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Molecular Methods in Microbial Ecology

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Erwin G. Zoetendal and Roderick I. Mackie

Abstract

It is now generally accepted in microbial ecology that cultivation-based approaches provide an incomplete picture of microbial diversity in the gastrointestinal (GI) tract because only a minority of microbes can be obtained in culture. Therefore, the application of molecular approaches, especially those focused on 16S ribosomal RNA (rRNA) sequence diversity, have become popular as they enable researchers to bypass the cultivation step. These approaches have provided considerable information about microbial ecosystems, including the GI tract. This chapter will summarize the different approaches and their impact on our knowledge of the ecology of the GI tract and provide guidelines for future research directions with a focus on pre- and probiotics.

Introduction

Every second of our life we are in contact with the microbes surrounding us. In fact, we cannot live without microbes as they are responsible for recycling the elements that are crucial for our life. Our GI tract is inhabited by large numbers of bacteria that collectively outnumber host cells by a factor of ten (Savage, 1977). The ecology of the GI tract is currently a hot research topic (The Gut, 2005). The complexity of interactions between these microbes and our intestinal cells varies tremendously and includes pathogenic, competitive and symbiotic interactions. Intriguingly, only one thin layer of epithelial cells separates the GI tract microbes from our other organs. The microbial community in the GI tract is very complex and consists of different groups of microbes, such as bacteria, archaea, ciliate and flagellate protozoa, anaerobic phycomycete fungi and bacteriophage; of these groups, bacteria have received most attention. An important function of the GI tract is the conversion of food into easily absorbable and digestible components. As a result, microbes are provided mainly with undigested dietary components. In addition, they have to deal with host-derived compounds such as mucopolysaccharides, mucins, epithelial cells and enzymes. Thus, the GI tract is an organ in which complex interactions occur between food, microbes and host cells. This interplay has a vital role in the normal nutritional, physiological, immunological and protective functions of the host animal, and therefore the GI tract has a special interest not only from a nutritional point of view but also from a commercial one (Saxelin *et al.*, 2005). Many probiotic and prebiotic products have been developed in order to improve our GI tract health. However, lit-

tle is known about the interactions that take place in the GI tract, and therefore the precise mode of action of pre- and probiotics is difficult to predict. However, in general, it is likely that probiotics affect the host cells (immune system) and intestinal microbes (competition) whereas prebiotics affect microbes directly because host cells cannot utilize prebiotic compounds. This gives microbes an opportunity to compete for the prebiotic compounds (Figure 1.1) while still influencing the host immune system indirectly.

Most frequently, the concept of symbiosis has been used to describe host–microbe interactions in the GI tract. However, the concept of détente would be more accurate as the host invests a lot of energy in a defence system to keep microbes away from the epithelial surface (Gaskins, 2001).

For more than a century, microbiologists have tried to isolate bacteria from all kinds of ecosystems, including the GI tract. Indeed, novel bacteria are still being isolated from the GI tract, e.g. butyrate-producing, cellobiose-degrading and mucin-utilizing bacteria (Barcenilla *et al.*, 2000; Pryde *et al.*, 2002; Zoetendal *et al.*, 2003; Derrien *et al.*, 2004). Despite these cultivation attempts, it has generally been accepted that only a minority of the GI tract microbes have been isolated in pure culture so far (Zoetendal *et al.*, 2004). Culture-independent approaches to study microbial ecosystems have clarified our limitations in isolating bacteria; as a result, a novel research area called *molecular microbial ecology* has developed. The difference between this novel research area and classical microbial ecology is that microbial ecosystems are now being studied

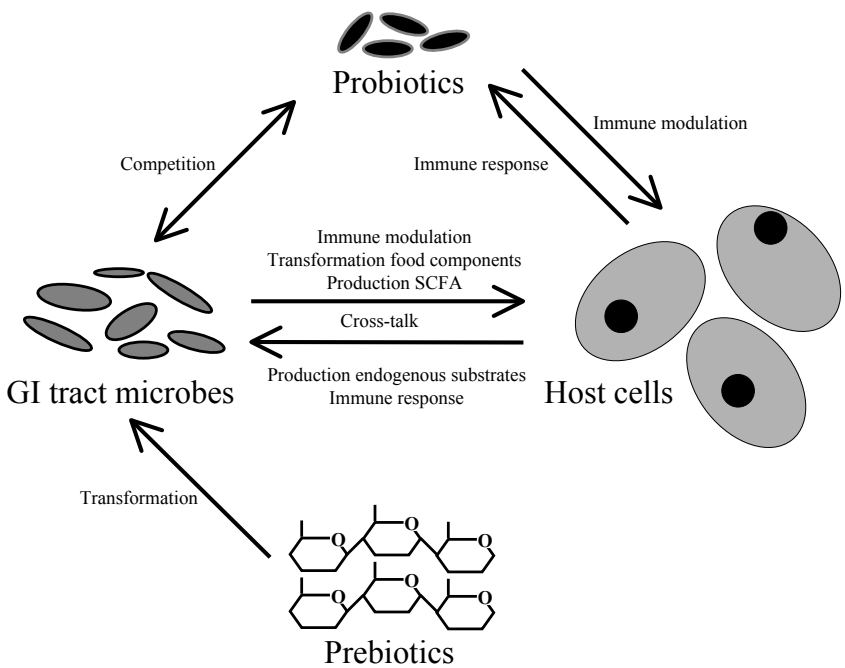

Figure 1.1 Host–microbe interactions and the hypothetical impact of pre- and probiotics on these interactions.

as a whole without the use of conventional cultivation procedures. It is already evident that molecular microbial ecology will enable us to obtain a complete description of the gastrointestinal ecosystem. This chapter will review progress made in microbial ecology with respect to the developments of novel methodologies to study microbes in a culture-independent way. In addition, it provides a brief overview of some major findings and suggestions for studying the impact of pre- and probiotics.

Culture-independent detection of GI tract microbes

The microbial ecology of the GI tract ecosystem involves several areas: (i) the investigation of the microbes present, (ii) their *in vivo* activity and (iii) their relationship with each other and the host animal (Hungate, 1960). This, and the fact that the majority of GI tract locations are inaccessible to sample, indicates that studying the microbial ecology of the GI tract is very complex. During recent decades, most of our knowledge concerning the ecology of the GI tract has been derived from the development of anaerobic culture techniques, the use of rodent and other animal models, and the development of gnotobiotic technology by which germ-free or animal models with a defined microbial community structure could be derived and maintained (Savage, 2001). Nowadays, *molecular microbial ecology* can be seen as the fourth major source of knowledge, offering great promise for the future.

First, ecologists must determine which bacteria are present in the GI tract, which is a complicated task. For many ecosystems, it is estimated that only a few per cent of microbes can be grown in culture (Amann *et al.*, 1995). The estimate of cultivability of GI tract bacteria is relatively high at between 10% and 50%, although it should be noted that this estimate is based on numbers and not diversity. Nevertheless, it is still a minority. For decades, the difference between total microscopic counts and colony-forming unit counts was explained by the number of dead cells in the sample, as cultivation of GI tract bacteria requires strict anoxic procedures. Using viability and dead stains, it has been shown that indeed one-third of the total bacterial community detected in faeces may be derived from dead cells (Apajalahti *et al.*, 2003; Ben-Amor, 2004). However, after the discovery that rRNA is present in every cell and that its nucleotide sequence can be used for phylogenetic classification (Woese, 1987; Woese *et al.*, 1990), the introduction of the so-called *16S rRNA approach* has demonstrated that the majority of GI tract bacteria are phylogenetically different from those described in culture. Therefore, it can be concluded that many bacteria escape cultivation procedures. Besides the difficulty of applying strict anoxic procedures, additional reasons for this cultivation anomaly may include the selectivity of the media that are used, unknown growth requirements and exposure to stress by the microbes during the cultivation procedures. In addition, it is also reasonable to assume that bacteria have adapted to GI tract conditions and, as a result, need specific interactions with other microbes and host cells. Therefore, avoiding these limitations when detecting and identifying GI tract microbes, and unravelling their function, requires the application of several culture-independent approaches (Table 1.1).

Phylogenetic identification of GI tract communities

Microbial communities cannot be accurately described without the use of culture-

Table 1.1 Overview of the current culture-independent approaches to studying microbial ecology

Approach	Target	Outcome experiment	Main limitations
16S rRNA gene sequencing	16S rRNA gene	16S rRNA gene sequence collection	Bias in NA extraction, PCR and cloning; laborious
BAC vector cloning	Genomic DNA	Gene sequences	Bias in NA extraction and cloning; laborious
Diversity arrays	16S rRNA genes; antibiotic resistance genes	Diversity profiles	Laborious; expensive; in early stages of development
DNA microarray	mRNA	Transcriptional fingerprints	Bias in NA extraction and NA labelling; expensive
Dot-blot hybridization	16S rRNA	Relative abundance of 16S rRNA	Laborious at species level; requires 16S rRNA gene sequence data
Fingerprinting	16S rRNA gene	Diversity profiles	Bias in NA extraction and PCR
FISH	16S rRNA	Enumeration of bacterial populations	Laborious at species level; requires 16S rRNA gene sequence data
In situ isotope tracking	Labelled biomarkers	Identification of substrate-utilizing microbes	Only suitable for simple pathways
Non-16S rRNA gene fingerprinting	Genomic DNA; cellular fatty acids	Diversity profiles	16S rRNA approaches required for indentification
Probe-based cell sorting	Genomic DNA, plasmid DNA, rRNA	Sorted cells containing certain gene sequences	Dependent on sequence data
Real-time PCR	16S rRNA gene	Relative abundance of 16S rRNA genes	Laborious and expensive in early stages of development
(R-)IVET	Promoter regions	Identification of induced promoters	Cultivation required
RT-PCR	mRNA	Specific gene expression	Bias in NA extraction and RT-PCR
Subtractive hybridization	Genomic DNA	Unique gene sequences	Bias in NA extraction, sensitive for false-positives

independent techniques, and sequencing of 16S rRNA genes has become a standard procedure in the identification of isolates. Currently, more than 100 000 16S rRNA sequences are available in the DNA databases, which is far more than for any other gene (Cole *et al.*, 2005). During the last decade, approaches based on sequence variability have frequently been applied to determine the microbial community structures in complex ecosystems, and developments are still ongoing. In addition, several

efforts are now being made to study the *in situ* activity of microbes within an ecosystem and subsequently link phylogeny and function. A summary of culture-independent techniques, including the latest developments, is given below.

Sequencing of 16S rRNA gene libraries

Most phylogenetic information from the GI tract has been gathered by sequencing of cloned 16S rRNA gene amplicons that have been obtained by polymerase chain reaction (PCR) of 16S rRNA genes present in the genomes of all bacteria. Amplification of rRNA by reverse transcription (RT)-PCR is also possible but has not been favoured for practical reasons. After creating the clone library, the sequences of the cloned amplicons are determined and compared with the available sequences in the DNA databases (http://rdp.cme.msu.edu/html), followed by phylogenetic analysis (Altschul *et al.*, 1990; Ludwig *et al.*, 2004; Cole *et al.*, 2005).

Sequencing of 16S rRNA gene clone libraries from human intestinal samples has indicated that a significant fraction of the bacteria have not been described previously. Most data have been retrieved from faecal samples owing to their accessibility (Wilson and Blitchington, 1996; Zoetendal *et al.*, 1998; Suau *et al.*, 1999; Eckburg *et al.*, 2005), but samples from the colon, ileum and oral cavity have also been characterized (Kroes *et al.*, 1999; Paster *et al.*, 2001; Hold *et al.*, 2002; Wang *et al.*, 2003; Eckburg *et al.*, 2005). Similar observations have been reported for a variety of animals, including pigs, horses, cows and chickens (Whitford *et al.*, 1998; Pryde *et al.*, 1999; Tajima *et al.*, 1999; Daly *et al.*, 2001; Gong *et al.*, 2002; Leser *et al.*, 2002a; Lu *et al.*, 2003). Interestingly, most of the novel sequences from GI tract samples are grouped in the low G + C Gram-positive phylum, indicating that this group is particularly under-represented by cultivation procedures.

Unfortunately, there is a lack of consistency between the molecular procedures (i.e. nucleic acids isolation, PCR), which complicates comparisons of data from different studies. More importantly, the thresholds used for operational taxonomic unit (OTU) determination vary from 1% to 5% sequence differences (Martin, 2002). This indicates that diversity estimates are very subjective. In addition, it has been demonstrated that source tracking of 16S rRNA genes is impossible because of these inconsistencies, as sequence depositions rely on the OTU determination and the researcher's opinion (Zoetendal *et al.*, 2004). Another problem concerned with the analysis of 16S rRNA gene clone libraries concerns the biases introduced by PCR and cloning, from which the OTU determination is frequently underestimated (Wilson and Blitchington ,1996; von Wintzingerode *et al.*, 1997; Polz and Cavanough, 1998; Whitford *et al.*, 1998; Bonnet *et al.*, 2002; Leser *et al.*, 2002a). Despite these limitations, it is evident that cloning and sequencing of 16S rRNA genes gives a more accurate picture of the bacterial composition in the GI tract than does culturing, and, therefore, the generation and analysis of clone libraries from various GI tract locations in different animals remain very important.

16S rRNA gene fingerprinting

Detailed phylogenetic information is obtained by cloning and sequencing of 16S rRNA genes, but for monitoring communities this approach is too laborious and expensive. Fingerprinting of 16S rRNA genes is more suitable for this, and several studies have reported its benefits in moni-

toring community shifts and comparing different communities. Denaturing gradient gel electrophoresis (DGGE) was first used in microbial ecology to study the bacterial diversity in a marine ecosystem (Muyzer *et al.*, 1993), and since then a variety of microbial ecosystems have been analysed using this or other fingerprinting techniques. Temperature gradient gel electrophoresis (TGGE) and temporal temperature gradient gel electrophoresis (TTGE) are other fingerprinting methods that are based on the same principle – sequence-specific melting behaviour of amplicons – but are less frequently used. Other fingerprinting techniques include single-strand conformation polymorphism (SSCP) and terminal-restriction fragment length polymorphism (T-RFLP). These fingerprinting techniques are all PCR-based, and their respective profiles represent the sequence diversity within ecosystems. For more detailed descriptions of these fingerprinting techniques, refer to the review papers by Muyzer and Smalla (1998), Vaughan *et al.* (2000) and Konstantinov *et al.* (2002). The development of software to analyse fingerprinting makes it a reliable approach for objective comparisons between communities, as similarity indices can be calculated and analysis of clustering profiles can be performed. Fingerprinting approaches are not quantitative, as PCR is involved. However, the possibility of absolute quantification of targets resulting in single amplicons in TGGE profiles has been demonstrated. Felske and colleagues (1998) mixed known concentrations of *Escherichia coli* RNA with RNA from soil and carried out combined competitive RT-PCR and TGGE analysis to quantify amplicons in the TGGE profiles. A similar quantification approach was performed by combining constant–denaturant capillary electrophoresis (CDCE) and quantitative PCR (Lim *et al.*, 2001). Remarkably, to our knowledge, these are the only quantitative studies so far.

DGGE and TGGE are reported to be sensitive enough to represent bacteria that make up greater than 1% of the total bacterial community, which means that only the most dominant bacteria will be represented in the profiles when domain-specific primers are used (Muyzer *et al.*, 1993; Zoetendal *et al.*, 1998). This can be overcome by using, for example, *Bifidobacterium* and *Lactobacillus* group-specific PCR-DGGE approaches (Simpson *et al.*, 2000; Satokari *et al.*, 2001a, b; Walter *et al.*, 2001; Heilig *et al.*, 2002; Temmerman *et al.*, 2003).

DGGE, TGGE and TTGE analyses of 16S rRNA genes have been successfully used to characterize and monitor the predominant GI tract bacterial communities in a variety of animals, including humans, pigs, dogs, cattle, rodents and chickens (Zoetendal *et al.*, 1998; Simpson *et al.*, 1999; Deplancke *et al.*, 2000; Simpson *et al.*, 2000; Tannock, *et al.*, 2000; Kocherginskaya *et al.*, 2001; McCracken *et al.*, 2001; Zoetendal *et al.*, 2001; Simpson *et al.*, 2002; Van der Wielen *et al.*, 2002; Zhu *et al.*, 2002; Zoetendal *et al.*, 2002a; Konstantinov *et al.*, 2003; Seksik *et al.*, 2003; Konstantinov *et al.*, 2004). T-RFLP has also demonstrated its success in monitoring GI tract communities (Leser *et al.*, 2000; Kaplan *et al.*, 2001; Nagashima *et al.*, 2003). These studies have already resulted in a substantial increase in the knowledge of factors that affect the community, as will be discussed in more detail later.

Non-16S rRNA-based profiling

As demonstrated above, most current profiling approaches used to describe bacterial communities have focused on 16S rRNA sequence diversity. Other approaches have also been used successfully to analyse and monitor bacterial communities, including

profiling of bacterial cellular fatty acids and determining community G + C content (Apajalahti *et al.*, 1998, 2001; Toivanen *et al.*, 2001; Apajalahti *et al.*, 2002; Vaahtovuo *et al.*, 2003). However, their application is limited to a few studies. The advantage of these other profiling approaches is that they do not need any amplification step, which is necessary for 16S rRNA profiling. Unfortunately, the observed shifts in bacterial cellular fatty acid and G + C profiles cannot be characterized phylogenetically and, therefore, they need validation by 16S rRNA approaches.

Quantification of 16S rRNA and its encoding genes

A major disadvantage of PCR-based approaches is that they do not provide quantitative data because of amplification biases. Therefore, other approaches are required to obtain quantitative data on 16S rRNA and its encoding gene. Dot-blot hybridization can be used to determine the relative amounts of rRNA from specified bacterial groups or species. Quantification by dot-blot hybridization is very accurate, as rRNA is directly targeted without any amplification procedure. This approach has been widely used to quantify rRNA from environmental samples, and successful analysis of GI tract samples from humans, ruminants and horses has been reported (Stahl *et al.*, 1988; Sghir *et al.*, 2000; Marteau *et al.*, 2001; Daly and Shirazi-Beechey 2003; Seksik *et al.*, 2003). Recently, an oligonucleotide database called ProbeBase was developed. This database has proved to be very useful in the search for existing probes when studying a target group of interest as it is now possible to link probe information to published literature (http://www.microbial-ecology.net/probebase/; Loy *et al.*, 2003).

A drawback of dot-blot hybridization concerns its limited sensitivity. Real-time PCR techniques (qrt-PCR) are currently very popular, as they combine the sensitivity of regular PCR with accurate quantification. Therefore, sequences that are of a very low concentration in environmental samples can best be quantified using this approach. qrt-PCR of 16S rRNA genes has been successfully applied in characterizing samples from ruminants, humans and pigs (Tajima *et al.*, 2001; Huijsdens *et al.*, 2002; Collier *et al.*, 2003; Malinen *et al.*, 2003; Matsuki *et al.*, 2004). However, the technique will be time consuming for complex ecosystems, as primers and PCR conditions have to be developed and validated for each group of sequences or OTU of interest. Competitive (RT-)PCR and most probable number (MPN)-PCR are other means of quantifying the bacterial 16S rRNA gene in environmental samples; however, after the introduction of the qrt-PCR technology, their application has drastically decreased.

Quantitative PCR approaches or dot-blot hybridization often emphasize their quantitative power. It must be noted that quantification is only relative (Rigottier-Gois *et al.*, 2003a). Extrapolation of data from isolated nucleic acids cannot be converted to cell numbers.

Fluorescence *in situ* hybridization

One frequently applied culture-independent approach to quantify bacterial cells in environmental samples is fluorescence *in situ* hybridization (FISH) using 16S rRNA-targeted oligonucleotide probes combined with epifluorescent light microscopy, confocal laser microscopy or flow cytometry (FCM) (Amann *et al.*, 1995). Compared with other quantitative 16S rRNA approaches, enumeration of bacteria by FISH is based on cell counts; there-

fore, FISH is more accurate for quantification. At present, the lowest level of detection is 10^6 cells per gram of faeces, which is less sensitive than qrt-PCR. FISH of intestinal bacteria has been mainly focused on faeces from humans, and probes targeting many phylogenetic groups of bacteria have already been developed and validated (Amann *et al.*, 1990; Schwiertz *et al.*, 2000; Harmsen *et al.*, 2002; Harmsen and Welling 2002; Zoetendal *et al.*, 2002b, Rigottier-Gois *et al.*, 2003b). Most counts have been performed using microscopy and, to facilitate enumeration, FISH has been automated and combined with image analysis. The results may then be analysed by computer software programs (Jansen *et al.*, 1999). Recently, flow cytometry has been shown to be another powerful tool for counting faecal bacteria with high-throughput possibilities (Zoetendal *et al.*, 2002b; Rigottier-Gois *et al.*, 2003a, b). In addition, flow cytometry enables target cells to be sorted and used for detailed characterization afterwards (Wallner *et al.*, 1997; Ben-Amor, 2004). The major disadvantages of FISH are that only a few probes can be used per analysis and that probe development is dependent on the 16S rRNA gene sequences deposited in the different databases. Other difficulties concerning FISH include the number of ribosomes per cell, the accessibility of the target and the permeability of the bacterial cell (De Vries *et al.*, 2004).

Diversity microarrays

DNA microarrays are very popular at present among researchers, and their development and application has grown explosively during recent years. DNA microarrays consist of glass or other surfaces spotted with a wide variety of covalently linked DNA probes that are available for hybridization. Most of the current applications of DNA microarrays include monitoring of gene expression (also called transcriptional profiling or transcriptomics). Less frequently, they are being used for detecting DNA sequence polymorphisms and mutations in genomic DNA. It is evident that DNA microarray analysis will be widely used in the near future in molecular biology (Rick *et al.*, 2001). The potential of DNA microarray technology in microbial ecology was first demonstrated by Guschin and colleagues (1997), who used microarrays containing oligonucleotides complementary to 16S rRNA sequences to detect and identify nitrifying bacteria in environmental samples. DNA microarray technology has since been optimized to study bacterial diversity in a variety of ecosystems in which the targets vary from 16S rRNA genes and genes involved in antibiotic resistance (Small *et al.*, 2001; Loy *et al.*, 2002; Call *et al.*, 2003; El Fantroussi *et al.*, 2003; Peplies *et al.*, 2003; Volokhov *et al.*, 2003). These types of microarrays are often termed *diversity arrays*. The first DNA microarrays for application to GI tract ecosystems look promising (Leser *et al.*, 2002b; Wang *et al.*, 2002; Wilson *et al.*, 2002).

Two of the main problems regarding DNA microarray analysis are hybridization specificity and quantification of signals. Often, very complicated software programs are involved in statistically determining signal–noise ratios. Currently, the most promising way to discriminate between specific and non-specific hybridization is the determination of thermal dissociation curves for each probe–target duplex (El Fantroussi *et al.*, 2003). Other approaches that minimize detection of false-positives include applying multiple probes for specific targets on the DNA microarray, although the problem with such approaches is the difficulty of explaining the outcome

when only one part of these multiple probes is hybridizing.

Quantification of hybridization signals is another complicated task. Loy and colleagues (2002) demonstrated that different targets which perfectly match the probes may vary significantly in signal intensities. Therefore, it may be concluded that DNA microarrays can currently be used as a qualitative screen rather than a quantification tool.

Culture-independent approaches to functionality analysis

The first important step in studying an ecosystem is to identify its members, and most molecular ecological studies have focused on answering the question of which bacteria are present. Unfortunately, identification of the microbes is not very valuable when they cannot be linked to the role that the different organisms play in establishing and maintaining a well-functioning ecosystem. Linking phylogeny to function is a very complicated and challenging task, given that (i) the majority of GI tract microbes cannot be cultured yet, (ii) the ability of a microbe to perform a certain function in culture does not mean that it performs the same function *in situ* and (iii) identifying the function of one type of microbe in a background of interactions within a complex ecosystem is very intractable. These three problems and ongoing research to tackle them will be discussed below.

The majority of GI tract microbes cannot be cultured

Insight into the physiological potential of microbes may be achieved by comparative genomics. In short, complete genome sequencing and subsequent comparative genomics provide researchers with an indication of the success of microbes. Recently, genome sequences from a variety of microbes of variable phylogenetic origins became available. As the number of sequences is growing exponentially, this makes the comparative genomics of microbes more reliable. The genome sequences of *Bacteroides thetaiotaomicron*, several *Lactobacillus* species, *Enterococcus faecalis*, *E. coli* and *Bifidobacterium longum*, all known to inhabit the intestine, have been determined. *In silico* comparisons between these and other bacteria have already provided remarkable observations of the features that could be crucial for their survival in specific niches in the GI tract (De Vos *et al.*, 2004). For more details concerning comparative genomics, see Chapter 5.

A major disadvantage of comparative genomics is that it is almost solely applied to well-studied isolates. Given the fact that most microbes have not been isolated, the full extent of microbial diversity cannot be accessed by genome sequencing and comparative genomics. One way to obtain genetic information about uncultured microbes is random cloning of DNA from environmental samples using bacterial artificial chromosome (BAC) or fosmid vectors. This strategy, often termed *metagenomics*, provides direct access to large genomic fragments (~100 kb for BACs, ~40 kb for fosmids) that are isolated directly from microbes in natural environments. The information on these fragments can be used to link 16S rRNA sequences and functional genes, from which the latter can be characterized experimentally. Recently, a metagenomic approach demonstrated that the viral diversity in human faeces consisted of approximately 1200 viral genotypes (Breitbart *et al.*, 2003). BAC technology has also been applied to prokaryotic genomics (Beja *et al.*, 2000; Rondon *et al.*, 2000; Liles *et al.*, 2003), and the number of metagenomic sequences is increasing, e.g.

the study of Venter and colleagues (2004), who randomly sequenced one billion base-pairs from the Sargasso sea. Therefore, metagenomic libraries can serve as an archive of DNA fragments for genomics purposes. However, it must be noted that the genomic information gathered by metagenomic approaches has to be validated experimentally by, for example, overexpression in *E. coli* or other hosts. One limitation of the BAC cloning approach is that only 2–3% of clones contain a 16S rRNA gene (Beja *et al.*, 2000). If more 16S rRNA gene sequences are required, the SuperPhyloBAC cloning vector might be an option for constructing libraries. This modified vector contains an I-*Ceu1* restriction site that is unique in the 23S rRNA gene.

Because the gene content of strains belonging to the same microbial species may differ by as much as 20%, it is evident that metagenomic libraries must be extremely large in order to cover the total diversity (Boucher *et al.*, 2001). This is an immense task. However, the number of clones required could be narrowed by subtracting genetic differences between microbes or ecosystems using techniques such as DNA microarray analysis and subtractive hybridization. The latter method is of special interest as it displays the difference between two organisms by excluding genes in common. In principle, subtractive hybridization involves hybridization of tester DNA that contains the target DNA fragments of interest with excessive driver DNA as a reference. Unhybridized target DNA is then separated from hybridized common sequences. At present, known techniques include representational difference analysis (RDA) and suppressive subtractive hybridization (SSH) (Lisitsyn, 1995; Diatchenko *et al.*, 1996; Felske, 2002). Both techniques have been successfully used in characterizing genetic differences between pathogenic and non-pathogenic strains of the same species, e.g. *E. coli* and *Helicobacter pylo*ri (Akopyants *et al.*, 1998; Janke *et al.*, 2001; Blanc-Potard *et al.*, 2002; Allen *et al.*, 2003). Subtractive hybridization has also been successfully used to identify genome fragments that were unique for *Ruminococcus flavefaciens* FD-1 with respect to *R. flavefaciens* JM1, two closely related cellulolytic rumen bacteria (Antonopoulos *et al.*, 2004). A study to display differences between two ruminal ecosystems has also demonstrated the possibility of retrieving *ecosystem-specific* genome fragments (Galbraith *et al.*, 2004).

The main drawback to these metagenomic approaches is that it is very difficult to link specific genes to their precise microbial origin. Mostly, it is possible to discriminate genes at a domain level, but linking genetic information to lower levels is a difficult task. However, a recent study offered some insight into how this problem might be solved. The approach described is based on the use of polynucleotide probes for whole cell hybridization; as it is suggested that part of the probe remains outside the cells because of network formation, the hybridized microbes can be captured on a microplate coated with DNA fragments complementary to these probes (Zwirglmaier *et al.*, 2004). In this way, it should be possible to link novel gene sequences to uncultured bacteria.

Culturing does not reflect *in situ* functionality

All of our basic knowledge of microbes has been obtained by characterizing their genetics and physiology in pure or well-controlled mixed cultures. This is still one of the most important areas of research for the future, as isolation of novel microbes and determination of genome sequences are ongoing. However, it must be noted

that physiological characterization of microbes in the laboratory does not mean that they perform in the same way *in situ*.

Studies with isotopically (stable or radioactive) labelled substrates provide insight into the metabolically active organisms in an ecosystem concerning this substrate. The isotopes of a substrate can be traced back by extracting biomarkers, such as DNA or lipids, or by combining microautoradiography and *in situ* hybridization, and in this way the organisms metabolizing the substrate can be identified (Radajewski *et al.*, 2003). For the first time, isotope tracking has been applied successfully to aquatic sediments, and the microbes involved in methane oxidation and acetate-coupled sulphate reduction could be identified (Boschker *et al.*, 1998). One of the better examples of the contradiction between phenotypic characterization and *in situ* isotope tracking has been described by Manefield and colleagues (2002). These authors showed that phenol degradation in the microbial community of a bioreactor was dominated by an uncultured member of the *Thauera* genus and not by the phenol-degrading bacteria isolated from this bioreactor. Recently, progress has also been made in combining isotope tracking with DNA microarray analysis, and the results look promising (Adamczyk *et al.*, 2003; Polz *et al.*, 2003). Using this approach, it will be possible to link immediately functional aspects to phylogenetic identification. Despite the value of isotope tracking, it must be remembered that some microbes might be able to discriminate between different isotopes, as has been demonstrated recently (Londry and Des Marais, 2003). Moreover, it will be very difficult to characterize the utilization of labelled substrates when their utilization requires complex pathways and/or multiple microbes. Last, but not least, the use of labelled substrates is rather expensive and is limited by their availability. To our knowledge, isotope tracking has not been used in GI tract ecology studies, but it will definitely be very useful when studying the *in situ* utilization of prebiotics or other components of the GI tract.

Identifying function in a complex ecosystem

DNA-based approaches and isotope tracking are very useful for obtaining information about genetic potential and substrate utilization in an ecosystem. However, they are too limited for any detailed analysis of *in situ* microbial activity. Therefore, other approaches are required.

One of the basic questions to answer in this respect is 'who is living?'. For the easily cultivable microbes, this can be determined by plate count analysis. However, for uncultured microbes, this has to be determined by different means. Ben-Amor and colleagues (2002) showed that pure cultures of bifidobacterial cells can be divided into active, injured or dead cell populations by FCM with functional probes. Using this FCM approach, Apajalahti and colleagues (2003) and Ben-Amor (2004) demonstrated that in human faeces up to one-third of certain bacteria are dead.

A more specific way of determining the *in situ* activity of bacteria in an ecosystem is to measure the expression of functional genes or proteins. In an excellent study, Hooper and colleagues (2001) demonstrated the power of transcriptomics in the investigation of host–microbe interactions in the murine GI tract. They studied the global transcriptional responses of germ-free mice to colonization by *B. thetaiotaomicron*, and even measured cell-specific responses by isolating RNA from cells captured by laser-capture microdissection. *B. thetaiotaomicron* clearly modu-

lated the expression of a wide variety of genes, including those involved in nutrient absorption and immune responses. When similar approaches are used to study bacterial responses, interactions between designated partners can be investigated on a molecular level in detail.

Transcriptional profiling is relatively straightforward for eukaryotes because they have stable poly(A)-tailed mRNA and are, therefore, easily converted into cDNA. Random hexamers are currently being used to obtain the cDNA of total RNA from prokaryotes. However, the bias introduced by this approach is not easy to determine. In addition, more than 95% of bacterial RNA consists of rRNA, which complicates the analysis of mRNA when converted to cDNA using random hexamers. Last, but not least, most messengers are poorly expressed or unstable; therefore, it remains a major challenge, especially for environmental samples. Nevertheless, different studies have shown that mRNA can be isolated from faecal samples (Deplancke *et al.*, 2000; Fitzsimons *et al.*, 2003), and even some success in quantifying mRNA has been reported. The expression level of four different genes of *H. pylori* was determined during its colonization of the gastric mucosa in humans and mice by means of qrt-RT-PCR (Rokbi *et al.*, 2001). Likewise, competitive RT-PCR was used to assess the germination level of genetically engineered *Bacillus subtilis* spores in mouse GI tract (Casula and Cutting, 2002). Recently, an excellent paper reported the simultaneous FISH detection of mRNA and rRNA in samples from pure cultures, bivalve symbionts and sediment (Pernthaler and Amann, 2004). It was demonstrated that *in situ* expression of *pmo*A could be monitored at single-cell level. The simultaneous detection of mRNA and rRNA in single cells is ideal for linking phylogeny and activity, and this provides many possibilities for the characterization of *in situ* gene expression of uncultured microbes in the GI tract.

A different approach for determining gene expression in ecosystems is *in vivo* expression technology (IVET). This strategy screens for promoters that are specifically induced when bacteria are exposed to certain environmental conditions (Rainey and Preston, 2000). The approach has mainly been used to study gene expression of pathogens and also recently for two *Lactobacillus* species. The colonization of *L. reuteri* in mouse GI tract revealed the expression of three genes, which might be less than one would expect in such a complex ecosystem (Walter *et al.*, 2003). In another study, the resolvase-based IVET (R-IVET) method enabled the identification of 72 induced genes of *Lactobacillus plantarum* during its passage through the GI tract of mice (Bron *et al.*, 2004). A major benefit of this R-IVET method compared with standard IVET is that promoter induction leads to an irreversible result, and therefore no selective pressure is required during the experiment. Interestingly, both studies reported a homologue of a gene with unknown function, and this may indicate its importance during passage through the GI tract by lactobacilli. Both IVET and R-IVET are approaches that will enable information about gene expression in a complex ecosystem, such as the GI tract, to be obtained. They do not provide information about expression levels and the location at which they are induced in the GI tract, but this information can be obtained using quantitative RT-PCR or other transcriptional approaches.

The remaining 'omics'

RNA-based approaches provide insight into gene regulation on a transcriptional

level. However, it must be noted that post-transcriptional regulation also occurs. The application of proteomics reflects activity at the protein level, and therefore probably represents activity more accurately. The main problem with proteomic approaches is that they are already very complex for pure cultures and therefore somewhat intractable for diverse ecosystems. However, a promising metaproteomic approach has been reported for activated sludge (Wilmes and Bond, 2004), which introduces the possibility of applying this technology to other ecosystems such as the GI tract. Metabolomics is another popular but complex 'omic' approach to profile activity but, to our knowledge, this has not yet been used in ecological studies. However, it is already clear that these non-nucleic acid-based approaches will complement genomic approaches in the near future. Genomics decodes the sequence information of an organism and transcriptomics gene expression, whereas proteomics and metabolomics attempt to elucidate the functions and relationships of these individual components. The main problem, however, with these 'omic' approaches is that technological improvements are rapidly evolving while the biological explanation of the results obtained – the main goal for biologists – remains for the most part unsolved.

GI tract ecology appears more complex than previously thought

The application of culture-independent approaches has already provided novel insights into GI tract ecology. As summarized in Table 1.2, the application of the 16S rRNA approach in GI tract ecosystems has answered some questions that could not have been answered by culture-dependent approaches, and studies focusing on GI tract functionality have been initiated as well. One of the main conclusions that can be drawn from these data is that the majority of bacteria in the GI tract have not yet been obtained in culture, which leaves their description and possible role in the GI tract still unknown. A major component of the uncultured bacteria belongs to clostridial clusters (originally described by Collins *et al.*, 1994), which indicates that these groups require further research in the near future. However, one should take into consideration that detection on the 16S rRNA level does not give any indication of the activity of the corresponding microbe. Using live–dead staining, Ben-Amor (2004) discovered that bacteria related to known butyrate-producing bacteria were more dominantly present in the active GI tract populations in humans, whereas bacteria affiliated to *Bacteroides*, *Ruminococccus* and *Eubacterium* were more numerically present in the dead fractions. Therefore, we should remember not only the power of 16S rRNA gene sequence-based technology, but also its weaknesses.

It has been observed that the predominant bacterial composition in faeces is relatively stable over time in healthy adult individuals (Franks *et al.*, 1998; Zoetendal *et al.*, 1998; Simpson *et al.*, 2000; Van der Wielen *et al.*, 2002; Konstantinov *et al.*, 2003). From cultivation data, it is already known that community shifts occur with ageing, especially in newborn babies and elderly people (Mitsuoka, 1992); some culture-independent studies have confirmed these findings (Harmsen *et al.*, 2000; Hopkins *et al.*, 2001; Favier *et al.*, 2002, 2003; Schwiertz *et al.*, 2003). However, it is remarkable that unstable faecal communities can often be correlated with GI tract disorders, as has been observed in humans suffering from Crohn's disease (Seksik *et al.*, 2003) and pigs experimentally infected with *Brachyspira hyodysenteriae*, the swine

Table 1.2 Overview of some of the major findings in GI tract ecology obtained by culture-independent approaches

Features of predominant GI tract bacterial community
Composed of numerous uncultured bacteria
Host specific
Affected by host genotype
Stable in time in healthy individuals
Unstable in time in individuals suffering from GI tract disorders
Affected by antibiotics
Affected by certain diets
Not significantly altered after consumption of pre- and probiotics
Changing during ageing of host
Different between niches within single individuals (faeces, GI tract location, lumen, mucosa)

dysentery-causing bacterium (Leser *et al.*, 2000). Similarly, an inverse relationship was found between ileal bacterial diversity in neonatal piglets and their susceptibility to colonization by the opportunistic pathogen *Clostridium perfringens* (Deplancke *et al.*, 2002). Although these studies suggest a relationship between GI tract disorders and the GI tract community structure, more studies are needed to characterize the mechanisms underlying this relationship.

One of the most reported observations from 16S rRNA gene sequence data is that the GI tract community structure differs between animal species and individual hosts of the same species (Zoetendal *et al.*, 1998; Tannock *et al.*, 2000; Simpson *et al.*, 2000; Toivanen *et al.*, 2001; Simpson *et al.*, 2002; Van der Wielen *et al.*, 2002; Konstantinov *et al.*, 2003; Seksik *et al.*, 2003; Vaahtovuo *et al.*, 2003). This sounds like a logical conclusion, but this phenomenon has been underestimated, as will be discussed in more detail in the following section. This *host specificity* phenomenon argues for a strong influence by the host genotype on bacterial diversity and community structure. Indeed, comparisons between DGGE profiles of faecal samples from human adults with differing genetic relatedness varying from unrelated persons to monozygotic twins indicated that the host genotype strongly influences the bacterial composition in the GI tract (Zoetendal *et al.*, 2001). Conversely, the impact of the environment was not so great. Similar conclusions have been drawn from mouse studies based on the bacterial fatty acid profiles (Toivanen *et al.*, 2001; Vaahtovuo *et al.*, 2003). These results are consistent with studies by Hackstein and colleagues in which they looked at methane production in the animal kingdom. These studies showed that the presence of methanogens in the GI tract of several vertebrate and invertebrate animals has a phylogenetic foundation (Hackstein and Stumm, 1994; Hackstein and van Alen, 1996). The exact nature of the host effect remains to be determined, but it is most likely to be found in specific host–microbe interactions (Hooper *et al.*, 2002). Recently, it has been observed that communication between host and microbes affects very important host functions, including fat absorption and innate immune development (Hooper *et al.*, 2003; Bäckhed *et al.*, 2004; Cash and Hooper, 2005). The importance

of the host on the bacterial community in the GI tract is a very important finding because it indicates that host-specific effects cannot simply be ignored. These marked interindividual differences in GI communities support a genetic linkage for the association of gastrointestinal infections and inflammatory bowel diseases among specific individuals, families and ethnic or other genetically related groups (Hooper and Gordon, 2001). This definitely deserves more attention in the near future.

Another factor complicating the study of the ecology of the GI tract is the fact that the community structure varies remarkably between GI tract niches. PCR-DGGE profiling of bacterial communities at several GI tract locations from the stomach to the colon were different from each other within single pigs (Simpson *et al.*, 1999). With the exception of the caecum, mucosal and luminal community structures were highly similar in all samples. Similar observations were made for *Bacteroides* and *Prevotella* species in a different study (Pryde *et al.*, 1999). PCR-DGGE profiling of the bacterial community at different GI tract regions of broiler chickens also revealed location-specific communities (Van der Wielen *et al.*, 2002), and contradictory conclusions were drawn in two studies on the differences between the bacterial community structure of mucosal and luminal samples from the caecum of chickens, which may be due to host or methodological differences (Gong *et al.*, 2002; Zhu *et al.*, 2002). For humans, differences in bacterial composition between colonic and faecal samples have been reported (Marteau *et al.*, 2001; Zoetendal *et al.*, 2002a; Nielsen *et al.*, 2003). In addition, mucosa-associated bacterial communities were found to be uniformly distributed along the colon (Zoetendal *et al.*, 2002a; Nielsen *et al.*, 2003). Although the number of comparisons are limited, and some studies are contradictory, they at least indicate that faecal samples do not necessarily reflect other parts of the GI tract, including the colon. This complicates studying the GI tract ecology.

In addition to host-specific factors, other factors such as geographical location and diet, are frequently reported to affect the GI tract community structure. Dietary effects are reported for a variety of animals, such as humans, pigs, mice, cows and chickens, especially in weaning animals or those using prebiotic-based diets (Kruse *et al.*, 1999; Harmsen *et al.*, 2000; Apajalahti *et al.*, 2001; McCracken *et al.*, 2001; Tajima *et al.*, 2001; Apajalahti *et al.*, 2002; Konstantinov *et al.*, 2003; 2004; Tannock *et al.*, 2004). The effect of geographical location on GI tract ecosystems has also been suggested and some results have been reported (Finegold *et al.*, 1983). However, it is very difficult to study the impact of geographic location as it is confounded by diet and other factors.

Lessons from culture-independent studies with respect to pro- and prebiotics

The previous section described the complexity of studying GI tract ecology. Individual- and location-specific communities in the GI tract make it a very difficult ecosystem to study and eventually to modulate towards beneficial interactions between host and microbes. It is therefore important to have host-specific factors minimized when studying the impact of pre- and probiotics. Some clear effects of probiotic and prebiotic trials have been observed, but in most cases the impact of prebiotics and probiotics on GI tract communities has not been found to be significant. For example, it was observed in individual dogs that bacterial

composition was stable over time, but that differences between dogs were physiologically (breed, size, age) related (Simpson *et al.*, 2002). The dog's diet was found to have less effect on the bacterial composition. Similarly, with the exception of the study by Konstantinov and colleagues (2004), the impact of pre- or probiotics on the GI tract community structure was found to be minimal (Simpson *et al.*, 2000; Tannock *et al.*, 2000, 2004). The main reason for this probably lies in host-specific differences in microbial diversity. Impey and colleagues (1984) have warned that translations of data from a 'model' animal system to human or another animal system should be carried out with caution because these authors observed that a competitive exclusion mixture, which was effective in chickens, did not show the same efficacy in turkeys. More importantly, many studies use random groups of individuals to study the impact of pre- and prebiotics, and therefore it is likely that host-specific differences outweigh the impact of pre- and probiotics. This does not mean that pre- and probiotics do not have an impact, but that their effect is less visible because of the large variation between individuals. Therefore, nutrition and feeding trials have to be designed in such a way that host and feed effects can be distinguished from each other. We suggest that each host should serve as its own control in a trial.

As most studies do not report a significant impact on GI tract community structure, we could alter our view of the impact of pre- and probiotics on this community. Seksik and colleagues (2003) reported that unstable communities are more frequently found in patients suffering from Crohn's disease. We may infer from this that the impact of pre- and probiotics on the community structure is not dramatic. We suggest that researchers should focus on how pre- and probiotics might improve GI tract functionality. For example, it is remarkable that there is such a wide diversity between microbial communities in different human hosts, whereas the concentrations of the various volatile fatty acids in faeces and the overall function of the GI tract community are very similar. With the availability of complete genome sequences of a variety of animal hosts and microbes, and the possibility of obtaining metagenomic libraries, it should be possible to identify biomarkers or indicators of a 'healthy GI tract'. When such indicators are known, the impact of pre- and probiotics on alterations of these indicators should be studied. This may ultimately prove or disprove the claims of pre- and probiotics made by industry. Therefore, we should shift our attention from phylogeny to functionality, also with respect to pre- and probiotics.

Concluding remarks

This chapter provides a brief overview of novel, mainly nucleic acid-based molecular techniques that are used in the study of GI tract ecology. It is clear that the application of these modern molecular techniques will enable researchers to obtain a complete description of the genetic diversity in the GI tract for the first time, which was not previously possible using conventional culture techniques. Ultimately, the combination of approaches targeting several biomarkers will enable GI tract microbial ecologists to determine the exact role or function of specific organisms in the GI tract ecosystem and its quantitative contribution to the whole process, which is the ultimate goal of the microbial ecologist. Currently, the focus of GI tract ecology is switching slowly from 16S rRNA approaches towards genomic and transcriptomic approaches, and perhaps our view of the impact of pre- and probiotics on the GI tract

community should follow this example. In the future, there will be developments towards high-throughput approaches in microbial ecology. In addition, we expect to see major developments in 'lab-on-a-chip' technology. Despite rapid developments in modern molecular techniques and all their challenges, a potential danger is that scientists may easily be tempted to perform descriptive rather than hypothesis-driven research. For example, at present there is an massive increase in the development of microarray technology and related statistical software. Biological interpretation of the available data often seems to be 'overlooked' – it should not be forgotten that microbial ecology is not a study of techniques but the study of life.

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