expressed from the *oxc* gene. Formate and carbon dioxide are the end products (Lung *et al.*, 1994), and the oxalate–formate antiporter, OxlT, catalyzes the export of the intracellular formate out of the cells (Abe *et al.*, 1996; Anantharam *et al.*, 1989). The generation of energy in *O. formigenes* is coupled to oxalate transport, mediated by the oxalate transport membrane protein OxlT (Anantharam *et al.*, 1989; Harold and Maloney, 1996; Kuhner *et al.*, 1996, Ruan *et al.*, 1992). The decarboxylation of oxalate generates a proton pump gradient, which generates one ATP molecule when it is coupled with oxalate–formate transport (Ruan *et al.*, 1992). *O. formigenes* uses oxalate as an exclusive source of energy, and may, therefore, be described as a ''specialist oxalotroph'' (Sahin, 2003).

B. *Enterococcus faecalis*

This Gram-positive facultative anaerobe, formerly known as *Streptococcus faecalis*, occurs as single cocci or in chains of various lengths. It is an intestinal commensal, but it is also a significant opportunistic pathogen

causing urinary tract and wound infections outside of this environment. In 2000, Hokama *et al.* isolated and described the first oxalate-degrading *E. faecalis* strain of human fecal origin. However, the bacteria only maintained the ability to degrade oxalate if they were cultured repeatedly in a poor nutritional environment. This implies that they are ''generalist oxalotrophs'' and would only utilize oxalate as a carbon source in the absence of alternative energy supplies. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analyses of protein lysates of the oxalate-degrading *E. faecalis* strain showed the presence of three proteins that were absent in non-oxalate-degrading strains of this bacterium (Hokama *et al.*, 2000). The proteins were also not expressed under conditions where the oxalate-degrading strains of *E. faecalis* failed to degrade oxalate. This suggested the presence of an inducible oxalate degradation system that might be used as a survival mechanism when preferred substrates become scarce. Western blot analyses of two of the putative *E. faecalis* oxalate-degrading proteins showed reactions with antibodies raised against *O. formigenes* Frc and Oxc proteins, suggesting that the oxalate-degrading enzymes of *E. faecalis* were similar to those found in *O. formigenes*. No oxalate transport protein was identified, and the exact mechanism of oxalate degradation in *E. faecalis* has not been reported.

C. *Providencia rettgeri*

An oxalate-degrading strain of *P. rettgeri* has been isolated from human feces. This bacterium is a rod-shaped, Gram-negative facultative anaerobe which shows weak virulence and can cause urinary tract infections (Hokama *et al.*, 2005). It is, therefore, unlikely that it plays a major role in oxalate metabolism in healthy subjects. Two putative oxalate-degrading proteins have been identified in the oxalate-degrading strains of *P. rettgeri* (Hokama *et al.*, 2005) which are not produced by strains of this bacterium that are unable to degrade oxalate. These two proteins cross-reacted immunologically with antibodies raised to the *O. formigenes* oxalate-degrading Oxc or Frc proteins and had similar molecular weights, suggesting that a similar mechanism of oxalate degradation might exist in these two species. It is interesting to note that sub-culturing of the *P. rettgeri* isolate in the absence of oxalate resulted in a loss of the ability to degrade oxalate as well as the disappearance of the two proteins, thus linking them strongly to the phenotype. Subsequent re-culturing of the *P. rettgeri* on an oxalate-rich medium did not allow recovery of the phenotype or the proteins (Hokama *et al.*, 2005). These results suggest a certain level of regulation of the oxalate-degrading activity in *P. rettgeri*, but the exact mechanism remains unclear.

D. *Eubacterium lentum*

The genus *Eubacterium* is the second most common bacterial genus found in the human GIT after the *Bacteroides* (Schwiertz *et al.*, 2000), and studies on oxalate-degradation by GIT bacteria identified *E. lentum* as an oxalate-catabolizing species (Ito *et al.*, 1996a,b). The genus is characterized as containing a wide range of phenotypically diverse species that are anaerobic and produce non-spore-forming Gram-positive rods. This makes the phenotypic identification of *Eubacterium* spp. extremely time consuming, and this has perhaps inhibited the detailed study of members of the genus isolated from fecal matter. Ito *et al.* (1996b), however, successfully isolated *E. lentum* WHY-1 from the feces of a Japanese male and showed that it could be used to reduce the oxalate content of tea *in vitro*. The oxalate-degrading proteins oxalyl-CoA decarboxylase and formyl-CoA transferase were reportedly isolated from this strain (Ito *et al.*, 1996a).

E. *Escherichia coli*

The YfdW protein of the Gram-negative facultative anaerobe *Escherichia coli* has been purified and confirmed to be functionally and structurally homologous to the *O. formigenes* formyl-CoA transferase (Gruez *et al.*, 2003; Toyota *et al.*, 2008). The *yfdW* gene is part of the *E. coli yfdXWUVE* operon, which has been shown to be involved in enhancing cell survival under acidic conditions (Foster, 2004), but the molecular mechanisms for this phenomenon are as yet unknown. Lewanika *et al.* (2007) found that the *yfdU* gene is a putative *oxc* homologue, and that *yfdV* encodes a putative membrane-bound transport protein. Although *E. coli* has not been reported in the scientific literature as being capable of oxalate degradation *in vitro*, certain freshly isolated fecal *E. coli* strains do initially show improved growth on an oxalate-enriched medium, but this phenotype is soon lost on further sub-culturing (unpublished data, our laboratory). These data, taken together with the information on transient oxalate degradation in *E. faecalis* and *P. rettgeri*, suggest that these GIT bacteria may have the capacity to degrade oxalate *in vivo* but that it is difficult to sustain and observe this phenomenon under currently used *in vitro* conditions. Simulated gut conditions might provide a more physiologically appropriate environment for investigations of oxalate catabolism in these organisms.

F. *Lactobacillus* and *Bifidobacterium* species

Lactobacillus and *Bifidobacterium* species are Gram-positive, non-spore-forming rods occurring in high numbers in the human gut. *Bifidobacterium* species are obligately anaerobic, whereas *Lactobacillus* spp. are often more

aerotolerant. Members of both genera have been used extensively as probiotics in promoting human health (Wolf *et al.*, 1998) since they are classified as GRAS (generally regarded as safe) for human consumption. Recently, there has been increased research interest in exploiting their possible oxalate-degrading capabilities as probiotics to manage kidney stone disease. Table 3.1 gives a summary of the major studies reporting oxalate degradation by strains of *Lactobacillus* and *Bifidobacterium*.

It has been noted that oxalate degradation in the lactobacilli and bifidobacteria is both species- and strain-specific (Table 3.1). Federici *et al.* (2004) investigated oxalate degradation in a range of *Bifidobacterium* species. They reported that *Bifidobacterium lactis* DSM 10140 showed the highest level of oxalate degradation at 61%, *Bifidobacterium longum* MB 282

TABLE 3.1 Reported oxalate-degrading *Lactobacillus* and *Bifidobacterium* species

Bacteria[a]	Reference
Lactobacillus plantarum ($n = 1/1$) *Lactobacillus brevis* ($n = 1/1$) *Lactobacillus acidophilus* ($n = 1/1$) *Bifidobacterium infantis* ($n = 1/1$)	Campieri *et al.* (2001)
Bifidobacterium lactis DSM 10140 ($n = 1/1$) *Bifidobacterium animalis* ATCC 27536 ($n = 1/1$) *Bifidobacterium breve* MB 283 ($n = 1/2$) *Bifidobacterium longum* MB 282 ($n = 4/5$) *Bifidobacterium infantis* MB 57 ($n = 1/2$) *Bifidobacterium adolescentis* MB 238 ($n = 1/1$)	Federici *et al.* (2004)
Lactobacillus casei ($n = 2/31$)	Kwak *et al.* (2006)
Lactobacillus acidophilus ($n = 1/1$)	Azcarate-Peril *et al.* (2006)
Lactobacillus gasseri Gasser AM63^T ($n = 1/1$)	Lewanika *et al.* (2007)
Lactobacillus acidophilus ($n = 32/32$) *Lactobacillus gasseri* ($n = 6/6$) *Lactobacillus plantarum* ($n = 3/7$) *Lactobacillus casei* ($n = 2/3$) *Lactobacillus rhamnosus* ($n = 2/2$) *Lactobacillus salivarius* ($n = 1/1$)	Turroni *et al.* (2007)
Lactobacillus gasseri ATCC 33323 ($n = 7/9$) (previously known as *L. gasseri* Gasser AM63^T)	Azcarate-Peril *et al.* (2008)

[a] ($n = x/y$): Bacterial numbers (n) in brackets indicate how many isolates showed the ability to degrade oxalate (x), relative to the total number of isolates tested in the study (y).

degraded 35.2% of oxalate, and *Bifidobacterium adolescentis* MB 238 showed 57% degradation. The oxalate-degrading ability of certain *Bifidobacterium* species was also strain-specific. For instance, *Bifidobacterium breve* MB 283 degraded 37.8% of available oxalate, while *B. breve* MB 151 degraded only 1%, and of the five *B. longum* strains tested in the study, only four were positive for oxalate degradation.

Previous *in vitro* experiments by Campieri *et al.* (2001) showed that the *Lactobacillus* and *Bifidobacterium* bacterial strains tested were ''generalist oxalotrophs'' since they were unable to utilize oxalate as a principal carbon source and could only degrade oxalate when the growth media contained glucose or lactose as well as oxalate. However, a polymerase chain reaction (PCR) investigation of these isolates using primers designed to the *O. formigenes* genes *oxlT*, *frc*, and *oxc* did not detect the presence of orthologs of these genes in any of the oxalate-degrading *Lactobacillus* and *Bifidobacterium* species reported. Subsequently, Federici *et al.* (2004), using a different set of primers to the *O. formigenes oxc* gene, successfully amplified a homologue from an oxalate-degrading strain of *Bifidobacterium lactis*. This *oxc* gene encoded a functional oxalyl-CoA decarboxylase enzyme when the recombinant protein was expressed in *E. coli*, and the protein cross-reacted with anti- *O. formigenes* oxalyl-CoA decarboxylase antibodies. However, no other molecular studies on the mechanisms of oxalate degradation in *Bifidobacterium* species have been reported and this genus would be of interest for further investigation.

Research on *Lactobacillus* spp. is at a more advanced stage. Genes encoding functional oxalate-degrading Frc and Oxc enzymes were identified in *Lactobacillus acidophilus* NCFM (Azcarate-Peril *et al.*, 2006). It was shown that oxalate-dependent induction of the genes occurred only when the cells were first adapted to subinhibitory concentrations of oxalate under mildly acidic conditions (pH 5.5). The molecular mechanism of oxalate degradation in *Lactobacillus gasseri* AM63^{T} has also been investigated (Lewanika *et al.*, 2007). The presence of *frc* and *oxc* genes was detected using PCR, and the genes were shown to be induced as an operon in the presence of oxalate under similar acid conditions to those reported in *L. acidophilus* (Azcarate-Peril *et al.*, 2006). A recent study by Turroni *et al.* (2007) identified a range of *Lactobacillus* spp. that could degrade oxalate (Table 3.1). The presence of *oxc* and *frc* genes, as demonstrated by gene-specific PCR, was shown in all *L. acidophilus* and *L. gasseri* isolates that degraded more than 50% oxalate. Specific functional characterization of the Oxc and Frc enzymes from one of the *L. acidophilus* isolates confirmed that these enzymes were responsible for oxalate degradation. The remaining oxalate-degrading species isolated in this study (Table 3.1) were not examined at the molecular level for the presence of the genes.

A summary of all the current data on *Lactobacillus* species that are able to degrade oxalate is presented in Table 3.1. This review shows that all strains of *L. acidophilus* tested (34 strains) are capable of degrading oxalate efficiently, while the phenotype was found in only 14 out of 16 strains of *L. gasseri*, 2 out of 31 strains of *Lactobacillus casei*, and 3 out of 7 *Lactobacillus plantarum* isolates tested. This inter-species and inter-strain variation is of interest from an evolutionary perspective, and it also impacts on the appropriate selection of bacterial isolates for future study and therapeutic applications.

IV. PHYLOGENY AND GENETIC ORGANIZATION OF THE GENES INVOLVED IN OXALATE DEGRADATION

A. Phylogeny of the Oxc and Frc proteins

Since functional studies on the oxalate-degrading abilities of different bacterial strains showed significant inter-species and inter-strain variation (Section II.F), we have performed a bioinformatic analysis to determine whether there are orthologous genes in the published genomes of various other bacteria. Amino acid sequences of experimentally confirmed functional Oxc and Frc proteins were used to identify a number of orthologous proteins in the databases by using the BLAST algorithm (Altschul *et al.*, 1997). Most of these were identified in newly sequenced genomes and were not yet annotated. Phylogenetic relationships between the Oxc and Frc proteins were established using the ClustalW algorithm of Thompson *et al.* (1994), and the multiple sequence alignments are shown as unrooted phylogenetic trees (Fig. 3.2A and B).

A similar pattern of evolutionary relationships between the orthologues of the two proteins can be seen. The Oxc and Frc proteins from the *Lactobacillus* and *Bifidobacterium* species form distinct clades, separate from each other and clearly different from the other oxalate-degrading bacterial species. This common ancestry is reflected in the *Lactobacillus* proteins having from 71% to 97% amino acid identity, while the *Bifidobacterium* proteins vary between 74% and 79%. The third cluster is comprised of orthologues from the *Enterobacteriaceae*, made up of *E. coli*, *Shigella boydii*, and *P. rettgeri*, and the β-proteobacteria, *O. formigenes* and *Burkolderia phytofirmans*, a plant-associated bacterium. Clustering with these orthologues are the Oxc and Frc from *Bradyrhizobium japonicum*, a rhizosphere plant-associated bacterium. This constitutes an interesting convergence of oxalate catabolism between plant- and human-associated bacteria. A group of high-GC Firmicutes, including *Mycobacterium tuberculosis* and *Streptomyces sviceus*, form a cluster that is more closely related

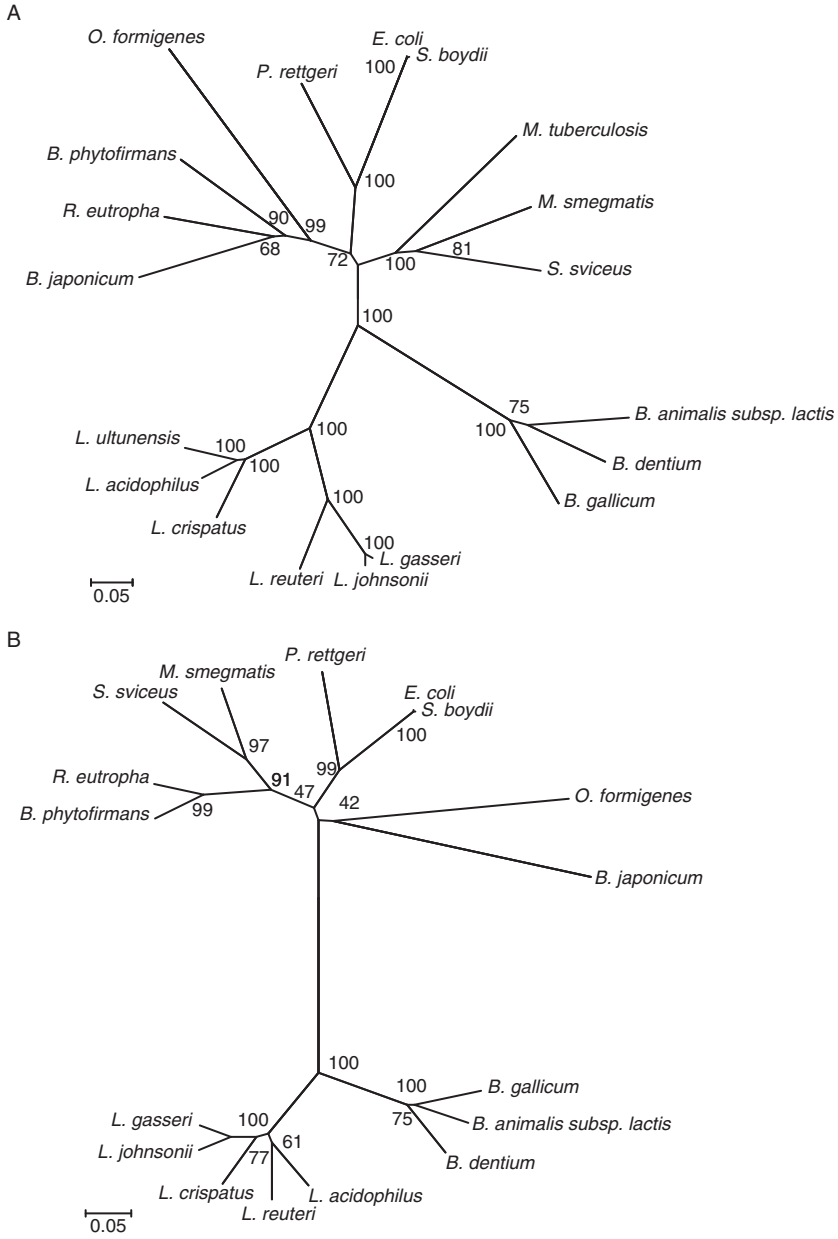

FIGURE 3.2 Phylogenetic analysis showing the relationships between the (A) oxalyl-CoA decarboxylases and (B) formyl-CoA transferases. Sequences were aligned with ClustalW, and the tree was drawn with the program MEGA4 with neighbor-joining and bootstrapping options. Figures indicate boot-strapping values after 500 iterations. NCBI accession numbers for the Oxc and Frc proteins for each species, respectively,

to these Gram-negative strains than to the high-GC *Bifidobacterium* strains, a possible indication of horizontal gene transfer (Ochman *et al.*, 2000).

B. Genetic organization of the oxalate loci

The first *oxc* and *frc* genes to be characterized were those from *O. formigenes*, and these were mapped to different regions of the genome (Lung *et al.*, 1994; Sidhu *et al.*, 1997b). The genome sequence of this bacterium has recently been completed, and confirms these findings. The *oxlT* gene, encoding the formate–oxalate antiporter protein, lies approximately 11 kb upstream of the *oxc* gene on the *O. formigenes* genome. The *frc* gene and a *lysR*-like transcriptional regulator gene lie in a different cluster, separated from *oxc* by approximately 580 kb (Fig. 3.3). In contrast to this, in all the other bacteria evaluated in this study, the *oxc* and *frc* genes are closely linked on the chromosome, and the same evolutionary relationships that are indicated by the phylogenetic studies are evident in the arrangement of the genes in the oxalate-degradation clusters (Fig. 3.3).

The gene arrangement in the lactobacilli is extremely conserved, with the *oxc* gene invariably lying upstream of the *frc* gene, suggesting a very close evolutionary relationship between the oxalate loci from these species. Genes encoding membrane-associated transport proteins or permeases are usually linked with the *oxc–frc* genes, but these vary in their position and orientation relative to the other genes. These permeases may possibly be involved in the transport of oxalate into the cell prior to catabolism, but this needs to be functionally confirmed. In contrast to this, the *Bifidobacterium* species possessing the oxalate gene cluster show close linkage between the *oxc* and the permease genes, while the *frc* gene is often separated from these by genes of unknown function and may be orientated in the opposite direction. It is interesting that in the case of *Bifidobacterium gallicum*, the *frc* gene is separated from the *oxc*-permease cluster by approximately 10 kb. The *oxc*-permease genes are followed by a gene encoding a transposase, suggesting that this region was acquired by a transposition event resulting in the separation of the *frc* gene from the oxc gene (Ochman *et al.*, 2000).

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are as follows: *L. gasseri*, YP_814095, YP_814094; *L. acidophilus*, BAF52673, YP_193317; *L. johnsonii*, ZP_04006707, ZP_04006708; *L. reuteri*, ZP_03074275, ZP_03074276; *L. crispatus*, ZP_03995673, ZP_05550023; *L. ultunensis*, ZP_04012429; *B. lactis*, *BAH23809*, ZP_02964132; *B. dentium*, ZP_02917880, ZP_02917882; *B. gallicum*, ZP_05965188, ZP_05965202; *M. tuberculosis oxc* AAK44350; *M. smegmatis*, YP_889543, YP_884574; *S. sviceus*, ZP_05023102, EDY61659; *E. coli*, ACX38958, AAN81360; *S. boydii*, YP_408788, ZP_408789; *P. rettgeri*, ZP_03640483, ZP_06127696; *O. formigenes*, AAA53683, AAC45298; *B. phytofirmans*, ACD21034, ACD21035; *R. eutropha*, YP_298857, AAZ64012; *B. japonicum*, NP_769797, Q89QH2.

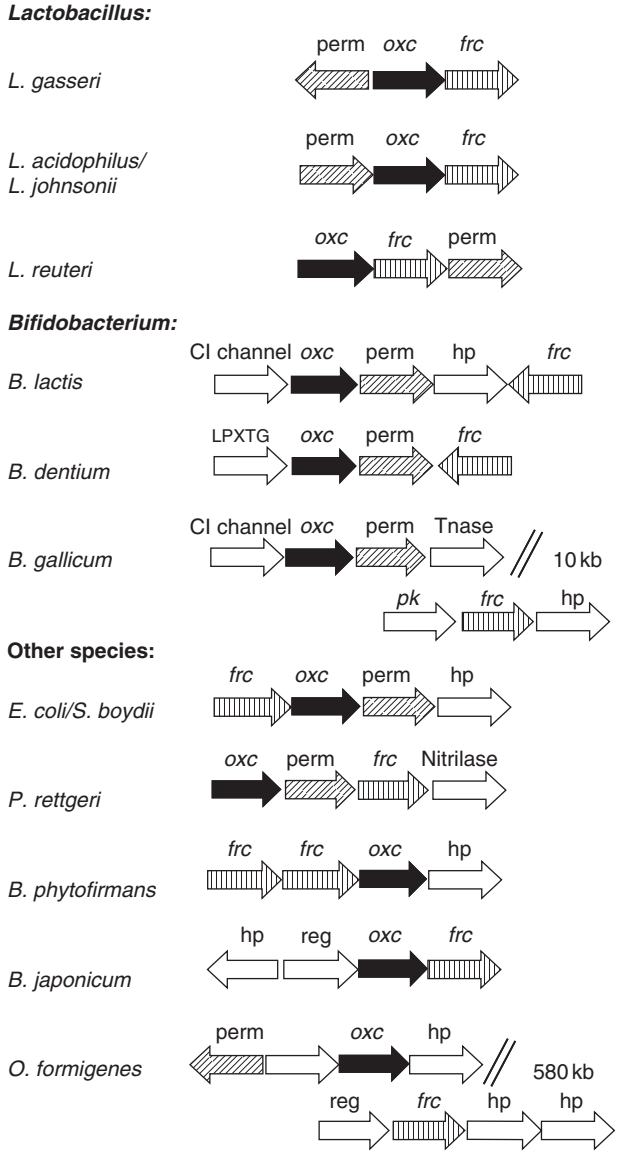

FIGURE 3.3 Comparison of the gene arrangement of the oxalate loci of different bacterial groups. Genes have been assigned identities on the basis of either their gene names or probable gene products: *oxc*, oxalyl-CoA decarboxylase; *frc*, formyl-CoA transferase; perm, membrane-associated permease of variable type; hp, hypothetical protein; Cl, channel protein; LPXTG, protein containing wall-binding motif; Tnase, transposase; pk, phosphoketolase; reg, transcriptional regulator. Strains presented are: *L. gasseri* Gasser AM63; *L. acidophilus* ATCC 4796; *L. johnsonii* ATCC 33200; *L. reuteri* 100-23; *B. lactis* DSM 10140; *B. dentium* ATCC 27678; *B. gallicum* DSM 20438; *E. coli* ATCC 8739; *S. boydii* Sb 227; *P. rettgeri* DSM 1131; *B. phytofirmans* PSJn; *B. japonicum* ORS278, and *O. formigenes* OXCC13.

The oxalate degradation gene clusters of the other bacterial species examined have very varied gene arrangements. The *oxc* gene of the different species is frequently closely linked to a gene encoding a membrane-associated permease, but these are not conserved and are not homologues of the *O. formigenes* OxlT. The nature of these permeases and their specific functions are not known. In most cases, other than that described earlier in *O. formigenes*, the *frc* gene is closely linked to these genes, and in the case of *B. phytofirmans*, the *frc* gene is duplicated in the cluster. Examination of the DNA sequences of the oxalate degradation clusters does not give an indication of how they may be regulated since a common gene encoding a regulatory protein is not evident. Only *O. formigenes* and *B. japonicum* have a gene encoding, a putative LysR-like transcriptional regulator lying upstream of the *frc* and *oxc* genes respectively, but this is absent in the other gene clusters.

As previously mentioned, many of these gene clusters have only been identified by DNA sequence identity, and functionality studies have not been performed. The situation is complicated by the finding that despite the presence of the oxalate gene cluster in the genome, some strains of a particular species are reported to show efficient degradation of oxalate, while others do not (Section II.F).The genes may be silent or only functional under certain conditions due to regulatory or genetic modifications. An interesting case is that of *Lactobacillus johnsonii*, where three different strains have been completely sequenced (ATCC 33200, F19785, and NCC 533) and the *oxc* and *frc* genes can only be detected in the genome of *L. johnsonii* ATCC 33200 (Fig. 3.3). However, the only *L. johnsonii* strain functionally tested for oxalate degradation to date is *L. johnsonii* NCC533, which was unable to utilize oxalate (Turroni *et al.*, 2007). This genetic variation between strains may represent the recent acquisition of the oxalate gene cluster by ATCC 33200. It is tempting to speculate that the genes were acquired from *L. gasseri* by horizontal gene transfer since the orthologues in these two species show 97% identity at the amino acid level.

On the other hand, a large number of *Lactobacillus* and *Bifidobacterium* strains, which have been reported to degrade oxalate, such as *L. plantarum*, *L. brevis*, *L. rhamnosus*, *L. salivarius*, *L. casei*, *B. longum*, *B. breve*, *B. adolescentis*, and *B. infantis* (Table 3.1), have not been genetically investigated for the presence of the *oxc* and *frc* genes. Homologues of these genes cannot be detected in the published genomes of these species. There are also reports of other species that are efficient degraders of oxalate, such as *E. faecalis* (Hokama *et al.*, 2000), *Eubacterium lentum* (Ito *et al.*, 1996a,b), and *Streptococcus thermophilus* (Goldfarb *et al.*, 2007), which also do not have homologues of the *oxc* and *frc* genes in their published genomes. This may be due to the fact that these species have a different mechanism for dealing with oxalate, controlled by genes with a different evolutionary origin which have not yet been identified.

V. DETECTION AND QUANTIFICATION OF OXALATE-DEGRADING BACTERIA IN THE GIT AND LINKS TO KIDNEY STONE DISEASE

Much of the knowledge about the identity of oxalate-degrading GIT microbial species has been obtained through bacteriological culture techniques. These have, however, been shown to be of limited value in isolating fastidious GIT bacteria since the selective media and growth conditions used often fail to mimic the conditions a particular bacterium needs for growth in its natural habitat (Hugenholtz *et al.*, 1998). It has been estimated that only 20% of naturally occurring bacteria have been isolated using culture techniques (Ward *et al.*, 1990). Culture-independent molecular techniques such as 16S rRNA gene sequence variation have, therefore, been used to infer the identity and phylogenetic relationships among microorganisms (Suau *et al.*, 1999). In addition, enumeration of specific species has been attempted using quantitative PCR techniques. This is an important aspect of kidney stone research since it might assist in finding correlations between the absence of certain oxalate-degrading bacterial species and the risk of developing calcium oxalate renal calculae.

Sidhu *et al.* (1999a) used a highly conserved region of the *O. formigenes* *oxc* gene as a target to enumerate this bacterial species in human fecal samples and showed that the results were reproducible and correlated with data obtained using culture-based techniques. A combined approach of using the 16S rRNA gene together with the *oxc* gene, in order to detect the presence or absence of *O. formigenes* in feces of patients, was used by Kwak *et al.* (2001) and Kodama *et al.* (2002), but neither of these groups attempted quantification of the actual numbers of bacteria present. Both studies reported a correlation between lower incidences of colonization in stone-forming patients as compared to healthy subjects. The use of molecular techniques to detect and accurately quantify *O. formigenes*, however, remains controversial since Prokopovich *et al.* (2007) found a high degree of variability in the numbers of *O. formigenes* detected in different samples from the same individual (nonuniform distribution) and between different individuals. Furthermore, even in cases where less variability was seen, they were not able to demonstrate a correlation between the amount of oxalate present in feces of patients and number of *O. formigenes* detected, suggesting that other bacteria in addition to *O. formigenes* may be involved in oxalate degradation. A recent review of the current status of detection and quantification of *O. formigenes* in the gut (Siva *et al.*, 2008) concludes that specificity of primers and heterogeneity of distribution of *O. formigenes* in the gut significantly affect the reliability of current methodologies and that future refinements are required.

Research on methods for the quantitation of oxalate-degrading *Lactobacillus* and *Bifidobacterium* species is not at an advanced stage. Although there are several reports on the use of real-time PCR to quantify these genera and species (Castillo *et al.*, 2006; Delroisse *et al.*, 2008), no studies have, as yet, been done in the context of linking them to gut oxalate management. One of the major difficulties in such studies is the design of sufficiently robust genus-specific 16S rRNA gene primers. In addition, variation in the numbers of 16S rRNA copies per strain could lead to severe over- or underestimation of numbers. As mentioned earlier (Sections II and III), there is a high degree of inter-species and inter-strain variation in the ability of the *Lactobacillus* and *Bifidobacterium* isolates to degrade oxalate, and simple measurement of the genus or species numbers would not necessarily reflect functionality in this context.

VI. PROBIOTIC POTENTIAL OF OXALATE-DEGRADING BACTERIA *IN VIVO*

The role of oxalate-degrading bacteria in the treatment of kidney stone disease has become a major research interest (Duncan *et al.*, 2002; Stewart *et al.*, 2004). Several studies have focused on ascertaining whether the oxalate-degrading capacity of various bacteria, as observed *in vitro*, can also be demonstrated in simulated gut continuous culture fermenters or *in vivo* using rat or human models. The current status of these studies in relation to the suitability of these bacteria as probiotics in the prophylactic treatment of kidney stone disease is reviewed below.

A. *Oxalobacter formigenes*

Several studies have reported an extensive variation in the degree to which *O. formigenes* colonizes the normal human gut. However, the data presented below need to be viewed with care in the light of the recent quantitation issues raised by Prokopovich *et al.* (2007) (Section IV). It has been reported that there may be undetectable levels of the bacterium and that it may also occur in numbers as high as 10^8 cfu/g wet weight of feces (Sidhu *et al.*, 1997a). The occurrence of *O. formigenes* is also reported to be affected by the age of an individual, reaching 100% detection in children, and only 40–60% in adults. Cystic fibrosis patients show a further reduction in *O. formigenes* numbers (Sidhu *et al.*, 1998), where the loss of the bacterium in these people has been associated with increased use of antibiotics and other drugs.

The functional role of *O. formigenes* in the treatment of kidney stone disease has recently been reviewed (Hatch and Freel, 2008; Siva *et al.*, 2008) and these articles should be considered together with the present

discussion. *O. formigenes* and its relevant oxalate-degrading enzymes have been used in the probiotic treatment of hyperoxaluria and hyperoxaluria-simulated conditions. Sidhu *et al.* (2001) fed a high-oxalate diet (2% ammonium oxalate) to laboratory rats together with enzyme replacement therapy in the form of encapsulated crude protein lysates of the two *O. formigenes* oxalate-degrading proteins, formyl-coenzyme A transferase and oxalyl-coenzyme A decarboxylase. They reported reduced urinary oxalate excretions in the test rats when compared to the control group. Further experiments involved feeding an *O. formigenes* probiotic preparation to rats placed on a high oxalate diet for 14 days and measuring the urinary oxalate as an indication of the oxalate-degrading ability of the probiotic (Sidhu *et al.*, 2001). Results showed that rats fed *O. formigenes* were able to maintain lower levels of urinary oxalate than the control group, which was not fed *O. formigenes*. In addition to the *O. formigenes* actively metabolizing the oxalate, it appears it also had an additional beneficial function via increasing oxalate secretion in the gut (Hatch and Freel, 2008).

The ability of *O. formigenes* to degrade oxalate in a simulated human gut environment was tested by Duncan *et al.* (2002) using a single-stage, continuous-culture fermenter inoculated with a human fecal sample that contained no detectable oxalate-degrading bacteria prior to the study. Results showed that *O. formigenes* strain OxB was able to colonize the fermenter and degrade all of the oxalate supplied in the culture medium in 2 days. In a follow-up investigation, four human subjects ingested oxalate together with an *O. formigenes* preparation and showed lower urinary oxalate levels 6 h after administration as compared to the experimental control group who had not received the probiotic (Duncan *et al.*, 2002). More recently, Hoppe *et al.* (2007) showed a similar reduction in urinary oxalate in patients suffering from primary oxaluria.

While these results show a promising trend towards the use of *O. formigenes* or its enzymes to control *in vivo* levels of oxalate in humans, studies are still at an early stage and the data need to be confirmed by larger scale, well-controlled trials (Hatch and Freel, 2008). In particular, investigations need to be done on whether the lack of *O. formigenes per se* is a direct cause of hyperoxaluria, or whether its absence might simply be considered a risk factor.

In this context, the absence of *O. formigenes* has been strongly linked to antibiotic treatment and not necessarily to stone formation (Sidhu *et al.*, 1999b). Furthermore, nearly 50% of healthy people who do not suffer from kidney stone disease are not colonized by *O. formigenes* (Sidhu *et al.*, 1998), suggesting the possible involvement of other organisms in the prevention of hyperoxaluria. This has led to investigations as to whether other oxalate-degrading gut bacteria might be of interest, and these are reviewed below.

B. *Lactobacillus* and *Bifidobacterium* species

Functional studies on the effectiveness of lactic acid bacteria in reducing hyperoxaluria *in vivo* are not at such an advanced stage as the *O. formigenes* research. The chief difficulties appear to be the great inter-strain and inter-species variations with respect to the ability of these bacteria to degrade oxalate *in vitro* and *in vivo.* The major functional studies using lactic acid bacteria are summarized here. However, it should be noted that these studies cannot be directly compared in the quantitative sense since there are several variations in the protocols used. For example, different sources and concentrations of oxalate (sodium vs. ammonium; range 5–20 mM) were used. In addition, assays were performed for different lengths of time (3–5 days). The general trends of the findings are, however, of interest.

Campieri *et al.* (2001) identified potential probiotic strains through evaluating oxalate degradation by pure cultures of *L. acidophilus*, *L. plantarum*, *L. brevis*, *S. thermophilus*, and *B. infantis*. Of the lactobacilli, *L. acidophilus* showed the highest percentage breakdown of 10 mM ammonium oxalate (11.8%), and *L. brevis* the lowest (0.9%). *S. thermophilus* and *B. infantis* degraded 2.3% and 5.3% ammonium oxalate, respectively. Although these *in vitro* values were relatively low, an *in vivo* trial that involved orally administering a freeze-dried preparation comprising all of these same strains ($>2 \times 10^{11}$ cfu/g), over a 4-week period, to six patients with a history of idiopathic calcium oxalate urolithiasis and mild hyperoxaluria was performed. There was a significant reduction in the urinary oxalate in these individuals (55.5 ± 19.6 mg/24 h reduced to 35.5 ± 15.9 mg/24 h), and they continued to show lower than baseline urinary oxalate levels 1 month after feeding had been discontinued.

Lieske *et al.* (2005) reported decreased urinary oxalate levels in 10 patients, with a history of calcium oxalate urolithiasis, after administration of Oxadrop®, a preparation comprising oxalate-degrading strains of *L. acidophilus*, *B. breve*, *S. thermophilus*, and *B. infantis*. The relative *in vitro* oxalate-degrading capacity of the individual or combined strains was not reported. In this study, 7 out of 10 patients showed decreased urinary oxalate secretion (mean 19%) after 1 month of a single daily dose of Oxadrop®. During the washout period of the study, when Oxadrop® feeding was discontinued, 5 of the 10 patients recorded decreased urinary oxalate levels (20% average) when compared to baseline values. However, in a follow-up randomized, placebo-control trial of the same Oxadrop® product, Goldfarb *et al.* (2007) were not able to demonstrate a significant reduction in urinary oxalate excretion in patients with idiopathic hyperoxaluria. Responses to this therapeutic product are, therefore, still controversial.

Kwak *et al.* (2006) used a mixture of oxalate-degrading *Lactobacillus casei* strains (HY2734 and HY7201) to investigate whether these bacteria

could prevent urolithiasis in high-risk stone-forming rats. They detected decreased levels of oxalate in the urine as well as less abundant crystal formation in the kidneys in the treatment group as compared to controls.

L. casei, together with *B. breve*, has also recently been tested as a treatment for hyperoxaluria by Ferraz *et al.* (2009). The experimental cohort consisted of 14 stone-forming patients who were first placed on an oxalate-free diet and then administered an oxalate-rich diet together with the bacterial mixture. Although seven of the patients showed a degree of reduction in oxaluria, the results were not significant and were highly variable. The report of this study, however, does not indicate whether the individual bacterial strains used showed *in vitro* oxalate-degrading capacity.

All these studies with the exception of that of Goldfarb *et al.* (2007) have only involved very small cohorts of participants and did not include placebo-controlled double-blind testing. These types of studies need to be carried out in order to verify the value of probiotic lactic acid bacteria in the treatment of hyperoxaluria.

VII. CONCLUDING COMMENTS

The high incidence of recurrent kidney stone disease has led to increased research on the possible role played by oxalate-degrading bacteria of the GIT in oxalate homeostasis with a view to the development of probiotic candidates for the management of hyperoxaluria. Most research to date has concentrated on the ''specialist oxalotroph'' *O. formigenes*. However, the absolute dependency of this bacterium on oxalate as an energy source may limit its use in preventive therapy where diets are low in oxalate. Furthermore, *O. formigenes* is a fastidious anaerobe which is highly sensitive to antibiotics, and this may reduce its usefulness for probiotic administration. The use of ''generalist oxalotrophs,'' particularly *Lactobacillus* and *Bifidobacterium* species, represents an alternative approach.

In order to develop a successful therapeutic regimen, it will be necessary to use a combination of functional and molecular approaches to identify the best oxalate-degrading bacterial species that also have additional good probiotic features such as the ability to colonize the gut effectively. This review of the current level of knowledge in the field has demonstrated that greater insight is needed into how bacteria regulate the expression of the genes encoding oxalate-degrading enzymes. It is also important to establish whether the presence of these genes on the genome always correlates with oxalate degradation capacity, especially in cases where the oxalate catabolic phenotype is only transiently expressed *in vitro* immediately after isolation and is lost on subsequent sub-culturing (Section II). This genetic and physiological information could be used to develop more accurate quantitation techniques to evaluate the correlation

between the oxalate levels, the presence or absence of oxalate-degrading bacteria, and the risk of developing kidney stone disease. Once candidate probiotic bacteria have been identified, it is essential that they be subjected to trials using rigorous *in vivo* placebo-controlled, double-blind tests.

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CHAPTER 4

Morphology and Rheology in Filamentous Cultivations

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Contents

Abstract

Because of their metabolic diversity, high production capacity, secretion efficiency, and capability of carrying out posttranslational modifications, filamentous fungi are widely exploited as efficient cell factories in the production of metabolites, bioactive substances, and native or heterologous proteins, respectively.

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There is, however, a complex relationship between the morphology of these microorganisms, transport phenomena, the viscosity of the cultivation broth, and related productivity. The morphological characteristics vary between freely dispersed mycelia and distinct pellets of aggregated biomass, every growth form having a distinct influence on broth rheology. Hence, the advantages and disadvantages for mycelial or pellet cultivation have to be balanced out carefully. Because of the still inadequate understanding of the morphogenesis of filamentous microorganisms, fungal morphology is often a bottleneck of productivity in industrial production. To obtain an optimized production process, it is of great importance to gain a better understanding of the molecular and cell biology of these microorganisms as well as the relevant approaches in biochemical engineering. In this chapter, morphology and growth of filamentous fungi are described, with special attention given to specific problems as they arise from fungal growth forms; growth and mass transfer in fungal biopellets are discussed as an example. To emphasize the importance of the flow behavior of filamentous cultivation broths, an introduction to rheology is also given, reviewing important rheological models and recent studies concerning rheological parameters. Furthermore, current knowledge on morphology and productivity in relation to the environom is outlined in the last section of this review.

LIST OF ABBREVIATIONS

μ_{hyphae}	specific length growth rate (h^{-1})
c_s	substrate concentration at the pellet surface (mol m^{-3})
CSTR	continuous stirred tank reactor
D	dilution rate (h^{-1})
D_{eff}	effective diffusion coefficient (m^2 s^{-1})
d_{hyphae}	diameter of a hyphal element (μm)
DO	dissolved oxygen (%)
GFP	green fluorescent protein
GOD	glucose oxidase
HEWL	hen egg white lysozyme
HGU	hyphal growth unit (μm)
K	consistency index (Pa s)
k_{bran}	branching rate
k_La	volumetric mass transfer coefficient
L_e*	mean dimensionless length
L_{HGU}	mean length of hyphal growth unit (μm)
L_{hyphae}	total average hyphal length (μm)
N	pellet concentration (m^{-3})

n_{stir}	agitation rate (min^{-1})/shaking intensity (min^{-1})
q_s	specific substrate uptake rate (mol g_{BM}^{-1} h^{-1})
q_{tip}	the tip extension rate ($\mu m\ h^{-1}$)
r_{crit}	critical radius (μm)
STR	stirred tank reactor
T	temperature (°C)
t	cultivation duration (h)
X_{BDM}	biomass concentration as dry cell weight ($g\ l^{-1}$)
X_{pellet}	biomass concentration of the pellet ($kg\ m^{-3}$)
γ	shear rate (s^{-1})
η	viscosity ($N\ m^{-2}\ s$)
ρ_{pellet}	pellet density ($kg\ m^{-3}$)
τ	shear stress ($N\ m^{-2}$)
τ_0	yield stress ($N\ m^{-2}$)

I. INTRODUCTION

Due to their metabolic diversity, high production capacity, secretion efficiency, and capability of carrying out posttranslational modifications, filamentous fungi are widely exploited as efficient cell factories in the production of metabolites, bioactive substances, and native or heterologous proteins, respectively. The commercial use of fungal microorganisms is reported for multiple sectors such as detergent, food and beverage, or pharmaceutical industries (Archer, 2000; Lubertozzi and Keasling, 2009; Meyer, 2008; Papagianni, 2004). However, one of the outstanding, and unfortunately often problematic, characteristics of filamentous fungi is their morphology, which is much more complex than that of unicellular bacteria and yeasts in submerged culture (Driouch *et al.*, 2009). Depending on the desired product, the optimal morphology for a given bioprocess varies (Gibbs *et al.*, 2000), optimal productivity here correlating with a specific morphological form (Kaup *et al.*, 2008; Zhang *et al.*, 2007).

In submerged cultivation, two distinct growth forms can be observed: the mycelial and the pelleted form (Casas López *et al.*, 2005; Gupta *et al.*, 2007; Kim *et al.*, 1983). Pellets are characterized by the mycelia developing into stable, spherical aggregates consisting of a more or less dense, branched, and partially intertwined network of hyphae (Berovič *et al.*, 1991; Kim *et al.*, 1983; Zhou *et al.*, 2000). At times, a pellet type of morphology is preferred in industrial cultivations and in downstream processing because of the non-viscous rheology of the broth (Atkinson and Daoud, 1976; Zhou *et al.*, 2000). Pellet growth seems favorable for production of citric acid (Gomez *et al.*, 1988; Papagianni, 2004), glucose oxidase (El-Enshasy *et al.*, 1999), glucoamylase (Lin *et al.*, 2010), or polygalacturonidase (Hemmersdorfer *et al.*, 1987). In these cultivations, the mass

transfer of oxygen and nutrients is considerably better and the subsequent separation of the pellets from the cultivation broth is simpler than in mycelia cultivations (Reichl *et al.*, 1992). Easy agitation and aeration ensure low operating costs, because much lower power input is needed (Oncu *et al.*, 2007). Although the heat and mass transfer within the bioreactor is not limited, concentration gradients within the pellet results in a depletion of nutrients, especially oxygen, in the central regions of the pellets (Hille *et al.*, 2005).

However, the use of freely dispersed mycelium in submerged cultures of fungi has become very popular recently, due to the fact that this morphology enhanced growth and production. Mycelial growth is preferred for the formation of fumaric acid or amylase (Gibbs *et al.*, 2000). Recently, studies have demonstrated many interesting biotechnological activities, including antitumour, anti-inflammatory, and cytotoxicity to hepatoma cells (Liu *et al.*, 2007). Furthermore, this holds for the biotransformation of steroids, where free mycelium facilitates downstream processing, the production of enzymes such as amylase, neo-fructosyltransferase and phytase (Teng *et al.*, 2009), or penicillin (Vecht-Lifshitz *et al.*, 1990). Filamentous growth increases the viscosity of the culture broth, which results in temperature and concentration gradients within the bioreactor as a result of transfer limitations (Kossen, 2000; Papagianni, 2004; Znidarsic and Pavko, 2001).

Fungal morphology in general is of interest, because it influences not only the productivity of the process, but through its impact on rheology it also has an influence on mixing and mass transfer within the bioreactor. To ensure high protein secretion and at the same time a low viscosity of the cultivation broth, it is desired by the industry to tailor-make the morphology of filamentous fungi (McIntyre *et al.*, 2001). The morphological type and the related physiology strongly depend on environmental conditions in the bioreactor (Znidarsic and Pavko, 2001), which can be controlled by regulation of the process parameters. These variable environmental conditions are nowadays often summarized under the term environom, including parameters such as inoculum concentration and spore viability, pH value, temperature, and dissolved oxygen concentration, as well as aeration- and stirring-induced mechanical stress (Deckwer *et al.*, 2006).

Figure 4.1 illustrates a biotechnological process, with the morphology of the biological system influenced by the environom. Morphology and environom as main process parameters are located in the center of the triangle, as they have an influence on all environmental parameters. Productivity is placed on the top of the triangle, because it is the central parameter to be optimized. Broth rheology and biomass growth form the base, completing the triangle. All environmental parameters on the side of the equilateral triangle have an influence on each other. Broth rheology causes mixing problems and influences transport phenomena, which in

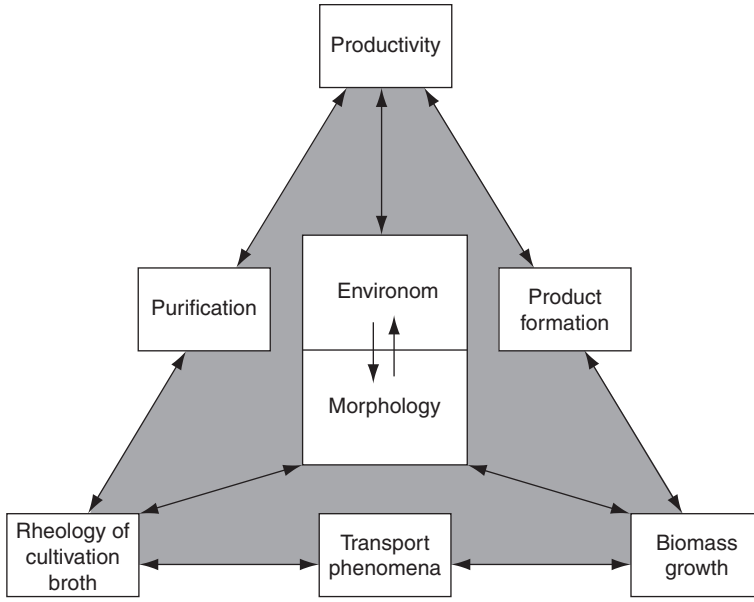

FIGURE 4.1 Schematic illustration of a biotechnological process, with the main parameters environom and morphology in the center, and the productivity, to be optimized, to the top of the triangle.

turn may lead to maintenance problems inhibiting cell growth. Product formation is closely related to the form of biomass growth, as the product is said to be mainly expressed at the tips of the hyphae. Optimized growth leads to extensive product formation and therefore high productivity. Furthermore, the rheology of the broth has an impact on the purification of the product, as high viscosities complicate the recovery of the product. A laborious and expensive purification procedure in turn leads to an uneconomical overall production process.

Overall, the relationship between morphology and production performance is obviously rather complex and each morphological form has certain advantages. The high importance of a suitable morphology for good performance has stimulated various attempts to manipulate the growth characteristics of filamentous fungi. For a complete characterization of a biotechnological process, it is crucial to consider the morphology and therefore the rheological behavior of the cultivation broth. In this article, growth and morphology of filamentous fungi will be described. Specific problems arise from fungal growth forms, and, as an example, growth and mass transfer in fungal biopellets will be discussed. Since automated image analysis is of considerable importance to link flow behavior to fungal morphology, a short historical overview will be

given in this work. To grasp the importance of flow behavior of filamentous cultivation broths, an introduction to rheology will be given, reviewing important rheological models and recent studies concerning rheological parameters. Furthermore, the rather new approach of rheology estimation from morphological parameters will be discussed. For a comprehensive characterization of a filamentous biotechnological process, the environom as a key variable has to be taken into account. The current knowledge on morphology and productivity as a function of the environom will be introduced in the last chapter of this work.

II. GROWTH OF FILAMENTOUS ORGANISMS

The morphology of filamentous organisms in submerged cultivation is a subject of considerable interest, due to the impact of morphology on process productivity (McIntyre *et al.*, 2001). Mycelial growth of filamentous fungi can be differentiated into micro- and macroscopic morphology. The first approache to describe fungal morphology from microscopic images were made by Metz and Kossen (1977). Characterization of fungal microscopic morphology in the early phase of cultivation can be described by the average and total hyphal length, which is obtained by the sum of all hyphal length in a mycelium, the number of tips, and the branching of individual hyphae (Metz and Kossen, 1977; Trinci, 1974). In submerged cultivations, the observed macroscopic morphology of filamentous fungi varies from freely dispersed mycelium over loose mycelial clumps to dense pellets (Papagianni, 2004; Paul and Thomas, 1998). Depending on the strain properties and chosen cultivation conditions, that is temperature, pH, mechanical power input, inoculum properties, or medium composition, the final macromorphology is determined (Papagianni, 2004; Reichl *et al.*, 1992; Znidarsic and Pavko, 2001).

A. Micromorphology

Microscopic morphology involves cell wall structure of the hyphae, tip extension, septum formation, and branching (Kossen, 2000; Nielsen, 1996). The rigid cell wall of filamentous fungi is essential to withstand the internal osmotic pressure and to maintain the shape and integrity of the fungal cell. Simultaneously, it has to preserve a certain formability to continue hyphal tip growth and branching. It provides the interaction of the fungal cell with its environment, transmits signals, and, therefore, activates intercellular signal transduction pathways, for example, as responses to environmental stress (Bowman and Free, 2006; de Nobel *et al.*, 2000; Roncero, 2002). Variations of the environment or cultivation conditions, respectively, lead to cell wall remodeling and changes of the

hyphal morphology due to activation of the cell wall integrity signaling pathway (Pel *et al.*, 2007).

After the activation of spores due to appropriate nutrient supply in the surrounding environment, spores begin to extend in an isotropic manner, and finally a singular germ tube with a tubular shape emerges (d'Enfert, 1997; Harris *et al.*, 1999; Osherov and May, 2001). Growth of the hypha occurs highly polarized, with a linear extension rate at the hyphal apex. Along the length of the hypha, precursors for the cell wall as well as proteins involved in cell-wall synthesis are transported in vesicles from the endoplasmatic reticulum (ER) to the hyphal tip. The ER network, as a result of its gradient distribution, supports the directed transport of the vesicles to the tip (Maruyama and Kitamoto, 2007; Maruyama *et al.*, 2006). At the apex, the vesicles accumulate as the so-called Spitzenkörper (SP) and account for up to 80% of the volume of the cytoplasm (Bartnicki-Garcia *et al.*, 1995; Howard, 1981; Kossen, 2000). The SP is shown to determine the tubular shape (Bartnicki-Garcia *et al.*, 1995) as well as the growth direction and rate of the hyphal cell (McIntyre *et al.*, 2001; Reynaga-Pena and Bartnicki-Garcia, 1997).

After an initial acceleration phase, the hyphae continue to grow with the maximal growth rate, as vesicle transport with its constant velocity is likely to be the limiting step of elongation (Grimm *et al.*, 2005a; Prosser and Trinci, 1979). Septation of the hyphae is linked to nuclear division. In *Aspergillus nidulans*, the first septum, dividing the hyphae into an apical and subapical compartment, is formed when the germ tube contains eight nuclei because of several duplication rounds (Grimm *et al.*, 2005a; Momany and Hamer, 1997). The apical compartment, located at the very hyphal tip, is able to exchange cytoplasm with the subapical compartment through the septum (Nielsen, 1993). Subapical compartments are constant in length and contain three to four nuclei (Kaminsky and Hamer, 1998). In contrast, the number of nuclei and length differ in apical compartments depending on the mitotic cycles and active growth (Kaminsky and Hamer, 1998). With continuing elongation, further septae are formed and an apical compartment becomes a subapical one, while the subapical one transforms into a hyphal compartment (Nielsen, 1993). Because of the accumulation and excess of transport vesicles, which cannot pass a septum, branching occurs in subapical compartments (Aynsley *et al.*, 1990; Prosser and Trinci, 1979), which then resume growth and nuclear division cycles (Kaminsky and Hamer, 1998; Nielsen, 1996). Further away from the apex, hyphal compartments are highly vacuolized and not involved in growth (Kaminsky and Hamer, 1998; McIntyre *et al.*, 2001; Nielsen, 1993). These compartments contain larger vacuoles with further distance from the apex and display a different metabolism compared to the apical and subapical zones of the hyphae (Nielsen, 1993; Thomas, 1992). With increasing cell age, vacuolization rises and, finally,

autolysis occurs (White *et al.*, 2002). The differentiation of the hyphal cell into compartments with different metabolic activities leads to the production of secondary metabolites in the growth-arrested parts of the cell, while growth-associated proteins are synthesized in the active compartments (McIntyre *et al.*, 2001; Nielsen, 1993; Thomas, 1992) (Fig. 4.2).

Based on the processes of elongation, septation, and branching, the microscopic morphology of the mycelium is determined, and can be characterized by the following parameters: the total average hyphal length L_{hyphae} (μm), which is the sum of all single hyphal lengths in the mycelium; $L_{\text{hyphae}} = \sum L_i$, the hyphal diameter of a hyphal element d_{hyphae} (μm); the number of tips n (–); the tip extension rate q_{tip} (μm h^{-1}); and the average branching rate k_{bran} (m^{-1} h^{-1}) of individual hypha (Grimm *et al.*, 2005a; Nielsen, 1996; Trinci, 1974). Although the extension of each hyphal tip proceeds with a constant rate and is therefore linear, as a result of branching the total mycelium shows exponential growth with a specific growth rate μ_{hyphae} (h^{-1}), as no substrate limitation occurs (Aynsley *et al.*, 1990; Grimm *et al.*, 2005a)

$$\frac{dL_{\text{hyphae}}}{dt} = \mu_{\text{hyphae}} L_{\text{hyphae}} = q_{\text{tip}} n. \tag{4.1}$$

Branching is a function of the total length of mycelium and can be described by the branching constant:

$$\frac{dn}{dt} = k_{\text{bran}} L_{\text{hyphae}}. \tag{4.2}$$

A fundamental relationship between tip growth and hyphal branching is the hyphal growth unit HGU (μm). The HGU was introduced by Caldwell and Trinci as the ratio between the total hyphal length and the number of tips (Caldwell and Trinci, 1973). It displays the relationship between elongation and branching:

$$\text{HGU} \equiv \frac{L_{\text{hyphae}}(t)}{n(t)} \tag{4.3}$$

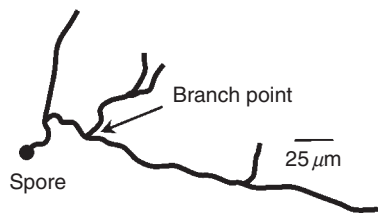

FIGURE 4.2 A growing mycelium of *A. niger*, comprising several tips. The total hyphal length is obtained by summing up the lengths of all branches (Grimm *et al.*, 2005a).

Using Eqs. (4.1)–(4.3), the time course of HGU is

$$\frac{\mathrm{dHGU}}{\mathrm{d}t} = q_{\mathrm{tip}} - k_{\mathrm{bran}}\left(\frac{L_{\mathrm{hyphae}}}{n}\right)^2 = q_{\mathrm{tip}} - k_{\mathrm{bran}}\mathrm{HGU}^2. \tag{4.4}$$

At the beginning of growth, with the extension of an individual hypha the HGU increases. Finally, it reaches a constant value as a result of incipient branching. This characteristic value can be interpreted as the average length of each tip in a mycelium. Under these constant process conditions ($\mathrm{dHGU}/\mathrm{d}t \approx 0$) follows:

$$\mathrm{HGU} = \sqrt{\frac{q_{\mathrm{tip}}}{k_{\mathrm{bran}}}} \tag{4.5}$$

From Eq. (4.1) follows subsequently

$$q_{\mathrm{tip}} = \mu_{\mathrm{hyphae}}\left(\frac{L_{\mathrm{hyphae}}}{n}\right) = \mu_{\mathrm{hyphae}}\mathrm{HGU}. \tag{4.6}$$

With Eqs. (4.5) and (4.6), the specific hyphal growth rate μ_{hyphae} can be correlated to the tip growth rate q_{tip} and the branching rate k_{bran} (Bergter, 1978; Grimm *et al.*, 2005a)

$$\mu_{\mathrm{hyphae}} = \sqrt{q_{\mathrm{tip}}k_{\mathrm{bran}}}. \tag{4.7}$$

A low HGU indicates an increased number of growing tips in the mycelium (Aynsley *et al.*, 1990). This might be preferred in industrial production processes, as secretion is primarily related to the apical zone of the hypha at the sites of cell wall synthesis (Barry *et al.*, 2009; McIntyre *et al.*, 2001; Peberdy, 1994; Wessels, 1992; Wongwicharn *et al.*, 1999b; Wösten *et al.*, 1991), which is assumed to contain larger pores due to the plasticity of the cell wall (White *et al.*, 2002). In another study, an increased secretion of the heterologous hen egg white lysozym by a more branched mycelium of *A. niger* was described, supporting this theory (Wongwicharn *et al.*, 1999a). However, further studies have found no correlation between the number of tips and protein secretion (Bocking *et al.*, 1999).

B. Macromorphology

The apparent macromorphology, which is discernible by the size and structure of the growth form, ultimately results from micromorphological events. Three different mechanisms of pellet formation have been reported (Metz and Kossen, 1977; Nielsen, 1996; Nielsen *et al.*, 1995). The coagulating type occurs at the beginning of the cultivation process when several spores aggregate and form pellets as a result of germination

of a fraction of the coagulated spores. The noncoagulating type is where a pellet originates from a single spore, while the hyphal element agglomeration type is where germinated hyphal elements form lose agglomerates, which possibly transform into a pellet.

Grimm *et al.* (2004) characterized conidial inocula and seeding cultures to assess the aggregation process by direct examination with an inline particle size analyzer (FBRM D600L, Lasentec, USA). The pellet formation of coagulating spores is described as a two-step mechanism (Fig. 4.3), which highly depends on the pH value and fluid dynamic conditions within the bioreactor (Grimm *et al.*, 2005a).

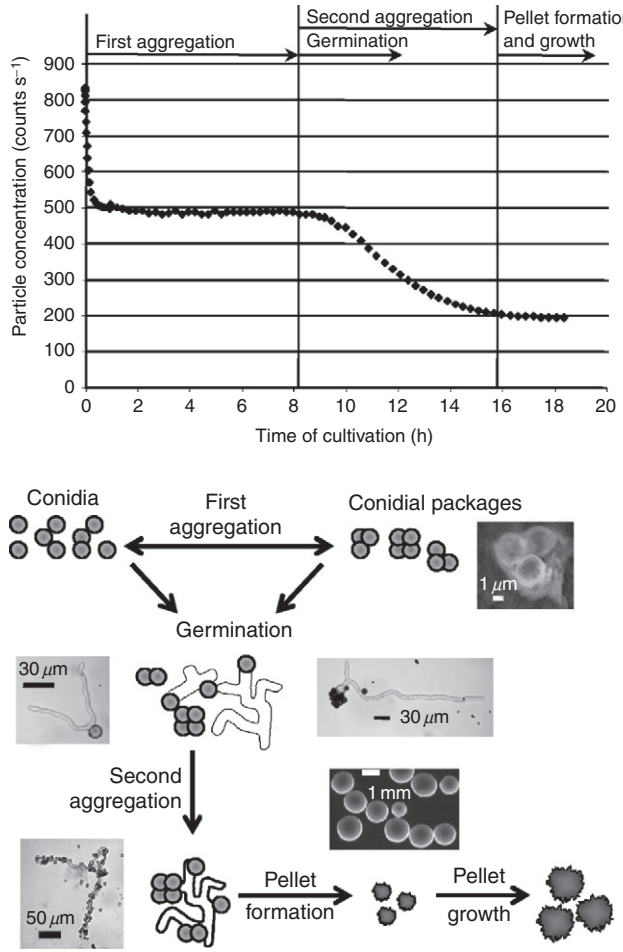

FIGURE 4.3 Aggregation model for coagulating filamentous microorganisms (Grimm *et al.*, 2004).

At the beginning of the cultivation, just after inoculation, spores aggregate until a steady-state condition between aggregation and disintegration of conidial packages is reached (Fig. 4.3). The steady state is disturbed by germination of conidia. Germination and hyphal growth of germ tubes subsequently increase the hyphal surface area, where conidia attach to and trigger the second aggregation step (Grimm *et al.*, 2005a). This leads to an overall decrease in particle concentration, which depends on the pH value as well as on the agitation- and aeration-induced power input (Amanullah *et al.*, 2001; Grimm *et al.*, 2004) Within the second aggregation step, further spores attach to germinating conidia and hyphal elements of the aggregates, which finally form pellets in later cultivation stages (Lin *et al.*, 2008).

C. Pellet growth and mass transfer

Pellets grow exponentially with a constant specific growth rate until a critical radius is reached, and mass transport limitations lead to a depletion of nutrients at the pellet core. The critical radius r_{crit} indicates the point where diffusion limitation occurs in the outer shell of the pellet, and it is given as the quotient of nutrient supply due to diffusion and the substrate uptake:

$$r_{crit} = \sqrt{\frac{6 D_{eff} c_s}{\rho_{pel} q_s}} \tag{4.8}$$

where D_{eff} is the effective diffusion coefficient ($m^2\ s^{-1}$), c_s is the substrate concentration at the pellet surface (mol m^{-3}), ρ_{pellet} is the pellet density (kg m^{-3}), and q_s is the specific substrate uptake rate (mol $g_{BM}^{-1}\ h^{-1}$) (Nielsen, 1996). Often, oxygen is the limited substrate, and the critical radius ranges from 50 to 200 μm depending on the density of the pellet (Hille *et al.*, 2005; Nielsen, 1996; Wittler *et al.*, 1986). Although fluffy pellets with a loose structure are shown to be penetrated with oxygen, they exhibit a lower productivity than dense pellets of 150–200 μm radius (Fig. 4.4).

Without substrate limitation, pellets grow exponentially. However, if pellet growth exceeds the critical radius r_{crit}, the substrate is consumed in the outer active pellet layer with the thickness

$$\Delta r = r_{pellet} - r_{crit}. \tag{4.9}$$

In pellets that exceed the critical radius, further growth is restricted to this active layer Δr. Growth is limited by substrate, if $r \leq r_{crit.}$ The pellet radius increases with the thickness of the active layer

$$\frac{dr_{pellet}}{dt} = \mu \Delta r. \tag{4.10}$$

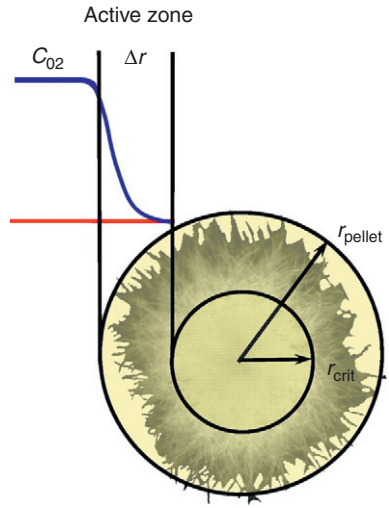

FIGURE 4.4 Principal sketch of radius and active layer within a pellet.

At constant growth in the active layer, integration yields

$$r_{\text{pellet}} = \mu \Delta r t + r_{\text{pellet},0} \tag{4.11}$$

where $r_{\text{pellet},0}$ is the pellet radius at $t = 0$. Assuming that the pellet structure is homogeneous and spherical, the biomass concentration of the pellet X_{pellet} (kg m ^{3}) and the pellet concentration N (m ^{3}) can be defined as

$$X_{\text{pellet}} = \frac{4}{3}\pi r_{\text{pellet}}^{3} \rho_{\text{pellet}} N. \tag{4.12}$$

Equation (4.12) solved to r_{pellet} and used in Eq. (4.11) yields the cubic root law of pellet growth:

$$X_{\text{pellet}}^{1/3} = kt + X_{\text{pellet},\,0}^{1/3} \tag{4.13}$$

where $X_{\text{pellet},0}$ is the biomass concentration at $t = 0$, and the strain-specific growth constant is

$$k \approx \left(\frac{4\pi \rho_{\text{pellet}} N}{3}\right)^{1/3} (\mu \Delta r). \tag{4.14}$$

The kinetic parameter k (kg$^{1/3}$m ^{1} h ^{1})) is determined by the thickness of the growing zone Δr, the pellet density ρ_{pellet}, and concentration N, as well as by the specific growth rate μ (Emerson, 1950; Kossen, 2000; Nielsen, 1996).

With continued exponential and unlimited growth of the peripheral layer, the inner zone, in which the oxygen supply is limited, increases, and finally autolysis occurs and pellets with a hollow core can be

observed (Barry *et al.*, 2009; White *et al.*, 2002). Different physiological zones within a pellet were quantified (Bizukojc and Ledakowicz, 2010). On the basis of staining with lactophenol blue, the active pellet region could be distinguished from the non-growing zones. As a result, the production of lovastatin is linked to the growing outer zone of the pellets and pellet size. El-Enshasy *et al.* (2006) visualized the zonal differentiation within fungal biomass by acridine orange staining and correlated the synthesis of glucose oxidase to the peripheral layer of pellets or the mycelial growth form.

Mass transfer in pellets of filamentous fungi in submerse cultivation can be broken down into three key processes: mass transfer from the bulk phase to the surface of the pellet, mass transport in the aggregate, and turnover rates within the pellet. Every single process has the potential to limit the biomass productivity of the pellet. The penetration depth of substrate and, therefore, the fraction of pellet biomass which contributes to turnover and production, are directly linked to mass transfer within these two regions (Hille *et al.*, 2009).

The majority of models of mass transport and turnover in fungal pellets depict mass transport as being purely diffusive on the basis of the fact that advection and turbulence are negligible in submerged cultivations (Cui *et al.*, 1998; Eleazar *et al.*, 2001; Lejeune and Baron, 1997). Following the assumption that diffusion is the dominating mass transport mechanism, the effective diffusion coefficient D_{eff} can be used as a parameter in equations for the description and calculation of mass transport and turnover (Hille *et al.*, 2009). Within pellets, D_{eff} depends on the space available for transport and is often expressed as a function of pellet porosity or biomass density. It has been shown that fungal pellet density varies over the radial coordinate (Hamanaka *et al.*, 2001) and changes with the time of cultivation (El-Enshasy *et al.*, 1999; Hellendoorn *et al.*, 1998). Pellet morphology, which is a result of the environmental conditions and pellet age, has an important influence on the effective diffusion coefficient and the penetration depth of oxygen into the pellet (Cui *et al.*, 1998; Hille *et al.*, 2009). These properties have been studied with oxygen microelectrode measurements. Oxygen concentration profiles have been presented for pellets of different filamentous fungi (Cronenberg *et al.*, 1994; Hille *et al.*, 2005, 2009; Huang and Bungay, 1973; Papagianni, 2004; Wittler *et al.*, 1986). By considering porosity to be the only influential parameter, several authors have described mass transport in fungal pellets (Cui *et al.*, 1998; Eleazar *et al.*, 2001; Miura and Miyamoto, 1977), where the effective diffusion coefficient is assumed to be directly proportional to the porosity. However, Hille *et al.* (2009) found in their study, focusing on oxygen as limiting substrate in submerged pellet cultivation of fungi, that diffusion alone is the sole transport mechanism within fungal biopellets as long as laminar conditions are prevalent and the biopellet structures are very

compact and there is laminar flow. Moreover, deformation of the pellet structure was found to have significant impact on penetration depth of substrates. Diffusion limitation of whole pellets was found to be mainly a function of size, with a pronounced influence of advection in the outer zone of pellets that is supplied with oxygen. It was concluded that the effective diffusion coefficient might not be sufficient for the description of mass transport within the pellet periphery for a broad range of realistic fluid dynamic conditions (Hille *et al.*, 2009).

D. Image analysis as a tool to determine morphology

The initial attempts for a quantitative description of fungal morphology were published in the 1950s (Dion *et al.*, 1954). In the 1970s, Fiddy and Trinci (1976) and later Prosser and Trinci (1979) investigated surface cultures of filamentous fungi, characterizing growth and branching. Quantification was then achieved by microscopy (direct measurement) or by photography. In 1981, the first semiautomatic method for quantitative representation of mold morphology was described (Metz *et al.*, 1981). With the help of a microscope, photographs of mycelial particles were made and projected on an electronic digitizing table attached to a computer, which determined the exact coordinates of any point touched by the cursor. This method for quantitative representation of hyphal morphology was found to be very accurate to control continuous cultivations. However, the reproducibility of the morphology data of batch cultures was poor (Metz *et al.*, 1981; Pazouki and Panda, 2000). Although the image analysis process was partly digitized, the process remained laborious and time consuming (Kossen, 2000). In 1988, an automated digital image analysis method was developed (Adams and Thomas, 1988), reducing the time needed for a single measurement. The method was still not completely automated, as it required manual intervention at times. A software for fully automated image analysis was introduced in 1990 (Packer and Thomas, 1990). With sufficient automation and speed, whole distributions of hyphal parameters could be tested. Using this method, significant amounts of partly aggregated mycelia material could be identified within mycelial cultivation broths (Papagianni, 2004). These so-called clumps were agglomerates much less stable and denser than pellets. Tucker *et al.* (1992) developed a method to measure clumps, presenting detailed information such as projected clump area, perimeter, compactness and roughness, circularity, and the degree of branching (Tucker *et al.*, 1992). The projected area of the clump was found to give the best correlations with broth rheology (Cox *et al.*, 1998). Furthermore, this method was used by Amanullah *et al.* (2001), to describe the dynamics of mycelial aggregation in batch and chemostat cultures of *Aspergillus oryzae* (Amanullah *et al.*, 2001). Paul *et al.* (1992) quantified the

structural complexity of the mycelium grown in dispersed form in submerged processes, using a fully automatic image analysis and differentiation method (Paul *et al.*, 1992, 1994). This model allowed a quantitative characterization of the biomass structure and, thereby, made it possible to develop a powerful structured model for the cultivation of *P. chrysogenum* (Paul and Thomas, 1996). Image analysis has also been used to describe pellet morphology. Reichl *et al.* (1992) measured mean sizes, content, and shape of pellets during cultivation of *Streptomyces tendae* and introduced a shape factor specifying the form of a pellet, which was the first qualitative discrimination between pellets (Reichl *et al.*, 1992). Another image analysis method characterized pellets based on the presence of a central core. Pellets were categorized into smooth and hairy types using automatic image analysis (Cox and Thomas, 1992).

Image analysis has become a suitable tool for characterizing and developing mathematical models that describe morphology and growth of filamentous microorganisms, such as characterization of pellet morphology, measurement and simulation of morphological development, and a mathematical model of apical growth, septation, and branching (Znidarsic and Pavko, 2001). Image analysis techniques permit the rapid and accurate measurement of simple cellular differentiation of fungi (Papagianni, 2004). As of today, image analysis is state of the art for the characterization of morphology and simple differentiation of filamentous microorganisms and is therefore of considerable significance for bioprocess control. The reduced cost of equipment, both hardware and software, help to make image analysis a routine laboratory tool (Znidarsic and Pavko, 2001). In current studies, image analysis of filamentous morphology is often performed with the software packages Image-Pro Plus (Media Cybernetics) (Gögus *et al.*, 2006; Gupta *et al.*, 2007; Lim *et al.*, 2006), or an image analyzer software by Matrox Electronic Systems (Cho *et al.*, 2002; Hwang *et al.*, 2004) and AxioVision (Carl Zeiss) (Ahamed and Vermette, 2009; Lin *et al.*, 2010).

III. RHEOLOGY

The flow behavior of cultivation broths containing filamentous organisms differs distinctly from broths with planctonic growing organisms. Several studies show that the complex morphology of filamentous fungi is responsible for highly viscous fermentation broths, characterized by shear-rate-dependent viscosities and by yield stress (Charles, 1978; Gupta *et al.*, 2007; Olsvik and Kristiansen, 1994; Papagianni, 2004; Riley *et al.*, 2000; Roels *et al.*, 1974). Variations of the broth characteristics affect the bioreactor hydrodynamic properties, which include mixing and mass transfer performances (Gavrilescu and Tudose, 1997; Moo-Young *et al.*,

1987; Sinha *et al.*, 2001b). The efficiency and productivity of the entire cultivation process depends on these hydrodynamic properties (Gupta *et al.*, 2007). An understanding of the broth flow behavior is necessary in order to develop design strategies that will help to overcome possible limitations in mass, momentum, and heat transfer in bioreactors (Papagianni, 2004).

In general mycelial growth rather than pellet growth increases the viscosity of the medium (Pazouki and Panda, 2000), although pelleted cultivation broth may be Newtonian, of low viscosity, and more economical in the separation of the biomass. Problems might arise with the transport of nutrients into pellet cores, thus reducing productivity. The dispersed forms therefore predominate in most industrial cultivations, because of increased cell growth and higher productivities (Oncu *et al.*, 2007; Riley *et al.*, 2000). However, these mycelia cultivation broths tend to show non-Newtonian flow behavior. Small increases in biomass concentration can lead to large increases in broth viscosity (Charles, 1978; Metz and Kossen, 1977; Riley *et al.*, 2000). This broth behavior is presumed to be caused by entanglement of the hyphae (Metz and Kossen, 1977). Filaments tend to grow uniquely to form three-dimensional networks by accumulating biomass at the growing tip of the hyphal network (Ruohang and Webb, 1995). The resulting highly branched network displays several fluid dynamic interactions, thus changing the properties of the cultivation broth from the initially Newtonian character to a non-Newtonian one (Gbewonyo and Wang, 1983). The much more viscous broth leads to heterogeneous, stagnant, non-mixed zone formations, which make the cultivation challenging and more expensive to operate (Metz and Kossen, 1977; Oncu *et al.*, 2007). The strong negative influence of broth viscosity on oxygen transfer has been demonstrated (Moo-Young *et al.*, 1987). To control cultivation performance, it is essential to know how the operating conditions influence the rheological properties of the filamentous cultivation broth. As these depend on the morphology of the biological system, the relationship between rheology and morphology is vitally important and has to be fully investigated. Such knowledge can be very valuable in the optimization and design of common cultivation processes (Oncu *et al.*, 2007; Papagianni, 2004).

A. Rheological models

The prediction of suspension rheology is a difficult and complex task (Barnes *et al.*, 1989; Petersen *et al.*, 2008). Fluid viscosity is related to mixing and mass transfer, including Reynolds number and correlations for the volumetric mass transfer coefficient $k_L a$ (Petersen *et al.*, 2008). Correlations using shear stress are also used to predict the size of caverns,

which can form around impellers in an otherwise unmixed broth (Amanullah *et al.*, 1998).

Broth rheology is mostly determined by offline viscosity measurements. Both the appropriate instrument and the method of representative sampling have to be considered carefully, because rheological properties are intrinsic properties of the fluid and should ideally not be influenced by the measuring technique or instrument (Olsvik and Kristiansen, 1994). Newton's law is the simplest of rheological models. Here, dynamic viscosity is defined as the relationship between the applied shear stress and the resulting movement of the fluid, defined as shear or strain rate

$$\eta = \frac{\tau}{\gamma}. \tag{4.15}$$

where η is the viscosity (N m ^{2} s), τ is the shear stress (N m ^{2}) and γ is the shear rate (s ^{1}). The viscosity of a Newtonian fluid is independent of the shear rate. For non-Newtonian fluids, this relationship is more complex. Mycelial cultivation broths generally have pronounced non-Newtonian rheological characteristics and show shear thinning or pseudoplastic behavior (Deindoerfer and John, 1960; Olsvik and Kristiansen, 1994). Pseudoplastic fluids exhibit a decrease in viscosity with increasing shear rate. Non-Newtonian viscosity is usually characterized using the ''apparent viscosity'' (Gupta *et al.*, 2007; Petersen *et al.*, 2008).

Four mathematical models commonly used for describing the rheology of mould suspensions are given in Table 4.1. These models describe cultivation broths in terms of time-independent, non-viscoelastic fluids (Bongenaar *et al.*, 1973; Deindoerfer and John, 1960). Since it is impossible to have complete knowledge of shear stress/shear rate relations and the elastic properties in the different flow fields for non-Newtonian fluids, the rheological models may be regarded as an empirical fit to the experimental data (Oolman *et al.*, 1986). Attempts have been made to best fit a number of rheological models to the rheological profile of a broth sample Table 4.1. In most studies, the power law is used to describe the rheological behavior of cultivation fluids due to its relative simplicity and easy handling (Deindoerfer and John, 1960; Gögus *et al.*, 2006; Gupta *et al.*, 2007;

TABLE 4.1 Rheological models applicable to cultivation broths

Bingham	$\tau = \tau_0 + \eta\,\gamma$
Casson	$\tau^{0.5} = \tau_0^{0.5} + K\gamma^{0.5}$
Herschel–Bulkley	$\tau = \tau_0 + K\gamma^n$
Power law	$\tau = K\gamma^n$
Ostwald–de Waele	

τ_0 is the yield stress (N m ^{2}), K is the consistency index (Pa s), and n is the flow index ( ).

Lim *et al.*, 2006; Oncu *et al.*, 2007; Petersen *et al.*, 2008; Rodríguez Porcel *et al.*, 2005, 2006; Swapnil *et al.*, 2005). This model does not include yield stress. The Herschel–Bulkley model additionally includes the yield stress and is therefore preferred by some authors (Petersen *et al.*, 2008).

The Bingham model also includes the often observed yield stress, which has to be exceeded before the fluid will flow. The existence of yield stress is important, because it will determine start-up power requirement in pumping through pipelines and mixers and the existence of dead regions in biorectors (Cheng, 1986; Olsvik and Kristiansen, 1994). The existence of yield stress in filamentous cultivation fluids was pointed out to be debatable by Cheng (1986). Moo-Young *et al.* showed that polymer solutions could be used for predicting the behavior of fluids, but only as long as no yield stress could be measured in these filamentous cultivation broths. However, no clear answers have emerged as to whether yield stress really exists in filamentous cultivation fluids. Several authors have used the Bingham model to describe the rheological properties of mould suspensions (Deindoerfer and John, 1960). The Casson model is also occasionally used to describe filamentous fermentation broths at low shear rates (Bongenaar *et al.*, 1973; Metz and Kossen, 1977; Oolman *et al.*, 1986; Roels *et al.*, 1974).

Typically, the power law of Ostwald-de Waele and the Herschel–Bulkley model most adequately describe the broth rheological profiles over the whole shear rate range (Pollard *et al.*, 2002). Pollard *et al.* (2002) showed a consistency index greater than 0.997 for these models, compared to the poorer fit of Casson and Bingham approximations. These four models were chosen over other potential models, as they are most commonly used for rheological analysis of mycelial cultivation broths and are widely used in non-Newtonian transport correlations (Pollard *et al.*, 2002). All models are understood to be empirical relationships and not physical laws (Karsheva *et al.*, 1997). In another study, the Casson, Herschel–Bulkley, and the power law plots for the apparent viscosity of a suspension of *Penicillium chrysogenum* were compared (Reuss *et al.*, 1982). The authors found that, within the relatively small range of shear rates measureable, it was not possible to distinguish between these models. A further study showed that a two-parameter model was sufficient for describing data measured in the shear rate range between 2 and 650 s^{-1}. Through the addition of a third parameter, the model fit to the data could not be significantly improved (Allen and Campbell, 1991).

In some studies, the consistency index K has been used as the sole indicator for the viscosity of filamentous cultivations (Olsvik and Kristiansen, 1992a,b). This seems to be a valid assumption, since the value of the flow index n is affected by the operational design to a much lesser extent than the value of K (Allen and Campbell, 1991). Because n is an exponent, changes in n will have a larger effect on shear stress than a

similar change in *K*, thus limiting the use of *K*-values as single indicator of fluid viscosity to datasets were n is constant (Olsvik and Kristiansen, 1994).

B. Characterization of important parameters and viscosity in filamentous processes

Rheology and morphology of filamentous fungi have been studied extensively but often separately. Early studies examining the broth rheology simply published apparent viscosity measured at a single speed in the viscometer (Warren *et al.*, 1995). Other more detailed analyses, however, showed a variable flow rate of filamentous cultivation broths (Charles, 1978; Roels *et al.*, 1974; Warren *et al.*, 1995).

Several studies were conducted on morphological changes and the coherent rheology of *Aspergillus terreus* during lovastatin production (Baños *et al.*, 2009; Casas López *et al.*, 2005; Gupta *et al.*, 2007; Rodríguez Porcel *et al.*, 2005, 2006). The process favored pelleted growth. The broth rheology was found to be affected by the biomass concentration, the pellet size, and the agitation rate (Casas López *et al.*, 2005; Gupta *et al.*, 2007; Rodríguez Porcel *et al.*, 2005, 2006). The viscosity, estimated using the Ostwald-de Waele power law approach, showed pseudoplastic non-Newtonian behavior (Gupta *et al.*, 2007; Rodriguez Porcel *et al.*, 2006). The broth rheology parameters *K* and *n* were found to be influenced by the concentration of the biomass, the average pellet size, and, to a lesser extent, the fluffiness of the pellet (Rodríguez Porcel *et al.*, 2005). The consistency index *K* was strongly influenced by an increasing size of pellets, but was not sensitive to an increase in the concentration of pellets of a fixed diameter (Rodriguez Porcel *et al.*, 2006). Casas López *et al.* (2005) found a dependency of the consistency index on agitation rate, suggesting that the consistency index of broth containing small pellets is not very sensitive to an increase in the concentration of the pellets.

Cho *et al.* (2002) studied the effect of carbon source and aeration rate on broth rheology and fungal morphology during red pigment production by *Paecilomyces sinclairii*. Sucrose and starch were used as carbon sources. The broth showed a clear pseudoplastic behavior as found in other fungal cultivations and was therefore described using the power-law approximation (Cho *et al.*, 2002; Goudar *et al.*, 1999; Sinha *et al.*, 2001a). *P. sinclairii* grown in sucrose medium showed longer hyphal length and a higher consistency index, due to a higher cell mass. In contradiction to Roukas and Mantzouridou (2001), who identified the aeration rate as one of the major determinants of the rheological properties of the cultivation broth, no significant variance in rheological parameters *K* and *n* between culture broths from different aeration conditions was found (Cho *et al.*, 2002).

Lim *et al.* investigated the relationship between morphology and the rheological properties of *Penicillium citrinum* to improve the production of

neo-fructosyltransferase (neo-FTase). The flow behavior of the cultivation broth was found to be pseudoplastic at all stages of cultivation. The rheology of neo-FTase fermentation by *P. citrinum* was dependent on cell growth and fungal morphology, as seen in previously introduced fungal cultivations (Lim *et al.*, 2006; Riley *et al.*, 2000; Rodríguez Porcel *et al.*, 2005; Sinha *et al.*, 2001a). The consistency index increased gradually during pellet growth and changes in the flow behavior index were inversely related to those of the consistency index (Lim *et al.*, 2006).

Oncu *et al.* (2007) produced polygalacturonase by a strain of *Aspergillus sojae* and studied the effect of various process parameters on morphology and rheology. The broth rheology was found to be not dependent on pH, but varied with agitation speed and dissolved oxygen tension. As previously introduced in other studies, Oncu *et al.* found the rheology to be dependent on biomass concentration and morphology. Non-Newtonian and pseudoplastic broth rheology was observed at 500 min ^{1} agitation speed; the broth rheology exhibited dilatant behavior at the lower agitation rate of 200 min ^{1}; and the broth was close to Newtonian at the medium agitation speed of 350 min^{-1}, making the broth at high and low agitation speeds more viscous (Oncu *et al.*, 2007). However, the specific power input was not mentioned, thereby making the description of the dependency between agitation rate and broth rheology somewhat incomplete. The consistency index was suggested to be sensitive to the pellet size and biomass concentration (Casas López *et al.*, 2005). In the process of polygalacturonase production, *A. sojae* morphology was furthermore argued to be sensitive to the dissolved oxygen concentration, and the rheology of the broth was directly affected by the morphology (Casas López *et al.*, 2005; Lim *et al.*, 2006).

Several studies were conducted concerning broth rheology and fungal morphology during exo-polymer production by various fungi (Hwang *et al.*, 2004; Sinha *et al.*, 2001a,b). The studies with *Paecilomyces japonica* showed pseudoplastic behavior of the broth, irrespective of biomass concentration, and a previously described dependency of rheological behavior on specific growth rate and fungal morphology. Pellets were the most predominant morphological form compared to free mycelia (Sinha *et al.*, 2001a,b). Noteworthy is also the shown variation of rheology with initial substrate concentration (Sinha *et al.*, 2001b). Hwang *et al.* (2004) compared the rheological properties of three different species of basidomycetes *Phellinus* in submerged cultures. Notably, high viscosities were measured for cultivations of *P. baumii* and *P. gilvus*, ranging from 5 to 7 N m^{-2} s. All species showed significantly different morphological and rheological properties, while the morphological variation was closely linked to productivity and the apparent viscosity of the culture broth. The apparent viscosity was primarily influenced by mycelial biomass rather than exopolysaccharide concentration (Hwang *et al.*, 2004).

For the most part, determination of flow behavior of cultivation broth is aimed at understanding and improving mass transfer, mixing, and power input. A reduction in apparent viscosity of the broth seems desirable to achieve this purpose. O'Cleirigh *et al.* (2005) took a notable opposite approach and used the microbial polysaccharide xanthan gum to artificially regulate the apparent viscosity of the actinomycete *S. hygroscopicus* cultivation broth, with the aim of controlling particle interaction, aggregation, and hence pellet formation. A supplement of xanthan gum significantly altered the fluid rheology by increasing the apparent viscosity and led to an increase in pellet count and biomass concentration, as well as a decrease in mean pellet volume.

C. Estimation of rheology

Comparatively few reports on correlations between morphological parameters and broth rheological properties exist. This is due to the large variability of mycelia growth in submerged cultures, which makes the characterization challenging (Metz and Kossen, 1977; Papagianni, 2004). Until the early 1990s, the appropriate instrumentation for making morphological measurements, not only on the micromorphological level but also on mycelia aggregates, was lacking (Papagianni, 2004). Tucker *et al.* (1992) and Tucker and Thomas (1993) were the first to use image analysis to investigate the separate influences of biomass concentration and mycelial morphology on broth rheology in the 1990s. The authors investigated the separate influences of biomass concentration and mycelial morphology on broth rheology, using an industrial strain of *Penicillium chrysogenum*. They proposed that the rheology of fungal cultivation broths should be related to clump properties, rather than to the morphology of small amounts of freely dispersed mycelia, and were able to investigate the effect of biomass concentration separately from that of morphology. The rheological parameter could be related with biomass concentration as dry cell weight X_{BDM}

$$\mathrm{RP} = \text{const.} \cdot X_{\mathrm{BDM}}^{\alpha} \tag{4.16}$$

where RP is the rheological parameter under examination and α is the exponent on the biomass concentration (Riley *et al.*, 2000; Tucker and Thomas, 1993). Having established the effect of biomass concentration on rheology of filamentous cultivation broths, the effect of mycelial morphology could be investigated. Morphological parameters, as found in Paul and Thomas, especially clump roughness R and compactness C proved to be significant, as they had a definite influence on broth rheology (Paul and Thomas, 1998; Tucker and Thomas, 1993). With these findings the following correlation was proposed:

$$\mathrm{RP} = \text{const.} \cdot X_{\mathrm{BDM}}^{\alpha}(R)^{\beta}(C)^{\gamma} \tag{4.17}$$

in which α, β, and γ are the exponents for each rheological parameter RP (Riley *et al.*, 2000). The resulting correlations were highly successful at predicting broth rheology for filamentous batch cultivations (Table 4.2) (Riley *et al.*, 2000; Tucker and Thomas, 1993). Olsvik *et al.* (1993) used the same image analysis method on *A. niger* broths from 7-L chemostats. They found that more than 89% of hyphae were in the form of clumps. Changes in biomass concentration and clump roughness led to different rheological properties of the broth, represented by the power-law consistency index (Table 4.2), which increased with the biomass. Olsvik and Kristiansen (1994) proposed a similar correlation for batch and fed-batch cultivations (Table 4.2). Mohseni and Allen (1995) used image analysis to examine the influence of biomass concentration and particle morphology on the yielding properties of filamentous broth of *Streptomyces levoris* and *A. niger*. They found correlations with the freely dispersed form using the biomass concentration, the mean dimensionless length, and the mean hyphal growth unit (Table 4.2). Furthermore, the biomass in form of aggregates was correlated with yield stress, showing that clumps with greater roughness caused greater yield stress (Mohseni and Allen, 1995; Olsvik and Kristiansen, 1994; Olsvik *et al.*, 1993; Riley *et al.*, 2000). All studies agree that clump morphology, particularly roughness, is important in determining fungal broth rheology (Mohseni and Allen, 1995; Olsvik and Kristiansen, 1994; Olsvik *et al.*, 1993). Riley *et al.* (2000) criticized earlier correlations based on clump morphology, stating that during later stages of cultivation, only 30–40% of biomass where in clumps. In this work, no clear relationship between the flow index and biomass concentration was found. The correlations found for the consistency index were based on the biomass concentration and the mycelial size represented by the mean maximum dimension of all the mycelia (Table 4.2) (Riley *et al.*, 2000). In all these correlations, the impact of biomass concentration varies with an index from 1.7 to 2.9, with morphological parameters, such as maximum dimension, compactness, or roughness, having less importance with indices between 0.96 to 1.2 (Petersen *et al.*, 2008). The measured morphological parameters were population averages based on selected samples and assumptions, with the drawback that much of the information from the image analysis data is lost by taking a population average, or excluding part of the population. A completely different approach for the prediction of rheological characteristics of filamentous cultivation broths was taken by Petersen *et al.* (2008). In that study, principal component analysis (PCA) and partial least squares regression (PLSR) (Geladi, 1986; Jackson, 1980) were used to extract information from particle size distribution data for the prediction

TABLE 4.2 Correlations for the prediction of rheology from biomass concentration and population averaged data from image analysis

Source	Correlation	Organism
Tucker and Thomas (1993)	$K = {X_{\mathrm{BDM}}}^{2.8} \times R^{0.7} \times C^{1.2} \times \mathrm{const.}^{a}$	*P. chrysogenum*
Tucker (1994)	$K = {X_{\mathrm{BDM}}}^{2.3} \times R^{-0.96} \times C^{0.79} \times 6.6 \times 10^{-5}$	*P. chrysogenum*
Olsvik *et al.* (1993)	$K = -0.56 + 0.0018 \times R \times {X_{\mathrm{BDM}}}^{1.7}$	*A. niger—continuous*
Olsvik and Kristiansen (1994)	$K = 0.38 + 4.8 \times 10^{-5} \times R \times {X_{\mathrm{BDM}}}^{2.9}$	*A. niger—fed batch*
Mohseni and Allen (1995)	$\tau_y = 4.2 \times 10^{-6} \times {X_{\mathrm{BDM}}}^{2.6} \times ({L_e}^*)^{2.2}$ $\tau_y = 7.2 \times 10^{-3} \times {X_{\mathrm{BDM}}}^{2.2} \times (\mathrm{HGU})^{0.65}$ $\tau_y = 4.8 \times 10^{-7} \times R^{3.2} \times {X_{\mathrm{BDM}}}^{2.5}$	*S. levoris* and *A. niger*
Riley *et al.* (2000)	$K = {X_{\mathrm{BDM}}}^{2} \times 5 \times 10^{-5} \times L - 10^{-5}$	*P. chrysogenum*

This table was composed by Riley *et al.* (2000); an altered form of this table can also be found in Petersen *et al.* (2008).K is the consistency index (Pa s), X_{BDM} is the biomass concentration as dry cell weight (g l^{-1}), R is the morphological parameter roughness, C is the morphological parameter compactness, ${L_e}^*$ is the mean dimensionless length, HGU is the hyphal growth unit (μm), and a is an arbitrary exponent.

of rheological properties (Petersen *et al.*, 2008). Using a PLSR model, the authors were able to obtain reasonable predictions of apparent viscosity, yield stress, and consistency index, from the size distribution and biomass concentration (Petersen *et al.*, 2008). However, these investigations were not the first to apply a multivariate approach to size distribution data for prediction of rheology, as such models had previously been used in the food industry (Shidara *et al.*, 1995).

IV. THE IMPACT OF THE ENVIRONOM ON MORPHOLOGY AND PRODUCTIVITY

Selected studies have focused on variations in the environom, changing operating parameters such as inoculum level, pH value, power input due to agitation and aeration, temperature, and medium nutrients. Distinct environmental conditions result in different morphological forms, thereby affecting the production yields of native as well as heterologous proteins (Table 4.3). Apart from these parameters, the inoculum level, pH value, and power input due to agitation and aeration are generally recognized as the most important operating parameters on morphology formation and productivity in submerged processes.

Morphology and productivity are correlated with the spore concentration of the inoculums, compare Table 4.3 (Bizukojc and Ledakowicz, 2010; Carlsen *et al.*, 1996; Carmichael and Pickard, 1989; Domingues *et al.*, 2000; Grimm *et al.*, 2004; Hemmersdorfer *et al.*, 1987; Liu *et al.*, 2008; Nielsen *et al.*, 1995; Papagianni, 2004; Papagianni and Mattey, 2006; Tucker *et al.*, 1992; Vecht-Lifshitz *et al.*, 1990; Xu *et al.*, 2000). Grimm *et al.* (2004) investigated the influence of spore concentration on the aggregation velocity. As result, the aggregation velocity increases with increasing inoculum spores until a maximum at a concentration of 3×10^6 ml^{-1} is reached. Besides, spore germination and hyphal growth rate are slowed down at higher conidia concentration. Xu *et al.* (2000) showed a decrease in pellet size with increasing conidia concentration. At values above 10^7 ml^{-1}, freely dispersed mycelium occurs. A maximum yield of heterologous proteins in *A. niger* was observed with a spore concentration of 4×10^6 ml^{-1}, and therefore in pelleted growth (Xu *et al.*, 2000).

The pH of the medium is also a cultivation parameter that is often neglected but can significantly affect the morphology and productivity (Bizukojc and Ledakowicz, 2009; Braun and Vecht-Lifshitz, 1991; Carlsen *et al.*, 1996; Lejeune *et al.*, 1995; Mainwaring *et al.*, 1999; O'Donnell *et al.*, 2001; Papagianni, 2004; Pirt and Callow, 1959; Vats *et al.*, 2004; Vecht-Lifshitz *et al.*, 1990). The chosen pH has an enormous effect on the aggregation of spores, especially at the beginning of the cultivation and therefore dictates the final morphology within submerged cultivations

TABLE 4.3 The influence of the environmental process parameters on morphology and productivity

	Organism	Investigated values	System	Morphology	Product	References
Inoculum	*A. niger*	Spore concentration 10 100 ml ^{1}	Shake flasks, pH 4.5, *T*: 25 °C, n_{stir}: 150 min ^{1}, *t*: 95 h	Pellet: diameter: 2 10 mm, smooth surface	*Polygalacturonase (homologous)*: increases with compactness of pellets	Hemmersdorfer *et al.* (1987)
		Spore concentration 10^{4} 10^{9} ml ^{1}	STR, pH 2.1, *T*: 28 °C, n_{stir}: 400 min ^{1}, *t*:150 h air flow: 1 l ^{1} min ^{1}	Pellets at concentrations of 10^{4} 10^{5} ml ^{1}, freely dispersed mycelium at concentration of 10^{8} 10^{9} ml ^{1}	*Citric acid (homologous)*: yield: >150 g l ^{1} with D glucose as substrate at 150 h of cultivation	Papagianni and Mattey (2006)
		Spore concentration 5×10^{5} 5×10^{6} ml ^{1} and 10^{7} ml ^{1}	Glass beaker and shake flasks, pH 5.5, *T*: 30 °C, n_{stir}: 240 min ^{1}	Increased aggregation velocity with rising spore concentration, maximal aggrgation velocity with a concentration of 3×10^{6} ml ^{1}, > 3×10^{6} ml ^{1} reduced spore germination and hyphal growth	Not specified	Grimm *et al.* (2004)
		Spore concentration 10^{3} 10^{7} ml ^{1}	Shake flasks, *T*: 24 °C, n_{stir}: 200 min ^{1}, *t*: 6 days	Decrease in pellet size with increasing conidia concentration, optimal pellet size of 1.6 mm for synthesis, freely dispersed mycelium $>10^{7}$ ml ^{1}	*Glucoamylase GFP fusion protein (heterologous)*: maximum yield of at spore concentration 4×10^{6} ml ^{1} (pellets of 1.6 mm diameter) due to reduction of protease synthesis	Xu *et al.* (2000)

(*continued*)

TABLE 4.3 (*continued*)

Organism	Investigated values	System	Morphology	Product	References
A. terreus	Spore concentration in preculture: 1.39×10^{9} 2.56×10^{10} l ^{1}	Shake flasks, inoculation with 10 ml, preculture culture, T: 30 °C, n_{stir}: 110 min ^{1}, t: 168 h	Inoculum concentration determines pellet size and growth rate, small pellets and high biomass concentration at 2×10^{10} l ^{1}	*Lovastatin and Geodin (homologous)*: increased Lovastatin synthesis with small pellets, no correlation of Geodin synthesis and pellet size	Bizukojc and Ledakowicz (2010)
C. fumago	Inoculation with preculture ($\sim 10^{3}$ pellet per reactor, 2 3 mm diameter)	Airlift reactor, T: 22 °C, air flow: 0.6 1.0 l ^{1} min ^{1}	Pellet (small volume of preculture) or amorphous, viscous mycelium (large volume)	*Heme glucoprotein chloroperoxidase (homologous)*: no influence of inoculum	Carmichael and Pickard (1989)
P. chrysogenum	Spore concentration 5×10^{4} 1 $\times$ 10^{6} ml ^{1}	Shake flasks, T: 26 °C, n_{stir}: 200 min ^{1}, t: 75 h	Pellets at low concentration, change in morphology between 5×10^{4} and 5×10^{5} ml ^{1} to dispersed mycelium	Penicillin (homologous)	Tucker *et al.* (1992)
	Spore concentration 4×10^{7} 6×10^{8} l ^{1}	STR, T: 25 °C, n_{stir}: 300 min ^{1} air flow: 1 l ^{1} min ^{1}, or controlled to keep a constant dissolved oxygen (DO) saturation of 45%	Freely dispersed mycelia, branching frequency linked to total hyphal length, fragmentation correlated to power input	Penicillin (homologous)	Nielsen and Krabben (1995)

	Spore concentration 10^4 10^6 ml ^{1}	Shake flasks and STR	Freely dispersed mycelium or aggregates and clumps, reduced aggregation and compactness at higher concentration	Penicillin (homologous)	Tucker and Thomas (1994)
	Spore concentration 2.4 8.6 $\times$ 10^7 l ^{1}	STR, T: 25 °C, n_{stir}: 500 min ^{1} air flow: 1 l ^{1} min ^{1},	Small pellets at low concentration, large pellets at intermediate concentrations, mycelial growth at high concentrations	*Penicillin (homologous)*: no relation of penicillin production and macroscopic morphology	Nielsen *et al.* (1995)
R. oryzae	Spore concentration 1×10^6 3×10^9 l ^{1}	Shake flasks, T: 27 °C, n_{stir}: 170 min ^{1}, t: 48 h	Pellet	Not specified	Liu *et al.* (2008)
S. tendae	Spore concentrations 7.5×10^2 2.3×10^8 l ^{1}	Shake flasks, n_{stir}: 100 min ^{1}, STR, n_{stir}: 400 min ^{1}, air flow: 1 l ^{1} min ^{1}, pH 4 8, T: 26 30 °C, t: 6 days	Concentration $<10^3$ l ^{1} large pellets with diameter >2 mm and cellular yield of 0.3 0.6 g ml ^{1}, higher conidia concentrations constant biomass yield (1.6 g l ^{1}), pellet size inversely proportional to concentration until a plateau is reached a at high concentrations	Not specified	Vecht Lifshitz *et al.* (1990)

(*continued*)

TABLE 4.3 (*continued*)

	Organism	Investigated values	System	Morphology	Product	References
	T. reesei	Spore concentration in preculture: 10^5 10^7 l ^{1}	Shake flasks, inoculation with seeding culture, pH 4.8, *T*: 28 °C, n_{stir}: 150 min ^{1}	Large pellets at low inoculum levels, small flocs at higher concentration, decrease of pellet size with increase of concentration	*Cellulase (homologous)*: increased synthesis with small flocs due to high inoculum concentration	Domingues *et al.* (2000)
pH	*A. niger*	pH 3.5 6.5	CSTR, *D*: 0.07 h ^{1}, 200 ml preculture, addition of 1.6 g l ^{1} Junlon (prop 2 enoic acid, $C_3H_4O_2$), inoculation with, n_{stir}:: 1000 min ^{1} air flow: 0.8 l ^{1} min ^{1},	Filamentous growth due to addition of Junlon	*Hen egg white lysozyme (HEWL) (heterologous)*: production optimum pH 4.5, specific production rate 0.65 mg g ^{1} h ^{1}	Mainwaring *et al.* (1999)
		pH 1.5 4.5	Shake flasks, inoculum: 8×10^6 spores per flask, n_{stir}: 150 min ^{1}, *T*: 27 °C	pH < 2.3 free dispersed mycelium, pellet formation under more alkaline conditions, no difference in biomass yield	Not specified	Galbraith and Smith (1969)
		pH 4 7	STR, inoculum: 2×10^6 ml ^{1}, *t*: 12 h n_{stir}: 200 min ^{1}, air flow: 0.5 l ^{1} min ^{1}, *T*: 30 °C	Pellets, first aggregation step of spores only affected by pH, higher number of pellets at pH4 compared to pH7, highest aggregation velocity at pH 4	*Glucoamylase (homologous)*: productivity per biomass lower at pH 7 compared to pH 4	Grimm *et al.* (2005b)

	Varying initial pH 3 7 without pH control and constant pH of 5.8 6.0	STR, inoculation with 3 days preculture, T: 30 °C, n_{stir}:: 400 min ^{1} air flow: 0.5 l ^{1} min ^{1}	Free dispersed mycelium and pellets, high initial pH favoured biomass growth	*Phytase (homologous)*: higher activity with lower pH, maximal synthesis with biomass growth at neutral pH with subsequent decline in pH	Vats *et al.* (2004)
	pH 3 7 and without pH control	STR, inoculum: 2.5 × 10^{5} ml ^{1}, T: 30 °C, n_{stir}: 200 min ^{1} air flow: 1 l ^{1} min ^{1},	Pellets	*Glucoamylse GFP fusion protein (heterologous)*: optimal pH 6.0 due to reduction of protease activity and increase of product	O'Donnell *et al.* (2001)
A. oryzae	pH 2.5 8.0	STR, inoculum: 3 6 × 10^{8} l ^{1} or mycelial preculture, T: 32 °C, air flow: 1 l ^{1} min ^{1}, n_{stir}: 300 800 min ^{1} (DO 70 100%)	pH < 3.5 mycelium, pH 4 5 both morphologies, pH > 5 pellets; increasing pellet radius with increasing pH value, no pellet formation with mycelium as inoculum	*α Amylase (homologous)*: production optimum at pH 6, production formation at pH < 4 and > 7 very low	Carlsen *et al.* (1996)
P. chrysogenum	pH 6.0 7.4	CSTR, n_{stir}: 1200 min ^{1}, vortex aeration, T: 25 °C, D: 0.05 h ^{1}, t: 200 2000 h	Decrease of hyphal length of mycelia with rise from pH 6 to 7.4, pellet formation and swollen hyphae with increase to pH > 7.0 or 6.7, resp.	*Penicillin (homologous)*: optimal pH of 7.4 for synthesis, two stages cultivation recommended: (1) mycelial growth at low pH values and (2) production of penicillin due to a shift to pH > 7	Pirt and Callow (1959)

(*continued*)

TABLE 4.3 (*continued*)

	Organism	Investigated values	System	Morphology	Product	References
	R. oryzae	pH control with or without $CaCO_3$	Shake flasks, *T*: 27 °C, n_{stir}: 170 min ^{1}, *t*: 48 h	Pellets	Not specified	Liu *et al.* (2008)
	S. tendae	pH 4 8	Shake flasks, n_{stir}: 100 min ^{1}, STR, n_{stir}: 400 min ^{1}, air flow: 1 l ^{1} min ^{1}, Inoculum: 7.5 × 10^{2} 2.3 × 10^{8} l ^{1}, *T*: 26 32 °C, *t*: 6 days	Small pellets (0.1 0.2 mm) at pH 7 8 optimal for growth, larger pellets (1.2 1.5 mm) at pH 4	Not specified	Vecht Lifshitz *et al.* (1990)
	T. reesei	pH 2.2 7.6	STR, inoculum: 2.0 × 10^{8} l ^{1}, *T*: 30 °C, n_{stir}: 300, 500, or 600 min ^{1}	Freely dispersed mycelium, maximum values of tip extension rate and branching frequencies at pH 4.5, time of spore germination influenced by pH	Not specified	Lejeune and Baron (1995)
Power input due to agitation and aeration	*A. awamori*	DO tension 5 330%	STR, inoculum: 6.3 × 10^{6} l ^{1}, pH 4.5, T: 25 °C, n_{stir}: 600 min ^{1}, constant *P/V* of 1.07 W kg ^{1}	Denser pellets at high DO concentration; weak, fluffly pellets at low DO concentration, DO concentration hardly influence pellet size and hairy length	Not specified	Cui *et al.* (1998)

A. niger	Agitation intensity n_{stir}: 100 300 min ^{1} or $(P/V)_{stir}$; 0.014 0.390 W kg ^{1}, resp. Or Power input due to aeration $(P/V)_{air}$: 0 0.5 m^3 h ^{1}	STR, Inoculum: 2 × 10^6 ml ^{1}, pH 4.8, *t*: 15 h, no aeration, *T*: 30 °C STR, Inoculum: 2 × 10^6 ml ^{1}, pH 4.8, *t*: 15 h, *T*: 30 °C n_{stir}: 150 min ^{1}	Pellets, no affect of agitation on first aggregation step, second aggregation step of conidia is dependent on agitation Neglible effect of aeration on spore agitation	Glucoamylase (homologous)	Grimm *et al.* (2005b)
	Agitation intensity n_{stir}: 100 600 min ^{1}	STR, inoculum: 40 h old preculture (initial spore concentration 1 × 10^7 ml ^{1}), pH 2.1, air flow: 1 l ^{1} min ^{1}, *T*: 28 °C	Clumps, no direct effect of agitation on biomass yield, decrease of clump size with increase of agitation intensity	*Citric acid*: increase of production with raising agitation intensity	Papagianni *et al.* (1998)
	Power input $(P/V)_{stir}$: 0.05 0.073 W kg ^{1} (n_{stir}: 200 600 min ^{1})	STR, inoculum: 1 × 10^6 ml ^{1}, pH 5.5 *T*: 30 °C, air flow: 0.5 l ^{1} min ^{1}	Pellets, higher $(P/V)_{stir}$ results in lower biomass concentration, smaller and compacter pellets a high $(P/V)_{stir}$ compared to large and fluffy pellets at low $(P/V)_{stir}$	Glucoamylase (homologous)	Kelly *et al.* (2006)

(*continued*)

TABLE 4.3 (*continued*)

Organism	Investigated values	System	Morphology	Product	References
	Power input $(P/V)_{stir}$: 0.056 0.099 W kg ^{1} (n_{stir}: 105 190 min ^{1})	STR, inoculum: 1×10^{6} ml ^{1}, pH 5.5 T: 30 °C, air flow: 0.5 l ^{1} min ^{1}	Pellet size and concentration affected by $(P/V)_{stir}$, minimal diameter at $(P/V)_{stir} \sim 0.075$ W kg ^{1}, decreasing pellet concentration with increasing $(P/V)_{stir}$	*Glucoamylase (homologous)*: maximal synthesis at ~0.065 W kg ^{1}, low production with smaller pellet diameter at power input of 0.075 W kg ^{1}	Kelly *et al.* (2004)
	Agitation intensity: n_{stir}: 150 300 min ^{1}	Shake flasks, pH 5.0 5.3, shift in viscosity due to addition of guar gum, T: 30 °C	Small pellets with entangled mycelium at 150 min ^{1}, freely dispersed mycelium at 300 min ^{1}, transition to filamentous growth with increasing viscosity	*Phytase (homologous)*: synthesis increased with increasing shaker speeds as well as with gum addition	Papagianni *et al.* (2001)
	Agitation intensity: n_{stir}: 200 min ^{1} increased to 400 min ^{1} after 13 h, raised to 600 min ^{1} after 15 h	STR, pH 5.5, T: 30 °C, air flow: 1 l ^{1} min ^{1}	Pellets, improvement of nutrient availability at the pellet core with decreasing size of dense pellets	*Glucose oxidase (GOD)*: *(recombinant homologous)* higher yields of GOD with smaller pellets with high density	El Enshasy *et al.* (1999)
	agitation intensity: n_{stir}: 200 800 min ^{1} or $(P/V)_{stir}$:0.1 8 W kg ^{1}, resp.	STR, inoculum: 1×10^{7} ml 1, pH 5.5, T: 30 °C, air flow: 1.5 l ^{1} min ^{1}, after initial 5 h of 0.75 l ^{1} min ^{1}	Range from large pellets at n_{stir}: 200 min ^{1} to a filamentous network with micropellets at n_{stir}: 800 min ^{1}, lower biomass concentration at higher agitation rates	*GOD (recombinant homologous)*: GOD increase with shift to mycelial growth at first, intermediate agitation favoured for prolonged cultivation	El Enshasy *et al.* (2006)

Agitation intensity: n_{stir}: 200 500 min ^{1}	STR, inoculation with 3 days preculture, no pH control (initial pH 5.5 6.0), T: 30 °C air flow: 0.5 l ^{1} min ^{1}	Mycelium and pellets, shear thinning with increasing stirring rate leading to short, highly fragmented hyphae compared to long, thin mycelia at lower agitation rates	*Phytase (homologous)*: maximum synthesis at n_{stir}: 300 min ^{1}, no significant influence of agitation on extracellular protein	Vats *et al.* (2004)
Varying levels of oxygen enrichment within aeration: 0%, 10%, 30%, and 50% O_2 (v/v)	CSTR, inoculation with 1% seeding culture, pH 4.0, T: 25 °C, air flow: 1 l ^{1} min ^{1}, D: 0.06 h ^{1}	Under oxygen limitation (0%, 10% O_2) long sparsely branched hyphal elements, shorter hyphal elements with high branch frequency with oxygen enrichment (30%, 50% O_2)	*HEWL (heterolog) and Glucoamylase (homologous)*: increased synthesis with rising oxygen supply	Wongwicharn *et al.* (1999b)
Constant $(P/V)_{total}$ with varying percentages of aeration (52.5 103.5 W m ^{3}) and agitation (90.5 39.5 W m ^{3})	STR, inoculum: 1×10^{6} ml ^{1}, pH 5.5, T: 30 °C, constant $(P/V)_{total}$ of 143 W m ^{3}	Pellets, with increasing proportion of aeration of $(P/V)_{total}$ decrease in pellet size and increase of pellet concentration as well as less dense surface structure	*Glucoamylase (GA) (homologous)*: increasing formation of GA with raising percentage of aeration and smaller pellets and high pellet concentration	Lin *et al.* (2010)
Increase of agitation intensity n_{stir} from 300 to 500 min ^{1} and increase of aeration from 0.5 to 1.2 l ^{1} h ^{1}	STR, no pH control, T: 32 °C, t: 90 160 h	Distinct pellets with long peripheral hyphae, no influence of varied parameters on morphology	*Pectolytic enzymes (homologous)*: growth associated synthesis of polygalacturonase, pectin esterase, pectinlyase	Friedrich *et al.* (1989)

(*continued*)

TABLE 4.3 (*continued*)

Organism	Investigated values	System	Morphology	Product	References
	Agitation intensity n_{stir}: 250 and 400 min ^{1}, DO tension: 15% and 35%	STR, inoculum 1 × 10^{6} ml ^{1}, pH 6.0, *T*: 32 °C, *t*: 90 160 h	Pellets	*Glucoamylse GFP fusion gene (heterologous)*: increase of GFP formation with increased agitation rate, decreased GFP production with raised DO concentration	Wang *et al.* (2003)
A. oryzae	Agitation intensity n_{stir}: 525 825 min ^{1}	STR, fed batch, pH 5.0, air flow: 1 l ^{1} min ^{1}, gas blending regulated to a constant DO level of 50%, *T*: 32 °C	Increase of biomass with increase of agitation rate in batch phase	*Amyloglucosidase (recombinant)*: increase of secretion with increase of agitation rate in batch phase, dependency on hyphal tip activity	Amanullah *et al.* (2002b)
A. terreus	Agitation rate n_{stir}: 300 800 min ^{1} aeration regimes with air or an oxygen enriched mixture (80% O_2)	STR, seeding cultures of 250 ml, *T*: 28 °C, *t*: 7 days, air flow: 1 l ^{1} min ^{1}	Pellets, no influence of investigated agitation rates and aeration regimes on biomass formation, but influence on pellet morphology	*Lovastatin (homologous)*: high production with large, fluffy pellets at low n_{stir} and with oxygen enriched aeration	Casas López *et al.* (2005)
P. chrysogenum	Agitation intensity: n_{stir}: 800 1200 min ^{1} (10 l) or	STR (10 l): 1.5 l seed medium, STR (100 l): 10 l seed medium,	Freely dispersed mycelium, 10 l cultivations: with higher n_{stir}, mean	*Penicillin (homologous)*: 10 l: reduced productivity at higher agitation rates, similar	Smith *et al.* (1990)

	n_{stir}: 350 565 min ^{1} (100 l)	T: 26 °C, exponential feed rate during the first 20 h and than linear	effective hyphal length decreases faster, similar power inputs in 100 l less influence, morphology not affected by aeration	power inputs in 100 l higher productivity	
	Agitation rate controlled by DO concentration (set point 45%)	STR, T: 25 °C, air flow: 1 l ^{1} min ^{1}	Reduction of hyphal agglomeration with increasing agitation rate n_{stir}	*Penicillin (homologous)*: no relation of penicillin production and macroscopic morphology	Nielsen *et al.* (1995)
R. oryzae	Agitation intensity: n_{stir}: 115 350 min ^{1} (orbital shaker)	Shake flasks, inoculum: 1×10^{9} ml ^{1}, T: 27 °C, t: 48 h	Pellet, low shaking speeds favour pellet formation, pellet size increases with lower shaking speeds	Not specified	Liu *et al.* (2008)
T. reeseie	Agitation intensity n_{stir}: 130 400 min ^{1}	STR, inoculum: 72 h old preculture (initial dry weight 0.5 g l ^{1}, initial pH $>$ 4.5, no pH control, air flow: 0.2 l ^{1} min ^{1}, aeration level regulated to a DO $>$ 30% by changing back pressure, T: 30 °C, Lactose as carbon source	Pellets, longer lag phase at n_{stir}: 400 min ^{1}, maximum dry weight not affected by agitation	*Endoglucanase*, *Xylanase*: decrease of enzyme production with increase of agitation rates	Lejeune and Baron (1995)

(*continued*)

TABLE 4.3 (*continued*)

	Organism	Investigated values	System	Morphology	Product	References
Others	*A. japonicus*	Effect of immobilization on different carriers	Shake flasks, different lignocellulosic materials as carriers, T: 28 °C inoculum: 1.8×10^7 ml $^{-1}$, no pH control, n_{stir}: 160 min $^{-1}$ t: 48 h	Corn cobs: material with highest level of microorganism immobilization	*Fructooligosaccharides (FOS) and β fructofuranosidase (FFase)*: with corn cobs as carrier maximal production of FOS and FFase	Mussatto *et al.* (2009)
	A. niger	Variation of size and concentration of added microparticles	Shake flasks, n_{stir}: 120 min $^{-1}$, STR, n_{stir}: 200 min $^{-1}$, air flow: 1 l $^{-1}$ min $^{-1}$ inoculum: 1×10^6 ml $^{-1}$, T: 30 and 37 °C	Formation of mycelium even at high pH values due to addition with talc particles, precise adjustment of growth to desired morphology with microparticles	*Fructofuranosidase (FFase), Glucoamylase (GA), GFP*: increased formation of GA and FFase with mycelial growth compared to pellets at identical process conditions	Driouch *et al.* (2009)
		Medium composition	STR, air flow: 1 l $^{-1}$ min $^{-1}$, pH 5.5, T: 30 °C, n_{stir}: 200 min $^{-1}$ increased to 400 min $^{-1}$ after13 h, raised to 600 min $^{-1}$ after 15 h	Pellets, increase in pellet size and decrease in density and number with raising yeast extract concentration	*GOD (recombinant homologous)*: higher yields of GOD with smaller pellets of high compactness	El Enshasy *et al.* (1999)

A. oryzae	Temperature (27 40 °C)	STR, inoculum: 3 6 × 10^8 l ^{1} or mycelial preculture, pH 5.0, *T*: 26 °C, air flow: 1 l ^{1} min ^{1}, n_{stir}: 300 800 min ^{1} (to maintain DO of 70 100%)	Temperature optimum for growth 35 °C, active growth layer of pellet about 145 μm	α Amylase (homologous)	Carlsen *et al.* (1996)
	Cultivation conditions in fed batch processes	STR, inoculum: 2 × 10^9 l ^{1}, pH 6.0, *T*: 30 °C, air flow: 1 l ^{1} min ^{1}, n_{stir}: 800 min ^{1}	Freely dispersed mycelium, increase of average hyphal length and diameter of tips in exponential batch phase, constant hyphal diameter in fedbatch phase	*Lipase (heterologous)*: increase of lipase activity linked to increase of hyphal diameter	Haack *et al.* (2006)
C. fumago	Supplementation and size variation of added microparticles (aluminium oxide and hydrous magnesium silicate)	Shake flasks, n_{stir}: 180 min ^{1} Inoculum: 7 days old preculture, pH 6.5, *t*: 15 days, micorparticle concentration: 0.05 25 g l ^{1}	No effect on morphology of particles with diameter of ∼500 μm, particles ≤42 result in freely dispersed mycelium up to dispersion to single hyphae, stimulation of single hyphae formation as result of added microparticels	*Chloroperoxidase (homologous)*: no effect on protein formation by particles with diameter of ∼500 μm, maximum specific productivity due to addition of particles with diameter of ≤42 μm	Kaup *et al.* (2008)

(*continued*)

TABLE 4.3 (*continued*)

Organism	Investigated values	System	Morphology	Product	References
R. oryzae	Temperature (22 38 °C) carbon source addition of biodegradable polymers	Shake flasks, inoculum:1 × 10^9 ml ^{1}, n_{stir}: 170 min ^{1}, *t*: 48 h	Pellets, no influence of T on biomass between 22 and 33 °C, biomass yield lower at 38 °C, significant influence of polymer concentration on biomass yield	Not specified	Liu *et al.* (2008)
S. tendae	Temperature 26 32 °C	Shake flasks, n_{stir}: 100 min ^{1}; STR, n_{stir}: 400 min ^{1}, air flow: 1 l ^{1} min ^{1}, Inoculum: 7.5 × 10^2 2.3 × 10^8 l ^{1}, pH 4 8, *t*: 6 days	Pulpy growth at 31 32 °C, pellets at lower temperature of 26 30 °C	Not specified	Vecht Lifshitz *et al.* (1990)
T. reesei	Medium composition	Shake flasks, inoculation with seeding culture, pH 4.8, *T*: 28 °C, n_{stir}: 150 min ^{1}	Freely dispersed mycelium in presence of Tween 80	*Cellulase (homologous)*: enzyme formation significantly influenced by addition of Tween 80	Domingues *et al.* (2000)

D, dilution rate (h ^{1}); DO, dissolved oxygen (%); GFP, green fluorescent protein; GOD, glucose oxidase; HEWL, hen egg white lysozyme; n_{stir}, agitation rate/shaking intensity (min ^{1}); (*P*/*V*), total volumetric power input (W m ^{3}); $(P/V)_{stir}$, volumetric power input due to agitation (W m ^{3}); $(P/V)_{air}$, volumetric power input due to aeration (W m ^{3}); *t*, cultivation time (h); *T*, temperature (°C); CSTR, continuous stirred tank reactor; STR, stirred tank reactor.

(Carlsen *et al.*, 1996; Galbraith and Smith, 1969; Grimm *et al.*, 2005a,b). Freely dispersed mycelium is linked to an acidic pH compared to distinct pellets at higher pH values. Pirt and Callow (1959) showed that a rise of the pH above 6 during cultivation is accompanied with decrease in the hyphal length. A further increase above a pH value of 7 results in swollen hyphae and pellet formation occurs (Pirt and Callow, 1959). In batch cultivations with *A. oryzae*, optimal α-amylase productivity was achieved at pH 6, as described by Carlsen *et al.* (1996). Vats *et al.* (2004) have reported an increase in phytase production of *A. niger* in an acidic environment of pH 1.5–1.8.

The influence of the agitation intensity (Amanullah *et al.*, 2002; Casas López *et al.*, 2005; Cui *et al.*, 1998; El-Enshasy *et al.*, 1999, 2006; Friedrich *et al.*, 1989; Kelly *et al.*, 2004, 2006; Lejeune and Baron, 1995; Liu *et al.*, 2008; Mitard and Riba, 1988; Nielsen *et al.*, 1995; O'Donnell *et al.*, 2001; Papagianni *et al.*, 1998; Smith *et al.*, 1990; Vats *et al.*, 2004; Wang *et al.*, 2003; Wongwicharn *et al.*, 1999b) as well as aeration (Casas López *et al.*, 2005; Cox *et al.*, 1998; Cui *et al.*, 1998; Friedrich *et al.*, 1989; Li *et al.*, 2008; Wongwicharn *et al.*, 1999b) and, therefore, varying mechanical power input (Lin *et al.*, 2010; Nielsen and Krabben, 1995) on morphology and productivity have been an objective in several studies (Gibbs *et al.*, 2000). However, in many studies the description of mechanical power input is incomplete and insufficient, because power input is confused with stirrer speeds. For an impartial description of power input, the geometry and type of reactor is needed in addition to the agitation rate. Moreover, is it possible to use parameters such as the volumetric power input or turbulent kinetic energy to characterize the mechanical stress on filamentous systems. Lin *et al.* (2010) described the alteration in pellet micromorphology (internal and surface structure) and macromorphology (pellet size and concentration) under different aeration and agitation intensities, keeping the total volumetric power input constant. As a result of the increased share of aeration in the total power, glucoamylase production is raised due to the high number of small and loosely structured pellets. Papagianni *et al.* (1998) found an increase in the production of citric acid with higher agitation intensities.

The other process parameters investigated were temperature (Carlsen *et al.*, 1996), the medium composition (Domingues *et al.*, 2000; El-Enshasy *et al.*, 1999; Liu *et al.*, 2008), and cultivation conditions (Haack *et al.*, 2006). In other studies, the effect of biodegradable polymers added to the medium (Liu *et al.*, 2008), or different carriers for immobilization (Mussatto *et al.*, 2009) was tested.

In pioneering studies, the use of inorganic microparticles added to the culture was recently introduced to control fungal morphology development (Driouch *et al.*, 2009; Kaup *et al.*, 2008). As shown for *Caldariomyces fumago*, the addition of microparticles consisting of aluminum oxide or

hydrous magnesium silicate caused a dispersion of the cells up to the level of single hypha and enhanced chloroperoxidase production (Kaup *et al.*, 2008). The authors observed that microparticles influenced the morphology of other fungi as well, suggesting that intentional supplementation to the culture might generally stimulate growth of these organisms. Additionally, supplementation with silicate microparticles was used as a novel approach to control the morphological development of *A. niger* in submerged culture. The authors demonstrate that, inoculated from spores, the morphology of *A. niger* can be precisely adjusted to a number different distinct morphological forms by the addition of silicate microparticles. Beyond previous findings, Driouch *et al.* (2009) showed that the use of microparticles does not only enable free mycelium but even a rather precise engineering of morphology through fine-tuned variation of particle size and concentration. These findings could open new possibilities to use microparticles for tailor-made morphology design in biotechnological processes.

V. CONCLUSIONS AND FUTURE PROSPECTS

As shown in various studies, there exists a close link between the operating environment of the bioprocess, the morphology, and the underlying metabolism of the individual cells. Currently, we are far from understanding of the underlying metabolic and regulatory mechanisms. Newly developing experimental and computational technologies in systems biology and systems biotechnology, however, now provide a powerful toolbox to move towards understanding this complex link between biological and engineering aspects of fungal cultures (Visser, 2009).

The connection of broth viscosity and morphology of the cultivated filamentous microorganism has been highlighted in countless studies as discussed in this review. With the help of image analysis, it was shown that it is possible to estimate the viscosity of the broth solely by processing morphological data. However, the methods introduced still leave room for improvement, as they depend heavily on partly agglomerated mycelia. Furthermore, imprecise morphological data, such as roughness or compactness, should be clearly defined. Various fractions of the biomass are unaccounted for, based on population averages and model assumptions, thus making it nearly impossible to describe the whole population. Eventually, new parameters such as microparticle size and concentration might be included in such models.

One upcoming challenge will be to tailor the morphology depending on the biotechnological process and the productivity desired. This will most likely be achieved by manipulation of the environom or cultivation additives such as microparticles. More powerful and automated image

analysis techniques will help to further categorize fungal morphology and eventually lead to a more certain prediction of the culture viscosity. All these techniques may be summarized by the term ''morphology engineering.''

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CHAPTER 5

Methanogenic Degradation of Petroleum Hydrocarbons in Subsurface Environments: Remediation, Heavy Oil Formation, and Energy Recovery

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Abstract

Hydrocarbons are common constituents of surface, shallow, and deep-subsurface environments. Under anaerobic conditions, hydrocarbons can be degraded to methane by methanogenic microbial consortia. This degradation process is widespread in the geosphere. In comparison with other anaerobic processes, methanogenic hydrocarbon degradation is more sustainable over geological time scales because replenishment of an exogenous electron acceptor is not required. As a consequence, this process has been responsible for the formation of the world's vast deposits of heavy oil, which far exceed conventional oil assets such as those found in the Middle East. Methanogenic degradation is also a potentially important component of attenuation in hydrocarbon contamination plumes. Studies of the organisms, syntrophic partnerships, mechanisms, and geochemical signatures associated with methanogenic hydrocarbon degradation have identified common themes and diagnostic markers for this process in the subsurface. These studies have also identified the potential to engineer methanogenic processes to enhance the recovery of energy assets as biogenic methane from residual oils stranded in petroleum systems.

I. INTRODUCTION

Subsurface methanogenic petroleum hydrocarbon degradation can be viewed from three distinct but overlapping perspectives. Methanogenic processes can play an important role in (1) heavy oil formation, (2) remediation of hydrocarbon contamination in anoxic environments, and (3) recovery of fossil energy assets. With respect to heavy oil formation, it is now believed that methanogenic processes are largely responsible for the alteration patterns observed in degraded petroleum reservoirs (Jones *et al.*, 2008). The world's huge heavy oil deposits are the result of this alteration and are known to far exceed conventional oil assets such as those found in the Middle East (Roadifer, 1987). Heavy oils are expensive to exploit because of their high viscosity, and have lower economic value due to their acidity and high bitumen and sulfur content. Given the declining inventory of conventional petroleum, it is clear that past microbiological degradation of oil has a current impact on the economies of industrialized and developing nations. Furthermore, technologies used to recover heavy oils (e.g., open cast mining and steam-assisted gravity drainage) produce two to three times more CO_2 per barrel than conventional oil production technologies (McKellar *et al.*, 2009). Accordingly, exploitation of heavy oils has significant environmental consequences. From a different and contrasting environmental perspective, methanogenic hydrocarbon-degrading processes may make an important contribution to amelioration of natural and anthropogenic hydrocarbon

releases in near-surface environments. Methanogenic hydrocarbon degradation—though slower than degradation via aerobic and alternative anaerobic respiratory pathways—is more sustainable in the subsurface because replenishment of an exogenous electron acceptor is not required. The third perspective on methanogenic hydrocarbon degradation is the enhanced recovery of stranded subsurface energy assets in the form of biogenic methane formed from the microbial degradation of residual oil *in situ*. This approach may soon be applied for the exploitation of partially degraded and residual oils *in situ* with less energy input than typically required for unconventional oil recovery. Key challenges both for energy recovery and contaminant remediation will be to determine the identity, activity of, and environmental constraints on methanogenic hydrocarbon-degrading microbial communities in relevant ecosystems. The current geochemical and microbiological understanding of subsurface hydrocarbon degradation is reviewed here, with a particular focus on methanogenic hydrocarbon-degrading consortia.

II. FORMATION, REACTIVITY, DISTRIBUTION, AND COMPOSITION OF HYDROCARBONS IN SUBSURFACE ENVIRONMENTS

Hydrocarbons are common constituents of surface, shallow, and deep-subsurface environments. These organic compounds are nonpolar and have low aqueous solubilities. Accordingly, they are some of the most reduced and chemically inert organic compounds in natural ecosystems. Hydrocarbons are formed naturally by diagenetic and catagenetic alteration of biomass over geological timescales (i.e., during oil and gas generation); however, they are also produced in living organisms as metabolites (e.g., leaf waxes) or as end products of energy-generating microbial metabolism (e.g., methanogenesis). Oil itself is an astonishingly complex mixture comprising approximately 11,000 individual compounds (Hughey *et al.*, 2002), many of which are unresolvable using conventional gas chromatography (a fraction of oil is described by geochemists as an unresolved complex mixture or UCM; Gough and Rowland, 1990). In addition, the majority of organic compounds in oil are macromolecular and not amenable to gas chromatographic analysis at all. With respect to the hydrocarbons in undegraded petroleum, saturated hydrocarbons are the most abundant compound class. The most abundant of these compounds are *n*-alkanes followed by isoprenoid hydrocarbons and to a lesser extent alkycyclohexanes; however, the UCM is also thought to contain large amounts of highly branched isoprenoids and alkanes (Gough and Rowland, 1990). Oils additionally contain substantial amounts of low molecular weight aromatic compounds for example BTEX (benzene, toluene, ethylbenzene, xylene)

and low molecular weight polyaromatic compounds (PAHs) such as napthalenes, phenanthrenes, and biphenyls. These compounds and many other hydrocarbons can be biodegraded in subsurface environments (Head *et al.*, 2003).

Oil generation in the subsurface involves the thermal degradation of buried macromolecular organic matter (kerogen) itself formed by biologically and geochemically mediated alteration processes during early burial (Deming and Baross, 1993; Tissot and Welte, 1984). Typically, the oil formed is expelled from kerogen-rich source rocks, with the final location and composition of the oil determined by migration and entrapment beneath an impermeable seal such as a mudstone to form a reservoir (Head *et al.*, 2003). Occasionally, migration and local geological circumstances (i.e., the absence of an effective seal, the presence of geological faults, or tectonic activity) result in the release of oil to surface environments. For instance, it has been estimated that as much as 45% of oil that is released into the world's oceans occurs through such natural hydrocarbon seepage (National Research Council, 2003). The balance of the oil released (55%) occurs as a result of industrial-scale oil extraction, transport, and consumption (National Research Council, 2002). Because of anthropogenic activities, many previously hydrocarbon-free locations are now heavily impacted to the detriment of human health, land use, and value (Hippensteel, 1997; Keith and Telliard, 1979). For instance, in urban environments, there is a large inventory of underground storage tanks that have leaked over extended periods of time (Pearce, 2002). On a larger scale, contamination of aquifers from leakage of pipelines has occurred (Bekins *et al.*, 2005).

Hydrocarbons are distributed ubiquitously from surface environments through the shallow subsurface to the deep subsurface and have been so for hundreds of millions if not billions of years. This distribution results in a range of very different environments and conditions where hydrocarbon degradation occurs. For instance, some petroleum reservoirs are deeply buried and hot, whereas others are shallow with lower in situ temperatures. Petroleum reservoirs are often functionally closed gas–oil–water systems, whereas contaminated aquifers or surface sediment and soil may be impacted by groundwater flow. Hydrological factors may have a significant impact on the redox chemistry of the degrading systems and hence the extent to which different redox processes contribute to hydrocarbon alteration. All of these environments and conditions may have very different salinity and nutrient availability and the nature of the specific hydrocarbons present may also be different. For example, oil reservoirs contain very complex mixtures of hydrocarbons, whereas near-surface systems are often contaminated by refined hydrocarbons such as fuel oils, diesel, or gasoline.

III. ECOPHYSIOLOGY OF METHANOGENIC HYDROCARBON DEGRADATION

In methane-producing environments, most organic carbon is mineralized by syntrophic partnerships of anaerobic bacteria and archaea. Distinct microbial components of these partnerships partition the overall degradation process into discreet steps. This is exemplified by the methanogenic degradation of hexadecane (Eq. (5.1)), which can involve different individual reactions (Eqs. (5.2)–(5.6)). The organisms involved in these partnerships obtain energy for growth by catalyzing pathways that operate close to thermodynamic equilibrium (Jackson and McInerney, 2002). They comprise different functional groups of bacteria and archaea (methanogens). Syntrophic fermenters convert complex organic compounds (hydrocarbons) into lower molecular weight intermediates, such as acetate, hydrogen, propionate, and formate (see Eq. (5.2)). For thermodynamic reasons, growth of syntrophic bacteria is sustainable only if these intermediates are efficiently removed; accordingly, syntrophs rely on methanogens (Eqs. (5.3) and (5.4)) that consume these compounds (Dolfing *et al.*, 2008). Syntrophic partnerships are further complicated by the potential transformation (under specific environmental conditions) of acetate to hydrogen (syntrophic acetate oxidation, Eq. (5.5)) and hydrogen to acetate (acetogenesis, Eq. (5.6)). Syntrophic acetate oxidation is more thermodynamically favorable at higher temperatures (Schink, 1997) but also occurs in mesophilic environments (e.g., Jones *et al.*, 2008; Nüsslein *et al.*, 2001). A recent examination of the thermodynamics of alkane degradation under methanogenic conditions indicated that at mesophilic temperatures, multiple pathways of alkane degradation are thermodynamically viable, including those involving syntrophic acetate oxidation, provided that hydrogen partial pressure remains low (Dolfing *et al.*, 2008). Different fates of acetate and hydrogen during hydrocarbon degradation (i.e., direct consumption of acetate and hydrogen by methanogens, or their interconversion by syntrophs and acetogens) have consequences for the interpretation of geochemical and microbiological data obtained from methanogenic hydrocarbon-degrading systems (see Section V).

Overall stoichiometry of alkane (hexadecane) degradation to methane:

$$4C_{16}H_{34} + 30H_2O \rightarrow 15CO_2 + 49CH_4 \quad (5.1)$$

Syntrophic alkane (hexadecane) oxidation to acetate and hydrogen:

$$4C_{16}H_{34} + 64H_2O \rightarrow 32CH_3COO^- + 32H^+ + 68H_2 \quad (5.2)$$

Hydrogenotrophic (CO_2 reducing) methanogenesis:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (5.3)$$

Acetoclastic methanogenesis:

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \quad (5.4)$$

Syntrophic acetate oxidation (SAO):

$$CH_3COO^- + H^+ + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad (5.5)$$

Acetogenesis:

$$4H_2 + 2CO_2 \rightarrow CH_3COO^- + 2H_2O \quad (5.6)$$

IV. EVIDENCE OF ANAEROBIC HYDROCARBON BIODEGRADATION IN NEAR-SURFACE ENVIRONMENTS

Aerobic hydrocarbon-degrading bacteria have been known for nearly a century (Söhngen, 1913), and the enzymes and mechanisms they employ are well described (Haddock, 2010; Rojo, 2010). Specifically, aerobic hydrocarbon degraders employ molecular oxygen as a terminal electron acceptor, and they also use it to overcome the chemical stability of hydrocarbons. These organisms use chemically aggressive mono- and dioxygenase enzymes to incorporate oxygen into alkanes and aromatic hydrocarbons, rendering them amenable to further degradation. In contrast, it was only in the 1980s that organisms capable of strictly anaerobic degradation of hydrocarbons were identified (Widdel and Rabus, 2001). To date, many nitrate-, iron-, and sulfate-reducing microorganisms that degrade hydrocarbons have been identified and isolated from near-surface sediments (Widdel *et al.*, 2010). The mechanisms employed by some of these anaerobes to overcome the chemically inert nature of hydrocarbons have been elucidated. For instance, a mechanism common to many different bacteria is the activation of alkyl and aryl hydrocarbons by fumarate addition with the formation of substituted succinates as catabolic intermediates (Boll and Heider, 2010).

Regardless of the initial activation mechanism, in the absence of exogenous electron acceptors, methanogenesis may be an important process in hydrocarbon degradation in many subsurface environments. It has been shown that aromatic hydrocarbons such as benzene (Grbic-Galic and Vogel, 1987; Ulrich and Edwards, 2003; Weiner and Lovley, 1998), toluene (Grbic-Galic and Vogel, 1987), xylene (Edwards and Grbic-Galic, 1994), and PAHs (Chang *et al.*, 2006; Natarajan *et al.*, 1999) are degraded methanogenically. The apolar sigma bonds of saturated hydrocarbons make them less reactive than aromatic hydrocarbons or alkenes, yet several laboratory studies have shown that saturated hydrocarbons can be degraded methanogenically with stoichiometric conversion to methane

and CO_2 (Anderson and Lovley, 2000; Gieg *et al.*, 2008; Jones *et al.*, 2008; Siddique *et al.*, 2006; Townsend *et al.*, 2003; Zengler et al., 1999). In the first study to demonstrate conversion of saturated hydrocarbons to methane, pure hexadecane was converted to CH_4 and CO_2 in anaerobic microcosms inoculated with mud from a freshwater ditch (Zengler *et al.*, 1999). This process occurred according to the overall stoichiometry shown in Eq. (5.1), but proceeded slowly with an initial 200-day lag phase. A later study (Jones *et al.*, 2008) confirmed the slow pace of methanogenic hydrocarbon degradation in comparison with sulfate-reducing microcosms incubated in parallel (Fig. 5.1A) and also demonstrated that the bulk of the *n*-alkanes in a crude oil (C_7–C_{34}) could be degraded to CH_4 and CO_2 (Fig. 5.1B). These results, in addition to the study of Townsend *et al.* (2003), show the potential for methanogenic degradation of the complex assemblages of hydrocarbons present within petroleum reservoirs or in anaerobic soils and sediments contaminated with crude oils. These studies have shown that growth rates of methanogenic hydrocarbon-degrading communities are slow but, critically, are stimulated by substrate amendment

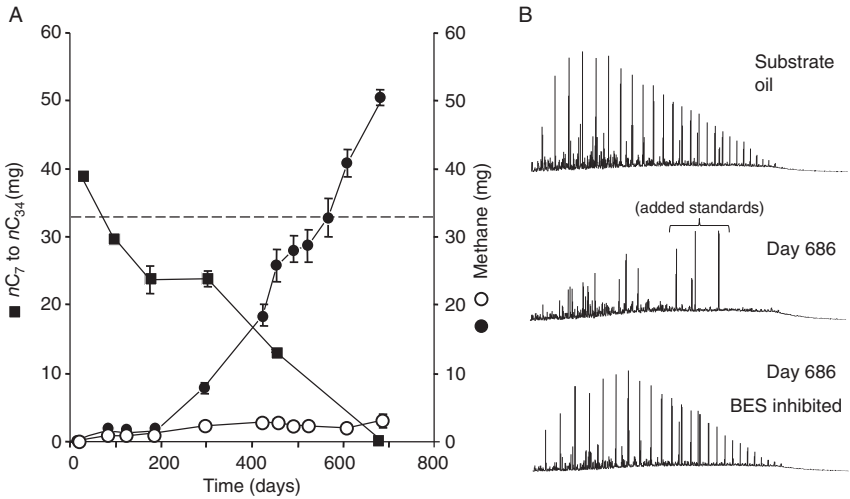

FIGURE 5.1 *n*-Alkane depletion and methane production during oil degradation. Panel A shows the loss of C_7–C_{34} *n*-alkanes (squares) and methane production (closed circles) from crude oil incubated under methanogenic conditions in replicate laboratory microcosms (100 ml) for 686 days. The horizontal dashed line represents the theoretical methane production if all crude oil *n*-alkanes were converted to methane according to Eq. (5.1). Methane production from control microcosms without oil (open circles) is shown for comparison and confirms the conversion of oil hydrocarbons to methane. Panel B shows the loss of *n*-alkanes over 686 days due to methanogenic crude oil degradation, as well as minimal hydrocarbon degradation over the same period in a control amended with bromoethanesulfonate (BES) to inhibit methanogenesis. Adapted from Head *et al.* (2010) and reproduced with kind permission from Springer.

with the buildup of sizable populations and hence faster degradation rates (Fig. 5.1). Therefore, on the decadal timescales required for the natural attenuation of hydrocarbon-contaminated environments or the implementation of enhanced oil recovery technologies, methane production rates will most likely be limited by those factors that limit growth, that is, nutrient supply and the toxicity of hydrocarbons and other organic compounds as well as metals present in the system (Head *et al.*, 2010).

Linking different anaerobic processes and their roles in hydrocarbon degradation *in situ* should be relatively straightforward in near-surface environments such as aquifers and contaminated soils. Samples can be easily obtained on a plume scale for anaerobic degradation tests under different redox conditions. For example, Kasai *et al.*, (2005) concluded that a fermentative/methanogenic community with hydrocarbon-degradation potential had become established in a petroleum-contaminated soil as opposed to an uncontaminated analog. Of particular interest was the finding that supplementing the same soil with electron acceptors (nitrate, sulphate, and oxygen) did not substantially stimulate hydrocarbon degradation. The authors suggested that other aerobic and anaerobic microorganisms were probably absent, implying that methanogenesis was quantitatively the most important degradation process. An alternative explanation, however, might be that hydrocarbon degradation was limited by a factor other than electron acceptor availability (e.g., toxicity or nutrient availability). Nevertheless, the rapid depletion of electron acceptors in many contaminated near-surface environments is a common feature of hydrocarbon contamination plumes (Christensen *et al.*, 2000). As a consequence, distinct partitioning of redox processes occurs and stimulation of methanogenic microbial communities in heavily contaminated zones has been observed (Bekins *et al.*, 1999; Haack *et al.*, 2004; Rooney-Varga *et al.*, 1999). Methanogenic hydrocarbon degradation can thus play an important role in natural attenuation. However, in many environments, several redox processes often appear to coexist. Gieg *et al.* (2008) demonstrated that crude oil alkanes in a petroleum reservoir sandstone were degraded by a methanogenic microbial consortium enriched from a gas-condensate-contaminated aquifer. Comparable rates of methane production were achieved regardless of the presence or absence of sulfate, even though molecular analyses based on 16S rRNA gene sequences identified putative sulfate-reducing bacteria (SRB) in the enrichment. Since these SRB-related sequences were present at low frequency within clone libraries, they are not indicative of a substantial direct contribution of sulfate reduction to hydrocarbon degradation; however, these organisms may make an important indirect contribution to methanogenic hydrocarbon degradation. Zengler *et al.* (1999) showed that the addition of low concentrations of sulfate (2 mM) to microcosms degrading hexadecane methanogenically had a stimulatory effect on methane production.

It was speculated that the SRB probably consumed part of the hydrogen formed by hydrocarbon-fermenting bacteria. Potentially, this removal of hydrogen may facilitate alkane fermentation reactions, which require low hydrogen partial pressures (Dolfing *et al.*, 2008).

V. EVIDENCE FOR HYDROCARBON BIODEGRADATION IN PETROLEUM RESERVOIRS AND THE FORMATION OF HEAVY OILS

Deeper subsurface environments such as petroleum reservoirs are logistically much more difficult to study than contaminated, shallow, subsurface environments. Nevertheless, it has been known for some time that oil biodegradation occurs in petroleum reservoirs (Connan, 1984) and recent geochemical evidence has provided a clearer picture of biodegradation patterns in petroleum systems. For instance, compositional gradients indicate that microbial activity is largely controlled in the subsurface by the availability of free water with its incumbent substrate and nutrient composition (Head *et al.*, 2003; Huang *et al.*, 2003). In biodegraded petroleum reservoirs where an oil leg overlies a substantial water leg, most biodegradation occurs close to the oil water transition zone, and in many cases, this is manifest as a decrease in saturated hydrocarbons and concomitant increase in poorly degradable polar components of oils such as ashphaltenes and resins (Head *et al.*, 2003; Huang *et al.*, 2003). It has been proposed that the oil–water transition zone (OWTZ) provides suitable physical and chemical conditions for microbial activity and that continuous diffusion of degradable hydrocarbons from the oil column coupled with their consumption at the OWTZ at a rate faster than they can be replenished by diffusion creates the compositional gradients observed (Head *et al.*, 2003).

A key question for petroleum geochemists in recent years has been—what are the processes occurring in petroleum reservoirs that drive oil biodegradation and hence are responsible for the formation of heavy oils? Despite well-documented examples of anaerobic hydrocarbon-degrading microbes present in near-surface environments, until recently, the mechanism of biodegradation in oil reservoirs was thought to be aerobic, requiring the flow of oxygenated ground water into deep petroleum systems (Palmer, 1993). Evidence for anaerobic rather than aerobic hydrocarbon degradation in petroleum reservoirs is now, however, substantial and compelling. The first microorganisms isolated from petroleum reservoirs were anaerobic (Bastin, 1926) and a wide diversity of anaerobes is now known from such systems (Magot, 2005, see Section VI). This is hardly surprising given that in many situations (e.g., organic-rich freshwater, estuarine and marine environments), oxygen is depleted within a few millimeters of the sediment

surface and results in only minor diagenetic alteration of the indigenous organic carbon. More direct evidence comes from the detection of metabolites indicative of anaerobic hydrocarbon degradation that are present in reservoir-degraded oils from around the world, but not present in ''pristine'' or aerobically degraded oils (Aitken *et al.*, 2004).

To date, anaerobic hydrocarbon-degrading microorganisms indigenous to petroleum reservoirs have not been isolated (Jeanthon *et al.*, 2005). This has largely been ascribed to the technical challenges associated with sampling from oil reservoir environments. However, nitrate- and sulfate-reducing, as well as fermentative and methanogenic, microorganisms have been commonly identified in and isolated from oil fields, though none of these have been implicated in hydrocarbon degradation. Nitrate, like oxygen, is highly reactive and would most likely be completely consumed before it could reach a deep petroleum reservoir. Inorganic nitrogen in petroleum reservoirs is primarily in the form of ammonium (Manning and Hutcheon, 2004). Sulfate will only be available in quantities required to explain the huge amounts of hydrocarbon removal seen in heavy-oil reservoirs in cases, where formation waters are rich in sulfate (e.g., in petroleum systems associated with evaporites). In contrast, there is substantial evidence for biogenic methane production in biodegrading reservoirs (Horstad and Larter, 1997; Larter and di Primio, 2005). The common occurrence of oil-rimmed gas accumulations (ORGAs) has been postulated to result from extensive methanogenic biodegradation of reservoired oil (Larter and di Primio, 2005). ORGAs represent reservoirs in which the heavy oil and dry gas are in equilibrium. In the giant Troll field located 60 km off the west coast of Norway, 74% of the accumulated petroleum is present as dry gas (93 mol% methane) with the remaining 26% present as heavy oil (Horstad and Larter, 1997). The heavy oil and dry gas are thought to be co-genetic and are to some extent formed from methanogenic biodegradation processes (Horstad and Larter, 1997). Evidence for this comes from the presence of methane in the gas that is isotopically light, suggesting it contains a relatively high proportion of biogenic gas compared to isotopically heavier thermogenic methane. The gas also contains isotopically heavy CO_2, indicative of closed-system reduction of CO_2 by hydrogen to methane (Larter *et al.*, 1999). The occurrence of anaerobic degradation of crude oils in subsurface reservoirs under methanogenic conditions would therefore explain the consistent hydrocarbon compositional patterns seen in degraded oils worldwide and their association with dry gas accumulations (Larter and di Primio, 2005). Recently, Jones *et al.* (2008) provided further evidence that the patterns of hydrocarbon degradation observed in biodegraded petroleum reservoirs were the result of methanogenic processes. The composition of oil in microcosms exhibiting methanogenic oil degradation was compared to patterns observed in a suite of biodegraded oils

from the Gullfaks field in the North Sea. Over a 2-year period, preferential degradation of *n*-alkanes in an initially undegraded North Sea oil was determined on the basis of pristane:nC_{17} ratios. This degradation pattern was similar to the patterns seen in a suite of Gullfaks oils exhibiting different degrees of biodegradation. Preferential *n*-alkane removal was not observed in analogous oil-degrading sulfate-reducing microcosoms. Furthermore, preferential removal of 3-methylbiphenyl relative to 4-methylbiphenyl was observed during oil degradation under sulfate-reducing conditions in the laboratory, whereas this was not observed under methanogenic conditions in the laboratory or in field-degraded Gullfaks oils. On the basis of these data, and data on oil and gas composition, including carbon isotope values of methane and carbon dioxide from biodegraded oil fields around the world, it has been proposed that methanogenic processes are responsible for the bulk of in-reservoir oil biodegradation (Jones *et al.*, 2008).

VI. METHANOGENIC HYDROCARBON-DEGRADING COMMUNITIES: ORGANISMS AND MECHANISMS

Many fundamental questions remain to be answered concerning the microbial ecology of oil-degrading methanogenic communities in petroleum reservoirs and contaminated groundwater and soil. For instance, what are the organisms and syntrophic partnerships involved and are there themes common to different settings? What are the pathways of degradation? What are the likely constraints on growth and gas production? Answers to these questions are of great importance with regard the potential utility of methanogenic hydrocarbon degradation in the remediation of hydrocarbon contamination or in enhancing the recovery of energy assets. A useful strategy for the identification of key organisms potentially involved in methanogen-rich hydrocarbon degradation is to survey the diversity present within a range of different methanogenic environments impacted by hydrocarbons to identify common themes and variations between these environments. We have collated data from published studies encompassing 26 culture-independent analyses of hydrocarbon impacted environments (Fig. 5.2). The 26 studies used bacterial 16S rRNA gene-based approaches to identify the dominant bacteria present in a range of high- and low-temperature petroleum reservoirs, contaminated aquifers, sediments, and soils (for references see Fig. 5.2). Our analysis considered over 3000 16S rRNA sequences recovered from these environments, which we classified at the level of major phylogenetic groups (phylum/sub-phylum). From these data, a distribution based on the average percentage representation of major phyla in clone libraries was plotted (Fig. 5.2A). Bacterial sequences affiliated with the phylum *Firmiucutes*

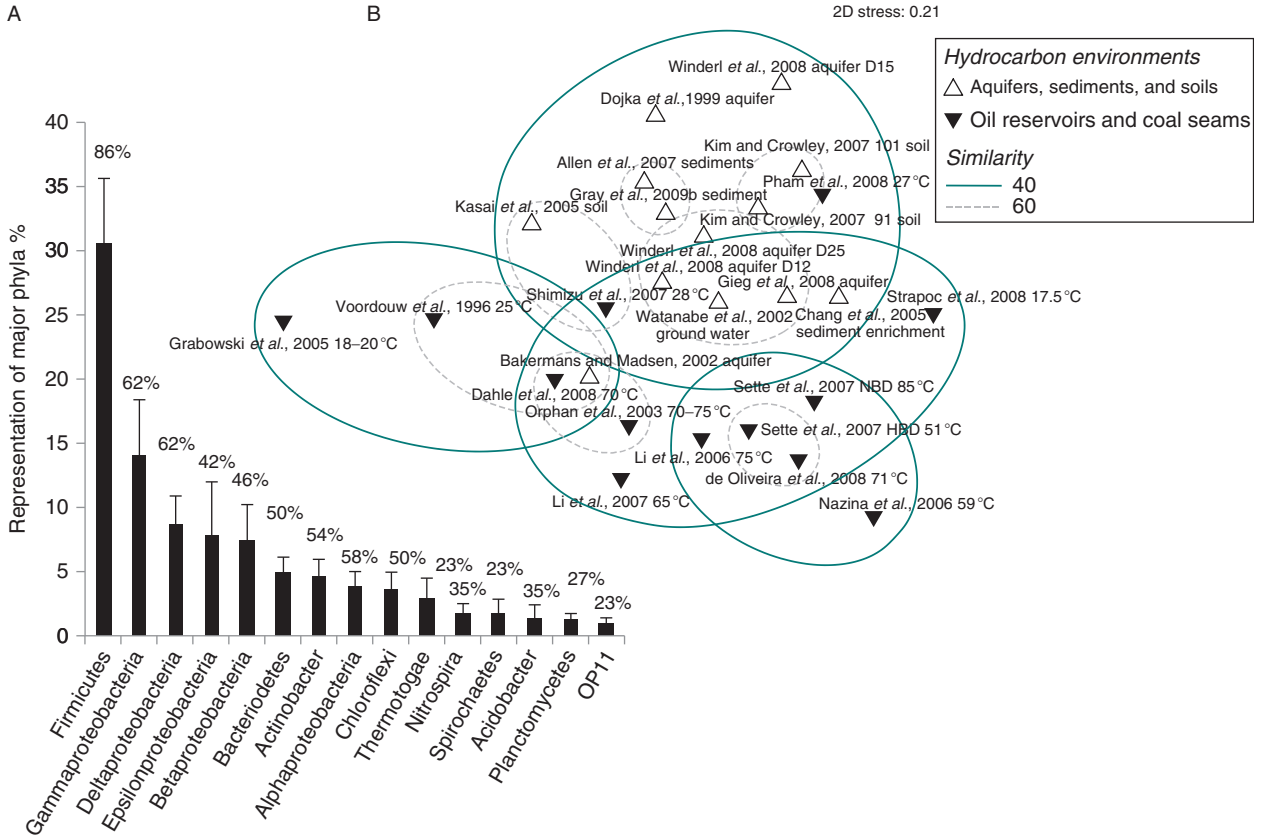

FIGURE 5.2 (A) Frequency distribution of 16S rRNA sequences (classified into major phylogenetic groups) recovered in clone libraries from hydrocarbon-impacted environments. Bars correspond to average percent representation of major phyla (1× standard error) based on a survey of 26 bacterial clone libraries. Values shown above the columns indicate the percentage of studies in which the phylum was identified. References for the 26 studies included in the survey are indicated in (B). (B) MDS analysis of Bray Curtis similarity coefficients calculated from normalized

were the most frequently detected sequence type (on average 31%, see Fig. 5.2A). Furthermore, *Firmicutes* were identified in 23 out of the 26 libraries—more than any other phylum. *Gammaproteobacteria* were detected at the second highest frequency (14% of sequences) and were detected in 16 of the libraries. In total, 15 major phyla accounted for 96% of the sequences. From these data, it is possible to infer that a wide diversity of bacterial species is present in hydrocarbon-impacted environments (Magot, 2005; Youssef *et al.*, 2009). It is striking that many of these phyla are predominantly comprised of bacteria that exhibit anaerobic and more specifically fermentative metabolism. Dominant groups within the *Firmicutes* were the classes *Clostridia*, *Thermoanaerobium*, and *Haloanaerobium*. Members of these groups are obligately anaerobic, as are the non-photsynthetic *Chloroflexi* (class *Anaerolinea*) that were also present at high frequency in many hydrocarbon-impacted environments. The *Bacteroidetes* also figure prominently in these microbial communities (Fig. 5.2A), and in addition to versatile aerobes related to *Flavobacteria*, the *Bacteroidetes* include obligately anaerobic organisms related to *Bacteroides*. Other major groups represented are characterized by organisms with predominantly anaerobic respiration or fermentation as modes of energy metabolism. For instance, the *Deltaproteobacteria* found in these studies include putative iron- and sulfate-reducing bacteria, as well as members of the family *Syntrophaceae* that are known to form syntrophic partnerships with methanogenic archaea.

The clone frequency data were also analyzed using multidimensional scaling (MDS) analysis (Fig. 5.2B). MDS plots are representations of how different datasets are from each other based on clustering of like samples. This comparison revealed distinct patterns within the different hydrocarbon-impacted environments (Fig. 5.2B). Particularly evident is the separate cluster representing high-temperature petroleum reservoir communities (Fig. 5.2B). *Firmicutes* were detected in all high-temperature oil field samples surveyed ($n = 8$) and also occurred at higher frequency in these clone libraries (average 50.2 $\pm$ 10.9%), relative to their frequency in clone libraries from other environments (average 20.9 $\pm$ 5.5%). *Gammaproteobacteria* (22.5 $\pm$ 7.6%) and *Thermotogae* (8.5 $\pm$ 2.7%) also had elevated clone frequencies when only the high-temperature petroleum reservoir clone libraries were considered. At low temperatures, these groups represented 12.6 $\pm$ 5.5 and 0.5 $\pm$ 2.0%, respectively.

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phylum/subphylum level clone library distributions. Analysis was conducted using Primer 6 for Windows (Version 6.1.5, Primer-E Ltd., UK). The distributions of oil field and coal seam samples are shown by inverted filled triangles, while the distributions of hydrocarbon-contaminated aquifer, sediment, and soil samples are shown by unfilled triangles. Similarity contours (60% and 40%) derived from a cluster analysis are superimposed on the MDS analysis.

While useful information can be gained from this general view of the microbial community compositions in hydrocarbon-impacted environments, such an approach is likely to be more useful if diagnostic markers (e.g., gene sequences) for specific hydrocarbon-degrading syntrophic bacteria and associated methanogens can be identified and their distribution patterns understood. The presence of these markers could then be used to infer methanogenic hydrocarbon degradation potential (see Section VII). A number of studies have identified partnerships between syntrophic bacteria and methanogenic archaea by the simple expedient of characterizing communities in hydrocarbon-amended enrichment cultures. For instance, a toluene-degrading methanogenic consortium enriched over a 10-year period from a creosote-contaminated aquifer was analyzed to determine the bacterial and archaeal community composition (Ficker *et al.*, 1999). We reclassified the bacteria identified in this study using the ribosomal database project (RDP) taxonomic classifier tool and the current RDP database (release 10; Cole *et al.*, 2007). Dominant bacterial sequences (i.e., those with >7% clone frequency) were affiliated to *Chloroflexi* (two phylotypes that were unclassfied in the original study), *Firmicutes* (genus *Desulfotomaculum*, one phylotype), *Deltaproteobacteria* (genus *Desulfovibrio*, one phylotype), and OP11 (one phylotype). OP11 has no cultured representatives but has a wide environmental distribution in surface, shallow, and deep-subsurface environments (Harris *et al.*, 2004) and has been identified in a range of hydrocarbon-impacted environments (Fig. 5.2A). On the basis of fluorescence *in situ* hybridization (FISH) analysis (using species-specific and universal bacterial probes), one of the *Chloroflexi* phylotypes (designated Eub 6) and the *Desulfotomaculum* sp. (designated Eub 1) together comprised 87% of all bacterial cells. These organisms were thus concluded to form the bacterial component of a syntrophic methanogenic consortium, with the *Chloroflexi* sp. responsible for the activation and primary fermentation of toluene and the *Desulfotomaculum* sp. responsible for the secondary fermentation of fatty acids to acetate and hydrogen (Ficker *et al.*, 1999). Interestingly, *Chloroflexi*-related sequences from hydrocarbon-impacted environments (Fig. 5.2A) are not closely related to the putative toluene-degrading syntrophic species described by Ficker *et al.* (1999) but form a distinct group within the *Anaerolinea*.

Other studies provide evidence of widely distributed bacterial components of methanogenic hydrocarbon-degrading consortia. Zengler *et al.* (1999) identified *Deltaproteobacteria* (*Syntrophus* spp.) as the dominant bacteria after prolonged methanogenic hexadecane degradation in freshwater mud-inoculated microcosms. Bacteria from the closely related genera *Syntrophus* and *Smithella* have since been identified in other methanogenic hydrocarbon-degrading enrichments (Gieg *et al.*, 2008; Jones *et al.*, 2008). *Syntrophus* and *Smithella* were also enriched in methangoenic

co-cultures inoculated with oil reservoir production water and fed with long-chain fatty acids (Grabowski *et al.*, 2005), and these organisms are closely related to the *Syntrophaceae* identified by Zengler et al. (1999) and Jones *et al.* (2008). This indicates that members of the *Syntrophaceae* are probably capable of the activation and fermentation of alkanes to lower molecular weight fatty acids or acetate and hydrogen. *Syntrophus*/*Smithella* have been detected in several hydrocarbon-impacted environments where methane is produced, and are often dominant members of microbial communities in these settings (Allen *et al.*, 2007; Bakermans and Madsen, 2002; Dojka *et al.*, 1998; Kasai *et al.*, 2005; Shimizu *et al.*, 2007). In a study of an aquifer contaminated with crude oil, JP4 jet fuel, and diesel fuel, *Syntrophus* spp. were found to represent 22% and 33% of bacterial 16S rRNA genes in clone libraries derived from methanogenic sediments with the highest levels of petroleum contamination (Allen *et al.*, 2007). A similar dominance of *Syntrophus* was observed in another aquifer contaminated with hydrocarbons (mainly jet fuel) and chlorinated solvents (Dojka et al., 1998).

Diverse methanogenic archaea have also been identified in deep and shallow hydrocarbon-impacted environments (e.g., Dojka, *et al.*, 1998; Jeanthon *et al.*, 2005; Kasai *et al.*, 2005; Watanabe *et al.*, 2002b). High-temperature oil reservoirs harbour a clear predominance of hydrogenotrophic (CO_2-reducing) methanogens, which has led to the suggestion that methanogenesis from acetate in these systems must be driven via syntrophic acetate oxidation (Eq. (5.5)) (Bonch-Osmolovskaya *et al.*, 2003; Davydova-charakhch'yan *et al.*, 1992; Nazina *et al.*, 2006; Nilsen and Torsvik, 1996). This is consistent with thermodynamic predictions that syntrophic acetate oxidation is more favorable at higher temperatures (Dolfing *et al.*, 2008; Schink, 1997). In some low-temperature environments, acetoclastic methanogens dominated archaeal clone libraries as well as a PCR-independent fosmid library (Dojka *et al.*, 1998; Grabowski *et al.*, 2005; Kasai *et al.*, 2005; Pham *et al.*, 2008), indicating that acetoclastic methanogenesis is important for terminal degradation. However, in other low-temperature environments, hydrogenotrophic (CO_2-reducing) methanogens dominate archaeal clone libraries (Grabowski *et al.*, 2005; Shimizu *et al.*, 2007; Watanabe *et al.*, 2002b). In a methanogenic oil-degrading enrichment dominated by a *Smithella* strain (Jones *et al.*, 2008, Gray *et al.* unpublished data), the most abundant archaeal clone was related to *Methanocalculus* – a member of the hydrogenotrophic *Methanomicrobiaceae*. The occurrence of syntrophic acetate oxidation (Eq. (5.5)) in the alkane-degrading microcosms required to account for the dominance of hydrogenotrophic methanogens (Eq. (5.3)) was confirmed using ^{13}C-labeled acetate, which demonstrated that the methyl group of the acetate was oxidized to CO_2. The contribution of syntrophic acetate oxidation to methanogenic hydrocarbon degradation in petroleum systems is supported by modeled $\delta^{13}CH_4$ and $\delta^{13}CO_2$ values

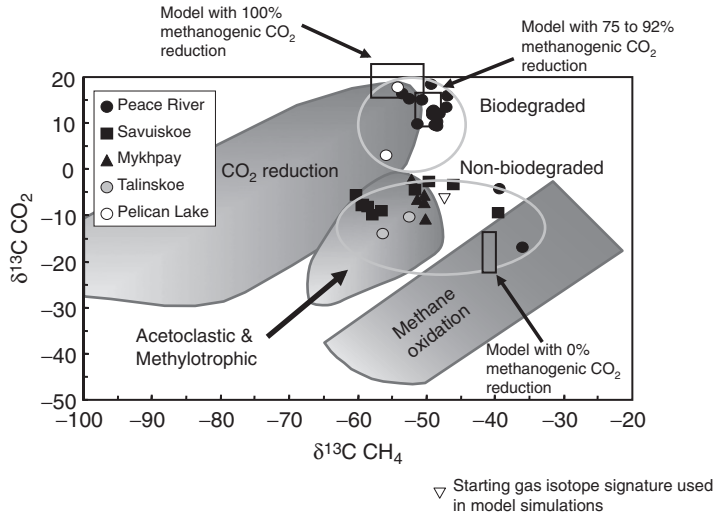

FIGURE 5.3 Measured and modeled methane and carbon dioxide ^{13}C isotopic composition from degraded and nondegraded oil reservoir gases (Grabowski *et al.*, 2005; Jones *et al.*, 2008; Nazina *et al.*, 1995; Rozanova *et al.*, 1995). These data have been superimposed onto to a plot of $\delta^{13}C$ CH_4 and $\delta^{13}C$ CO_2 showing isotope fractionation ranges (gray shaded areas) for methanogensis by CO_2 reduction; acetoclastic/methylotrophic methanogenesis; and methane oxidation (adapted from Whiticar, 1999). The measured gas isotope data (see symbol legend on graph) was derived from oil reservoirs from western Canada and western Siberia. Symbols indicate individual gas sample measurements for biodegraded (encompassed by a gray edged circle) and non-biodegraded oils (encompassed by a grey edged ellipse). Modeled methane and CO_2 compositions were derived from Rayleigh isotope fractionation (Jones *et al.*, 2008) using a defined starting gas isotope signature (inverted open triangle) and simulated for biodegradation of petroleum via alkane oxidation to acetate, with acetate degraded by syntrophic acetate oxidation and acetoclastic methanogenesis in different proportions and hydrogenotrophic methanogenesis. Three simulated ranges for gas isotope composition (black edged boxes) were derived assuming 0%, 75%, or 100% contribution of syntrophic acetate oxidation to acetate degradation and, thus, the same proportions of methanogenesis contributed to by CO_2 reduction.

based on Rayleigh isotope fractionation during methanogenic *n*-alkane degradation (Jones *et al.*, 2008). Figure 5.3 shows the isotopic composition of gases present in low-temperature oil reservoirs in western Canada and Western Siberia (Grabowski *et al.*, 2005; Jones *et al.*, 2008; Nazina *et al.*, 1995; Rozanova *et al.*, 1995) has been presented in comparison with known isotope fractionation ranges for methanogenesis by CO_2 reduction, acetoclastic/methylotrophic methanogenesis and methane oxidation (Whiticar, 1999). The modeled data indicate that a high proportion of methanogenic CO_2

reduction and hence the dominance of syntrophic acetate oxidation are required to match the relatively light $\delta^{13}CH_4$ and relatively heavy $\delta^{13}CO_2$ signatures seen in gases associated with biodegraded reservoirs. Gases associated with non-degraded oils do not show this enrichment in $^{13}CO_2$. These data together suggest that the terminal oxidation processes for subsurface hydrocarbons may often involve syntrophic acetate oxidation and that methanogenic alkane degradation may often be dominated by CO_2 reduction (Jones *et al.*, 2008).

VII. PUTTING METHANOGENIC HYDROCARBON-DEGRADING COMMUNITIES TO GOOD USE

The wide distribution of methanogenic petroleum hydrocarbon degradation in both shallow and deep-subsurface environments offers the potential to engineer this process for enhancing recovery of stranded energy assets. Evidence for methanogenic degradation of saturated hydrocarbons (Zengler *et al.*, 1999), the finding that the patterns of changes in oil chemistry in biodegrading petroleum systems are consistent with methanogenic oil degradation, and gas isotope evidence that the process occurs *in situ* in petroleum reservoirs (Jones *et al.*, 2008) have opened the way for a subsurface biotechnology to enhance recovery of stranded energy assets (Gieg *et al.*, 2008; Gray *et al.*, 2009; Head *et al.*, 2003; Jones *et al.*, 2008; Luca technologies, 2005; Parkes, 1999). Microbial conversion of residual stranded oil to methane in situ would not only allow the recovery of energy from residual oil as methane (typically $\gg$50% of oil remains in place after secondary production, whereas approximately 70% of gas can be recovered) but could also improve oil yield by reducing oil viscosity and repressurizing the reservoir with gas (Gray *et al.*, 2009; Grigoryan and Voordouw, 2008). The development and implementation of such a technology is timely given the dwindling inventory and declining discovery of conventional oil reserves and the ongoing issues relating to security of energy supply. Furthermore, the production of methane in the subsurface from partially degraded heavy oils would reduce the need for energy-intensive extraction techniques such as steam injection, which is currently used to recover heavily degraded oils. However, technological challenges must be overcome before biogenic methane from petroleum can make a serious contribution to global energy supply. These challenges are likely to differ on a reservoir-by-reservoir basis, but can be categorized into two distinct areas. One challenge is the engineering of the subsurface petroleum reservoir environments to effectively stimulate the oil-degrading methanogenic consortia and recover any gas and oil produced. The other challenge is to understand the physiology and ecology of subsurface methanogenic microbial communities.

The engineering challenges include the logistics of moving fluids, gases, and biostimulants through heterogeneous reservoir systems and the development of technologies for rapid *in situ* assessments of the success of such interventions. A claimed advantage of biogenic methane as a fuel is that it is clean-burning compared to other hydrocarbons. The amount of carbon dioxide that results from combustion is fixed by the hydrogen-to-carbon ratio in a given fuel (MacDonald, 1990). As such, the combustion of gasoline and diesel produces, respectively, 1.4 and 1.46 times more CO_2 per kilojoule than the combustion of methane. However, this ''clean fuel'' claim would only be valid if the CO_2 produced during the degradation of the source hydrocarbons (Eq. (5.1)) is naturally scrubbed and retained in reservoir formation waters or captured during gas production. Given the very different properties of methane and carbon dioxide, this is a realizable ambition. A detailed assessment of the engineering challenges is beyond the scope of this review, but the petroleum industry has considerable relevant expertise and infrastructure already available as a result of the development of secondary and tertiary oil recovery technologies.

The key microbiological challenge for enhanced oil recovery as methane is the stimulation of indigenous (Gray *et al.*, 2009) or establishment of exogenous (Gieg *et al.*, 2008) hydrocarbon-degrading microbial consortia within target reservoirs. A realistic assessment of their activities and growth characteristics is required. With respect to indigenous communities, past and probable ongoing biodegradation has been identified from geochemical signatures in numerous oil reservoirs. For instance, the common occurrence of ORGAs has been postulated to result from extensive methanogenic biodegradation of reservoired oil (Larter and di Primio, 2005), and methanogenic biodegradation process may be occurring in many ORGAs today (Gray *et al.*, 2009). On this basis, energy recovery from these biologically active reservoirs would rely on acceleration of natural methanogenic biodegradation (Head *et al.*, 2003). A recent study of formation waters from a high-temperature (70 °C) ORGA (where methanogenic hydrocarbon degradation has been implicated as a major source of methane in the reservoir (Gray *et al.*, 2009)) attempted to determine the activity of communities present. Critically, this ORGA had not undergone any water injection during oil recovery, making formation waters unlikely to have been contaminated with exogenous organisms or alternative electron acceptors. Although long-term incubations did not result in the enrichment of an indigenous oil-degrading methanogenic consortium, methanogenesis could be accelerated by the addition of H_2 and CO_2, and further stimulation of methanogenesis could be achieved by addition of inorganic nutrients (Fig. 5.4). These results indicate that stimulation of methanogenic oil biodegradation with inorganic nutrients could be an important strategy for implementing enhanced in situ

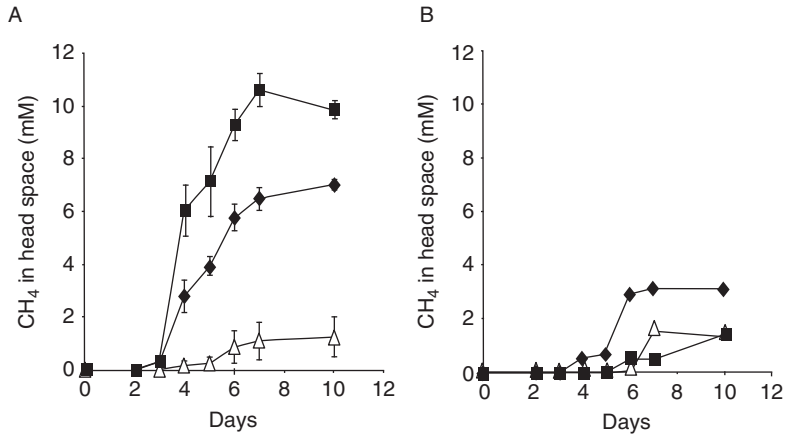

FIGURE 5.4 (A) The influence of inorganic nutrient and yeast extract amendments on methanogenesis from H_2/CO_2 in formation waters from a biodegraded North Sea oil and gas reservoir. Triangles (formation waters + H_2/CO_2), circles (formation waters + inorganic nutrients H_2/CO_2), and squares (formation waters + inorganic nutrients + yeast extract + H_2/CO_2). Errors bars denote 1× standard error. (B) Methane generation from formation waters amended with yeast extract alone. Different symbols (triangles, circles, and squares) represent individual replicates of the same experiment (formation waters + inorganic nutrients + yeast extract). Redrawn from Gray *et al.* (2009) with kind permission from Springer.

methanogenesis in oil reservoirs. However, enrichment of an oil-degrading community from such high-temperature reservoirs has yet to be achieved and has been identified as a major challenge for petroleum microbiologists necessitating rigorous and innovative sampling and culturing techniques (Jeanthon *et al.*, 2005). Furthermore, there is evidence that relatively modest temperatures, by the standards of known hyperthermophiles, may be sufficient to inactivate anaerobic crude oil-degrading bacteria (Wilhelms *et al.*, 2001). By contrast, an indigenous methanogenic oil-degrading community has been reported from a low-temperature petroleum system, namely the Monument Butte area of the Uinta basin of Utah (Luca Technologies, 2005). In this study, methane was generated in produced waters amended with crude oil, relative to sterile controls. Furthermore, *Clostridia* and *Thermotoga* were identified in the produced waters, suggestive of a fermentative capacity within the microbial community. However, a direct link between hydrocarbon degradation and methane production was not unequivocally determined in this study. For instance, the methanogenic degradation of non-hydrocarbon substrates was not assessed by the inclusion of controls that comprised oil-free production waters. Additionally, the rapid initial production of methane reported by Luca (Luca technologies, 2005) is inconsistent with

the long lag phases and considerably higher methane yields reported in other studies of methanogenic degradation of hydrocarbons (e.g., Jones *et al.*, 2008; Zengler et al., 1999). These differences may be attributable to the initial size and composition of the respective methanogenic consortia, the availability of nutrients, and the type of hydrocarbons available for degradation; however, such ambiguities also serve to highlight the need for rigorous and universally applied protocols in this research area.

The use of exogenous microorganisms (i.e., inoculating oil reservoirs with a methanogenic hydrocarbon-degrading consortium) to stimulate methane production has also been considered (Gieg *et al.*, 2008). One driver for such an undertaking is the finding that crude oil present within some petroleum reservoir samples could be converted to methane only by the addition of an exogenous microbial community. In this study, a consortium enriched from a gas condensate-contaminated aquifer yielded methane from the oil present in the reservoir sands. However, this bioaugmentation approach has some clear technical challenges. First, there are the logistics of producing and introducing a viable consortium with sufficient cell numbers into a target system. For instance, the syntrophic partnerships between fermenters and methanogens are known to be sensitive to physical disturbance and may not necessarily survive the physical and chemical stresses involved in fluid transfer (Hatamoto *et al.*, 2007). The second and perhaps more difficult challenge is the potential incompatibility of an exogenous methanogenic consortium with the *in situ* conditions of the target reservoir. Differences in temperature, salinity, pressure, or oil composition may all play a role in limiting the activities of the introduced organisms.

Despite the challenges to be overcome, there is little doubt as to the benefits of enhancing methanogenic hydrocarbon degradation in petroleum systems. In preliminary laboratory studies that have produced methane from hydrocarbons, some attempts have been made to extrapolate methane yields for a realistic field-scale application. Gieg *et al.* (2008) calculated the amount of methane that could theoretically be recovered from known domestic U.S. reservoirs (estimated at 375 billion barrels). This was done using methanogenesis rates from enrichment cultures, the average residual oil saturation of a marginal core, the density of the model formation oil, and an assumption that 1% of residual oil supplies would be amenable to biological transformation. The resulting production was estimated to be 3–13 billion cubic feet (Bcf) of CH_4 per day or 1–5 trillion cubic feet (Tcf) of CH_4 per year. The upper limit of this number represents 17% of current natural gas consumption in the United States. Using similar calculations and assumptions, estimates of short and longer term methane production rates for 40 wells drilled in the Monument Butte oil field were determined from laboratory experiments (without nutrient amendments). This exercise estimated production of 132 thousand cubic

feet (Mcf))/day/well (averaged over 87 days) and 50 Mcf/day/well (averaged over 297 days). Based on the price of natural gas in April 2010 (ca. $4 per Mcf), these levels of production equate to a revenue of $200–528 per well per day. However, to date, no field-scale applications of enhanced oil recovery as biogenic methane have been reported. This potentially beneficial technology therefore remains an exciting possibility rather than a proven reality.

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INDEX

CONTENTS OF PREVIOUS VOLUMES

Volume 40

Volume 41

Volume 42

Volume 43

Volume 44

Volume 45

Volume 46

Volume 47

Volume 48

Volume 49

Volume 50

Volume 51

Volume 52

Volume 53

Volume 54

Volume 55

Volume 56

Volume 57

Volume 58

Volume 59

Volume 60

Volume 61

Volume 62

Volume 63

Volume 64

Volume 65

Volume 66

Volume 67

Volume 68

Volume 69

Volume 70

Volume 71