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# Molecular Microbial Ecology of the Gastrointestinal Tract: From Phylogeny to Function

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## Abstract

During the past decade it became evident that anaerobic cultivation-based approaches provides an incomplete picture of the microbial diversity in the GI tract, since at present only a minority of microbes can be obtained in culture. The application of molecular, mainly 16S ribosomal RNA (rRNA)-based approaches enables researchers to bypass the cultivation step and has proven its usefulness in studying the microbial composition in a variety of ecosystems, including the gastrointestinal (GI) tract. This critical review summarizes the impact of these culture-independent approaches on our knowledge of the ecology of the GI tract and provides directions for future studies which should emphasize function of specific strains, species and groups of microbes.

## Introduction

The prokaryotic life form is most abundant on earth (Whitman *et al.*, 1998) and as a consequence we are adapted to life in a world surrounded by microbes. The complexity of interactions between animals and microbes varies tremendously and includes pathogenic, competitive, and symbiotic interactions. All these interactions can be found in the animal body, especially the gastrointestinal (GI) tract. The animal GI tract is a specialized tube divided into various well-defined anatomical regions extending from the lips to the anus which includes the stomach (rumen-reticulum, crop, gizzard), small intestine (duodenum, jejunum, ileum), and large intestine (cecum and colon). The main function of the GI tract is the conversion of food into components which can be digested and absorbed by the host. On the other hand, the GI tract is also an ecosystem in which host cells and microbes interact. Only one thin layer of epithelial cells interacts with GI tract microbes, which collectively outnumber the total number of animal cells by a factor of 10 (Savage, 1977). This complex microbial community in the GI tract consists of different groups of microbes, such as bacteria, archaea, ciliate and flagellate protozoa, anaerobic phycomycete fungi, and bacteriophage. Bacteria are the most widely studied group of microbes. These microbes are provided with undigested dietary components, and host-derived compounds such as mucopolysaccharides, mucins, epithelial cells, and enzymes. Because of this, the GI tract can be considered the most metabolically adaptable and rapidly renewable organ of the body, which

plays a vital role in the normal nutritional, physiological, immunological and protective functions of the host animal. The concept of symbiosis has been used most commonly to describe the host-microbe interactions, but based on the costly investments of the host in a defense system to keep microbes away from the epithelial surface the concept of détente has been proposed to describe these interactions more accurately (Gaskins, 2001).

Since the isolation of "*Bacterium coli commune*" in 1885 (Escherich, 1885) many attempts have been made to isolate bacteria from the GI tract and study their function. Although Pasteur (1860) first described microbes that live without air, a major breakthrough came after the introduction of the roll tube technique by Hungate (1950) and the development of the anaerobic glove box (Aranki *et al.*, 1969). These anaerobic cultivation techniques have lead to the isolation and characterization of many novel anaerobic GI tract bacteria, notably from humans and ruminants (Hungate, 1950; Moore and Holdeman, 1974; Finegold *et al.*, 1983) and are still being used as standard tools in the isolation and cultivation of anaerobic microbes in many research areas including clinical microbiology. Moreover, novel bacteria are still being isolated from the GI tract, including butyrate-producing and cellobiose-degrading bacteria (Barcenilla *et al.*, 2000; Pryde *et al.*, 2002; Zoetendal *et al.*, 2003).

Despite the major cultivation attempts of the past decades, it is generally accepted that only a minority of the GI tract microbes have been isolated in pure culture yet (Vaughan *et al.*, 2000). The majority of microbes have only been detected using molecular detection methods and the recognition of our limitations in isolating bacteria resulted in the development of a novel research area called "Molecular Microbial Ecology". The difference between this novel research area and classical microbial ecology is that microbial ecosystems are now being studied as a whole without the use of conventional cultivation procedures. Since this research area is relatively young, molecular microbial ecological studies have mainly been focused on the detection and identification of uncultured bacteria. However, it is already evident that the use of molecular microbial ecology as well as 'omics' technologies, such as genomics, proteomics and metabolomics, will generate the next major advance in our knowledge. For the first time it will be possible to gather, not simply a refinement or increased understanding but, a complete description of the gastrointestinal ecosystem. The current review will provide a critical summary of the data gathered during the past years using molecular techniques for studying GI tract ecosystems and will discuss future directions.

## From Cultures to Molecules

Microbial ecology has developed as a specialized research field in microbiology and focuses on studying the role of microbes in a variety of ecosystems, including the GI

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tract. It is worthwhile reiterating that microbial ecology in GI tract ecosystems involves the investigation of the microbes present, their *in vivo* activity, and their relationship with each other and the host animal (Hungate, 1960). This indicates that studying the microbial ecology of the GI tract is very complex because of all the interactions between animal host cells, microbes, and diet. It could be argued that the technological impetus for major advances in our knowledge of gastrointestinal ecology during the last decades has been derived from three major sources: the development of anaerobic culture techniques; the use of rodent experimental 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).

A key part in studying the microbial ecology of the GI tract is knowledge of "who are present" in the GI tract and "what is their function"? Although these questions sound very logical, identification of the key players is already a complicated task. For many ecosystems it is estimated that only a few percent of bacteria can currently be obtained in culture (Amann *et al.*, 1995). Compared to other ecosystems, the estimate of culturability of GI tract bacteria is relatively high (10-50%). It should be noted that this estimate is based on numbers and not diversity. Nevertheless, the estimated culturable fraction from the GI tract is still a minority. The difference between total viable counts and total microscopic counts was previously thought to be due to the number of dead cells. Indeed, one third of the total bacterial community detected in feces may be derived from dead cells (Apajalahti *et al.*, 2003). However, nucleic acid based studies have indicated that a majority of bacteria in a variety of ecosystems are different from those described in culture. Therefore, it is reasonable to assume that the differences between microscopic and total viable counts are due to an inability to culture the majority of the bacteria. Reasons for this cultivation anomaly include selectivity of the media that are used, unknown growth requirements of the bacteria, stress caused by the cultivation procedures, necessity of strictly anoxic conditions, and interactions of the bacteria with other microbes and host cells. The circumvention of these limitations in studying the GI tract requires the application of culture-independent approaches. After the discovery that ribosomal RNA is present in every cell and that its nucleotide sequence can be used for phylogenetic classification (Woese 1987, Woese *et al.*, 1990), a dramatic increase in the application of approaches based on the sequence diversity of 16S ribosomal RNA (rRNA) and its encoding gene have been made during the past decade to explore the diversity of bacterial communities in a variety of ecosystems, including the mammalian GI tract (Vaughan *et al.*, 2000). The major development in these technologies and their contribution have lead to novel insights into our knowledge about the GI tract ecology as outlined below.

#### **Application of Culture-Independent Characterization of GI Tract Communities**

The application of culture-independent, mainly 16S rRNA-based approaches has given us novel insights into the ecology of GI tract. In addition, sequencing of 16S rRNA genes has become a standard procedure in

the identification of isolates and it is now impossible to adequately describe microbial communities without 16S rRNA sequence data. Currently, more than 97,000 16S rRNA sequences are available in the DNA databases, which is far more than for any other gene. Although the use of 16S rRNA sequence data has been strongly advocated by Pace (1997) this technique has not been as widely applied to the GI tract when compared to other ecosystems. However, several different strategies including the application of 16S rRNA sequence data to study GI tract ecosystems have been used successfully as described below.

#### *Sequencing of 16S rRNA Gene Clone Libraries*

Most phylogenetic information from the GI tract has been gathered by sequencing of cloned 16S rRNA gene amplicons. Ribosomal RNA sequences can be obtained either directly by reverse transcriptase (RT-) or regular PCR from rRNA or from the encoding genes located at various positions in the genome, respectively. For practical reasons sequences of 16S rRNA genes are determined by creating rRNA gene clone libraries rather than cDNA libraries from rRNA. After creating the clone library, the sequences of the cloned amplicons are determined and compared to the available sequences in the DNA databases (<http://rdp.cme.msu.edu/html>), followed by phylogenetic analysis (Altschul *et al.*, 1990; Cole *et al.*, 2003; Ludwig *et al.*, 2004).

Sequencing of 16S rRNA gene clone libraries from the human feces (Wilson and Blichington 1996; Zoetendal *et al.*, 1998; Suau *et al.*, 1999), colonic samples (Hold *et al.*, 2002; Wang *et al.*, 2003), ileal samples (Wang *et al.*, 2003) and oral cavity (Kroes *et al.*, 1999, Paster *et al.*, 2001) have indicated that a significant fraction of the bacteria have not been described previously. Similar results have been reported for several GI tract locations in 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 grouped in the low G+C Gram positive phylum, indicating that this group is particularly underrepresented by cultivation procedures.

Despite the wealth of information gathered from 16S rRNA sequences, there is unfortunately a lack of consistency between the molecular procedures (i.e. nucleic acid isolation, PCR), which makes comparisons between data from different studies difficult. Moreover, it is known that PCR and cloning steps are not without biases (von Wintzingerode *et al.*, 1997; Leser *et al.*, 2002a). Comparative analysis of 16S rRNA gene clone libraries from several GI tract studies indicated that the number of cycles during PCR should be minimized, based on the estimated diversity represented by the libraries (Wilson and Blichington 1996; Whitford *et al.*, 1998; Bonnet *et al.*, 2002) and several steps to minimize PCR bias have been suggested (von Wintzingerode *et al.*, 1997; Polz and Cavanaugh 1998).

Unfortunately, for practical reasons replicates are often absent when comparing clone libraries of amplicons generated after different PCR cycles. Leser *et al.* (2002a) reported that cloning is also not without bias, which indicates that comparisons between two libraries only

are not sufficient to determine the bias caused by PCR step. In addition, there is also no consistency in the OTU determination (with thresholds for sequence differences within OTUs varying from 1 to 5%), while this determination is crucial to estimate how much of the actual diversity is represented by the clone libraries (Martin, 2002). This inconsistency makes quantification of diversity and statistical comparisons between clone libraries impossible. Despite the above-mentioned limitations, it is evident that cloning and sequencing of 16S rRNA gene gives a more accurate picture of the bacterial composition in the GI tract than does culturing. Therefore, the generation and analysis of clone libraries from more animals and different GI tract locations and the subsequent deposition of novel sequences in the DNA databases remains very important. Each novel sequence gives an indication of what types of bacteria we are currently overlooking and can be taken into account in subsequent studies.

#### *16S rRNA Gene Fingerprinting*

Cloning and sequencing of 16S rRNA gene sequences is suitable for analysis of bacterial communities in a culture-independent way, but for monitoring communities this approach is unsuitable because the analysis is very laborious and expensive. Fingerprinting of the 16S rRNA gene has been successfully performed to monitor community shifts and compare different communities. Denaturing Gradient Gel Electrophoresis (DGGE) was first applied in microbial ecology to study the bacterial diversity in a marine ecosystem (Muyzer *et al.*, 1993) and since this pioneering study, a variety of microbial ecosystems have been analyzed using DGGE or similar techniques, such as Temperature Gradient Gel Electrophoresis (TGGE) and Temporal Temperature Gradient Gel Electrophoresis (TTGE). Other fingerprinting techniques used in the analysis of microbial communities are single strand conformation polymorphism (SSCP) and Terminal-Restriction Fragment Length Polymorphism (T-RFLP). These fingerprinting techniques are all PCR based and result in profiles representing the sequence diversity within ecosystems, but the principles and technical procedures are different. DGGE, TGGE, and TTGE are based on sequence specific melting behavior of amplicons, SSCP on the secondary structure of single stranded DNA and T-RFLP on specific target sites for restriction enzymes. For more detailed descriptions of these fingerprinting techniques we refer to previous review papers (Muyzer and Smalla 1998; Vaughan *et al.*, 2000; Konstantinov *et al.*, 2002).

Interestingly, with the exception of T-RFLP, all other techniques have been used successfully in mutation detection in clinical research before they were applied to microbial ecology, which demonstrates their discriminative power. With ongoing improvements of fingerprinting analyzing software similarity indices can be calculated and clustering analysis of profiles can be performed. This makes the use of fingerprinting in analyzing and monitoring microbial communities very reliable. Since PCR is involved in fingerprinting analysis of the 16S rRNA gene, it is not accurate to draw quantitative conclusions. However, the possibility of absolute quantification of targets resulting in single amplicons in TGGE profiles was demonstrated using a combination of competitive RT-PCR and TGGE

analysis (Felske *et al.*, 1998). Similar observations were made when constant-denaturant capillary electrophoresis (CDCE) and quantitative PCR were combined (Lim *et al.*, 2001). Remarkably, these are to our knowledge the only quantitative studies so far.

DGGE, TGGE, and TTGE analysis of 16S rRNA genes have been the most widely applied fingerprinting techniques in microbial ecology and have been successfully used to characterize and monitor predominant GI tract bacterial communities in human (Zoetendal *et al.*, 1998, 2001a, 2002a; Tannock, *et al.*, 2000; Seksik *et al.*, 2003), pig (Simpson *et al.*, 1999, 2000; Konstantinov *et al.*, 2003), cattle (Kocherginskaya *et al.*, 2001), dog (Simpson *et al.*, 2002), rodent (Deplancke *et al.*, 2000; McCracken *et al.*, 2001) and chicken (Van der Wielen *et al.*, 2002; Zhu *et al.*, 2002). Also T-RFLP, although less frequently used, has proved to be a useful fingerprinting technique to monitor GI tract communities (Leser *et al.*, 2000; Kaplan *et al.*, 2001; Nagashima *et al.*, 2003). These studies have already resulted in substantial knowledge concerning factors that affect the community structure such as environmental perturbations, physiological conditions, GI tract location, and the genetic background of the host as will be discussed later.

It has been reported that DGGE or TGGE are sensitive enough to represent bacteria that constitute up to 1% of the total bacterial community (Muyzer *et al.*, 1993; Zoetendal *et al.*, 1998). This means that only the most dominant bacteria will be represented in the profiles when domain-specific primers are used. On the other hand, *Bifidobacterium* and *Lactobacillus* group-specific PCR-DGGE approaches have also been developed to specifically amplify monitor these groups (Simpson *et al.*, 2000; Satokari *et al.*, 2001a,b; Walter *et al.*, 2001; Heilig *et al.*, 2002; Temmerman *et al.*, 2003). It was demonstrated that probiotics could be detected and monitored in feces using group-specific PCR-DGGE, which appeared to be difficult or impossible using general bacteria-specific primers (Simpson *et al.*, 2000; Satokari *et al.*, 2001b; Walter *et al.*, 2001; Heilig *et al.*, 2002). These group-specific PCR-DGGE approaches will definitely be very helpful when implemented as a routine in large probiotic trials.

#### *Quantification of 16S rRNA and its Encoding Genes*

The disadvantage of the PCR-based approaches described above is that they do not provide quantitative data because of amplification biases. However, several approaches have been developed to quantify 16S rRNA and its encoding gene. Dot blot hybridization has been developed to quantify the amount of specific 16S rRNA in a mixture relative to the total amount of rRNA. In brief, total RNA is isolated from the samples of interest, cross-linked to a filter using a dot or slot blot manifold device and hybridized with labeled oligonucleotide probes. The relative amount of rRNA is estimated by dividing the amount of specific probe by the amount of labeled universal probe hybridized, after normalization of the signals with rRNA from control strains. This approach has been widely used to quantify rRNA from samples including those from the human GI tract, rumen, and horse (Stahl *et al.*, 1988; Sghir *et al.*, 2000; Marteau *et al.*, 2001; Daly and Shirazi-Beechey 2003; Seksik *et al.*, 2003). Since no amplification procedures

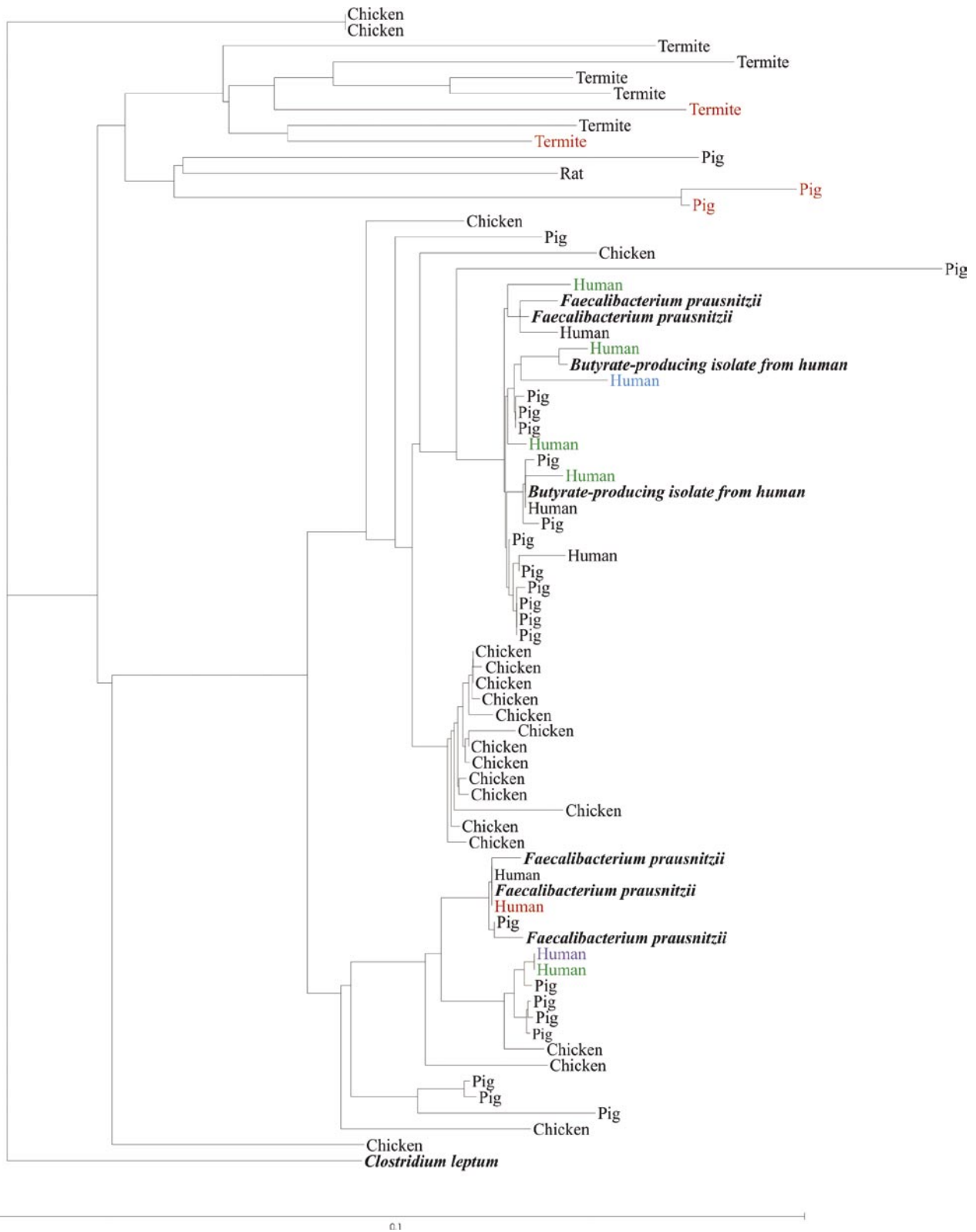


Figure 1. Phylogenetic representation of the *Faecalibacterium* group. The tree was prepared using PHYLIP with nearly complete 16S rRNA gene sequences which were aligned in RDP II database (Cole *et al* 2003). Isolates are indicated in bold typeface, the sequences from uncultured bacteria are encoded as the host animal species they were derived from. Sequences of each host animal from different studies are distinguished by their color. *Clostridium leptum* was used as outgroup to root the tree. The scale bar represents the sequence divergence.

are involved the quantification is more accurate than PCR-based approaches. During the past decades many oligonucleotide probes have been developed, validated and successfully used, and recently many of those probes have been published in a database called ProbeBase ([www.probebase.net](http://www.probebase.net); Loy *et al.*, 2003).

As indicated before, PCR is the most sensitive technique to detect sequences that are present in very low concentrations in the environment, but provides no quantitative information, because many factors can cause amplification biases (von Wintzingerode *et al.*, 1997). However, several approaches have been developed to determine quantitatively 16S rRNA genes or 16S rRNA concentrations using PCR. Competitive (RT-)PCR is one way to quantify the target and this has initially been used to quantify messenger RNA (mRNA) from human cells (Wang *et al.*, 1989). With competitive PCR, a specific standard distinguishable from the target is added in different concentrations to the target followed by PCR amplification and this allows discrimination and subsequent quantification on agarose gel. Using competitive PCR, the 16S rRNA genes of a variety of bacterial species could be quantified in rumen samples (Reilly and Attwood 1998; Koike and Kobayashi 2001; Reilly *et al.*, 2002). As described above, this quantification principle has also been used to quantify targets corresponding to single amplicons in community fingerprints (Felske *et al.*, 1998; Lim *et al.*, 2001). The benefit of these approaches is that the target and standard have similar sizes and can be discriminated by their sequence.

Most probable number (MPN) PCR is another way to quantify bacterial 16S rRNA gene in environmental samples and has successfully been used to analyze fecal samples (Wang *et al.*, 1996). The principle is similar to MPN-counting of bacteria. Target DNA is diluted until extinction and used as template for PCR using species or group specific primers. The method is quite fast and easy to quantify major groups of bacteria, but is less attractive for analysis of complex communities at the species level. This approach can also be used to determine cell lysis efficiencies of DNA isolation protocols by diluting bacterial cells followed by DNA extraction instead of diluting target DNA (Zoetendal *et al.*, 2001b).

A relatively new quantitative PCR method is the real-time PCR approach which has successfully been applied in characterizing samples from rumen and GI tract of humans and pigs (Tajima *et al.*, 2001; Huijsdens *et al.*, 2002; Collier *et al.*, 2003; Malinen *et al.*, 2003; Matsuki *et al.*, 2004). Although real-time PCR has still to prove its suitability in analyzing complex bacterial communities, this approach looks very promising, since bacterial targets in very low concentration can be quantified, which is difficult using other approaches.

An important reminder is that studies describing quantitative PCR or dot blot hybridization often highlight the quantitative power of the approaches. However, it has to be kept in mind that the quantification is only relative as has been reported recently (Rigottier-Gois *et al.*, 2003a). The amount of 16S rRNA or ribosomes per cell depends on the bacterial species and also varies with growth phase and activity of the cell. In addition, bacterial species differ in genome sizes and numbers of 16S rRNA gene copies

per genome. This makes extrapolation of the data to cell numbers inaccurate.

#### *Fluorescent In Situ Hybridization*

One frequently applied culture-independent approach to quantify bacterial cells in environmental samples is fluorescent *in situ* hybridization (FISH) using 16S rRNA-targeted oligonucleotide probes (Amann *et al.*, 1995). FISH combines the power of 16S rRNA probe hybridization with epifluorescent light microscopy, Confocal Laser Microscopy, or flow cytometry for direct quantification of individual cells. FISH has increasingly been used to study the bacterial composition in the GI tract and several probes have already been developed and validated to quantify bacteria belonging to various genera. These include *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Lactobacillus*, *Collinsella*, *Eubacterium*, *Fusobacterium*, *Clostridium*, *Veillonella*, *Fibrobacter*, and *Ruminococcus* (Amann *et al.*, 1990; Schwartz *et al.*, 2000; Harmsen *et al.*, 2002; Harmsen and Welling 2002; Zoetendal *et al.*, 2002b, Rigottier-Gois *et al.*, 2003b). It is remarkably that with the exception of the murine cecum (Amann *et al.*, 1990), FISH has mainly been used to quantify major groups of bacteria in human feces. To facilitate enumeration, FISH has been automated and combined with image analysis that is analyzed by computer software programs (Jansen *et al.*, 1999). Compared to other 16S rRNA approaches, enumeration of bacteria is best addressed using this approach. The lowest level of detection is  $10^6$  cells per gram of feces at present. FISH enables microbial ecologists to address five ecological themes simultaneously, I) to bypass cultivation problems, II) to obtain information on community structure by using varying sets of probes, III) to accurately enumerate cell populations, IV) to identify sub-populations in natural ecosystems and to locate their niche, and V) to determine *in situ* cellular rRNA content (Vaughan *et al.*, 2000). Most counts have been performed using microscopy, but recently flow cytometry has also demonstrated as a powerful high-throughput method to count fecal bacteria (Zoetendal *et al.*, 2002b; Rigottier-Gois *et al.*, 2003a,b). Statistical analysis indicated that counts retrieved by microscopy and flow cytometry were similar (Zoetendal *et al.*, 2002b). The combination of FISH and flow cytometry is a very promising approach since it is possible to sort uncultured bacteria (Wallner *et al.*, 1997). Although these sorted bacteria are not viable, they can be used for molecular genetic studies. The major disadvantage of FISH is that it is dependent on the available 16S rRNA gene sequences in the databases and that only a few probes can be used per analysis. Furthermore, it is dependent on the permeability of the bacterial cell, the number of ribosomes per cell and the accessibility of the target.

#### *Diversity Microarrays*

A new and popular way to detect bacteria in environmental samples is the use of DNA microarrays (also called biochips, gene chips or DNA chips). DNA microarrays are basically glass surfaces spotted with numerous covalently linked DNA fragments (probes) that are available for hybridization. Current applications of DNA microarrays include monitoring gene expression (transcriptional profiling) or detecting DNA sequence polymorphisms or mutations in genomic DNA.

Since the number of genome sequences from a variety of organisms is rapidly increasing, DNA microarray analysis becomes the technique of the coming decade in molecular biology (Rick *et al.*, 2001). The potential of DNA microarray technology in microbial ecology was first demonstrated using microarrays containing oligonucleotides complementary to 16S rRNA sequences of nitrifying bacteria. These bacteria could be detected and identified in environmental samples on the basis of their DNA or RNA hybridizing the probes on the DNA microarray (Guschin *et al.*, 1997). Thereafter, DNA microarray technology is being optimized to study bacterial diversity in a variety of ecosystems (Small *et al.*, 2001; Loy *et al.*, 2002; El Fantroussi *et al.*, 2003; Peplies *et al.*, 2003). Not only the 16S rRNA gene is used as target for developing diversity microarrays, but also other genes, such as those involved in antibiotic resistance are used as targets (Call *et al.*, 2003; Volokhof *et al.*, 2003).

Besides the expense of the technology, the two of the main problems regarding DNA microarray analysis are the hybridization specificity and quantification of the signals. El Fantroussi *et al.* (2003) demonstrated that specific and non-specific hybridization can be discriminated by determining the thermal dissociation curve for each probe-target duplex. Another approach to minimize detection of false positives was performed by applying multiple probes for specific targets on the DNA microarray. Quantification of hybridization signals seems to be a complicated task at present since it has been shown that the signal intensities may vary significantly between targets even those perfectly matching the probes (Loy *et al.*, 2002). The first attempts to generate DNA microarrays for application to GI tract ecosystems have been performed and look promising (Leser *et al.*, 2002b; Wang *et al.*, 2002; Wilson *et al.*, 2002). It is already evident that the application of DNA microarray technology in studying the ecology of the GI tract will be expanded and extended in the near future.

#### *Non-16S rRNA-Based Profiling*

Most culture-independent techniques used to describe bacterial communities have focused on the sequence diversity of 16S rRNA. However, other approaches to analyze and monitor bacterial communities have also been used successfully, such as profiling of bacterial cellular fatty acids (Toivanen *et al.*, 2001; Vaahtovuori *et al.*, 2003) and determining G+C content of the total community (Apajalahti *et al.*, 1998, 2001, 2002). The advantage of these types of profiling is that they are direct without any amplification step. However, the identification of bacteria causing the shifts in the profiles is difficult, because the data lacks phylogenetic information. Therefore, 16S rRNA approaches are needed for the validation of these alternative methods. In addition, these methodologies have not been widely applied at the moment, which may restrict researchers to use them.

#### **Major Findings on GI Tract Communities from Culture-Independent Data**

Despite the fact that culture-independent approaches have been used less frequently in studying the ecology of the GI tract compared to other ecosystems, their application has already provided novel insights. In fact, it is generally

accepted that it is now impossible to adequately describe microbial communities without 16S rRNA sequence data. Currently, more than 97,000 16S rRNA sequences are available in the DNA databases, which is far more than for any other gene. As outlined below, the application of the 16S rRNA approach in GI tract ecosystems has answered some questions which could not have been answered by culture-dependent approaches.

One of the main conclusions which can be drawn from these data is that the majority of bacteria in the GI tract have not been obtained in culture yet. This leaves their description and possible role in the GI tract still unknown. Interestingly, a major part of the "to-be-cultured-bacteria" cluster within the low G+C bacterial phylum, especially in the different *Clostridium* clusters described by Collins *et al.* (1994). This indicates that the development of new cultivation procedures should focus on these groups. Another clear observation from 16S rRNA gene sequence data is that the GI tract community structure differs between animal species. Although this conclusion sounds very logical, it indicates that microbial ecologists should be very careful when making generalizations. Translations of data from a "model" animal system to the human or other animal system should therefore be performed with caution as discussed by Impey *et al.* (1984). They observed that a competitive exclusion mixture, which was effective in chickens did not show the same efficacy in turkeys. This observation demonstrates the limitation of making predictions for probiotics when they are studied in animal models.

The number of 16S rRNA gene sequences directly retrieved from several GI tract locations in a variety of animals is already quite large and still growing. We wondered if any host species-specific clusters could be defined within a selected phylogenetic group using this existing database. The *Faecalibacterium* group is of particular interest since only a few isolates have been described while a large number of sequences from uncultured GI tract bacteria of several animal species cluster within this group (Duncan *et al.*, 2002). Using RDP II (Cole *et al.*, 2003), we constructed a phylogenetic tree of this group (Fig 1). Unfortunately and rather unexpectedly, the phylogenetic analysis raises more confusion than clarity which is mainly due to the inconsistency of sequence deposition. A majority of sequences found within the *Faecalibacterium* group were partial and frequently without overlap with each other and therefore could not be used for the tree construction. Moreover, the inconsistency between laboratories concerning OTU definition and subsequently the random number of deposited representative sequences from each OTU into the DNA databases resulted in a biased tree. For example, the sequences from the human GI tract were single representatives of each OTU found in different studies, while the sequences from the chicken were from the same study and represented multiple representatives of different OTUs (Fig 1). We used the *Faecalibacterium* group as example, but we predict that the result of these inconsistencies is even worse in other groups with larger numbers of sequences. These inconsistencies seriously limit researchers to perform detailed phylogenetic analysis. Therefore, we argue that clear and uniform guidelines must be defined in order for sequence submissions to have

ecological relevance and comparative value, as well as to provide an unbiased representation of diversity. These guidelines should include 1) a precise description of the ecosystem/samples from which the sequences are derived, 2) a uniform OTU definition, 3) submission of full length sequences only, and 4) submission of only the two most divergent sequences within each OTU.

The first culture-independent studies based on FISH and TGGE profiling of 16S rRNA demonstrated that the predominant bacterial composition in human feces remains relatively stable over time (Franks *et al.*, 1998; Zoetendal *et al.*, 1998). Whether this compositional stability also indicates a functional stability remains to be investigated, since it has been observed in methanogenic reactor fed with glucose that functional and compositional stability do not necessarily correlate (Fernández *et al.*, 1999). In humans structural stability has only been observed in fecal bacterial populations of healthy adults. From cultivation data, it is already known that community shifts occur with aging and that these changes happen 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; Schwartz *et al.*, 2003). For other animals such data is limited. Feeding trials with weaning piglets indicated a clear succession of their GI tract communities in time (Simpson *et al.*, 2000; Konstantinov *et al.*, 2003). Similar observations were made for broiler chickens (Van der Wielen *et al.*, 2002). In addition, it was observed in individual dogs that the bacterial composition was stable in time, but that differences between dogs were related to physiology (breed, size, and age) (Simpson *et al.*, 2002). Remarkably, unstable fecal communities can often be correlated with GI tract disorders. This was observed with humans suffering from Crohn's disease (Seksik *et al.*, 2003) and pigs experimentally infected with the swine dysentery causing bacterium *Brachyspira hyodysenteriae* (Leser *et al.*, 2000). Moreover, a recent study on the ileal bacterial community in neonatal piglets revealed an inverse relationship between bacterial diversity and susceptibility to colonization by the opportunistic pathogen *Clostridium perfringens* (Deplancke *et al.*, 2002). Although these studies demonstrate a relationship between GI tract disorders and the GI tract community structure, more studies are needed to characterize the mechanisms underlying this relationship.

The first comparison of TGGE profiles from fecal samples of human adults has demonstrated that the composition of the predominant bacterial community is host-specific (Zoetendal *et al.*, 1998). This host-specificity has been observed in other human fecal samples (Tannock *et al.*, 2000; Seksik *et al.*, 2003), as well as in other animals, including pigs, dogs, chicken, and mice (Simpson *et al.*, 2000, 2002; Toivanen *et al.*, 2001; Van der Wielen *et al.*, 2002; Konstantinov *et al.*, 2003; Vaahtovuori *et al.*, 2003). These studies clearly indicate that host-specificity of bacterial communities in the GI tract is a general phenomenon and not restricted to one animal species. This phenomenon argues for a strong influence of the host genotype on the bacterial diversity and community structure. The first studies addressing this hypothesis showed that the presence of methanogens in

the GI tract of several vertebrate and invertebrate animals has a phylogenetic foundation indicating indeed a strong host genotype influence (Hackstein and Stumm 1994; Hackstein and van Alen 1996). Moreover, comparisons between DGGE profiles of fecal 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.*, 2001a). The similarity between DGGE profiles of monozygotic twins was significantly higher than that for unrelated individuals, while those of marital partners were not, indicating that the genetic background had more prominent influence on the predominant GI tract bacteria than did a shared environment. Similar conclusions have been drawn from mice studies based on the bacterial fatty acid profiles (Toivanen *et al.*, 2001; Vaahtovuori *et al.*, 2003). 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). 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 neglected. These marked interpersonal differences in GI bacterial populations support a genetic linkage for the association of gastrointestinal infections and inflammatory bowel diseases (ulcerative colitis and Crohn's disease) with specific individuals, families or ethnic and other genetically related groups (Hooper and Gordon 2001). This means that the design of nutrition and feeding trials have to be performed in such a way that host- and feed effects can be distinguished from each other. Otherwise, in random trials a potential diet or other non-host related effect might become invisible or underrepresented due to differences between host genotypes.

A factor which complicates studying the ecology of the GI tract is the fact that the community structure varies between GI tract regions, including those within the same organ. The first molecular data showing these differences were obtained by Simpson *et al.*, (1999). PCR-DGGE profiling of bacterial communities at several GI tract sites from stomach to colon were different from each other within single pigs. On the other hand, mucosal and luminal community structures were highly similar in all samples with the exception of the cecum. Similar observations were made for *Bacteroides* and *Prevotella* species (Pryde *et al.*, 1999). Moreover, 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). Contradictory conclusions were made in two studies on differences between the bacterial community structure of mucosal and luminal samples from the cecum of chickens, which may be due to host or methodological differences (Gong *et al.*, 2002; Zhu *et al.*, 2002). No differences between mucosa and lumen were found in the equine colon (Daly *et al.*, 2001). For humans differences in bacterial composition between colonic and fecal samples were reported (Marteau *et al.*, 2001; Zoetendal *et al.*, 2002a, Nielsen *et al.*, 2003). In addition, it was found that the predominant mucosa-associated bacterial community was uniformly distributed along the colon (Zoetendal *et al.*, 2002a, Nielsen *et al.*, 2003). Although the number of comparative analyses at different locations in the animal GI tract is limited and the

conclusions sometimes contradict, they all indicate that fecal samples do not necessarily reflect other parts of the GI tract, including the colon.

In addition to the host-specific factors, other factors, such as geographical location and diets are frequently reported to affect the GI tract community structure. Diet effects are reported for a variety of animals, such as humans, pigs, mice, cows, and chickens (Kruse *et al.*, 1999; Harmsen *et al.*, 2000; Apajalahti *et al.*, 2001, 2002; McCracken *et al.*, 2001; Tajima *et al.*, 2001; Konstantinov *et al.*, 2003). Interestingly, most effects were observed in studies with weaning animals or using prebiotic-based diets. The effect of geographical location on GI tract ecosystems is also suggested, but difficult to test, since it can hardly be separated from other factors, such as diet (Finegold *et al.*, 1983).

The term “normal microbiota” which is being used by many GI tract researchers refers to microbes which inhabit the GI tract in the healthy host animal. However, the “normal microbiota” is often linked to a composition of bacteria which are “expected to be present”. Based on 16S rRNA data described above, it is already evident that it is hard, if not impossible, to define this term by determining which bacteria belong to the “normal microbiota”. The community shifts in very young and aging animals, the difference in composition is evident between hosts even within the same host species, and the variation between GI tract locations as well as mucosa and lumen is clear, which makes this fine tuning impossible. Therefore, we reiterate that a “normal microbiota” should only refer to bacteria associated with the healthy animal host in general, not to an inventory of names of microbes.

### From Structure to Function

The first important step in studying ecosystems is to identify who are present. Until now, most molecular ecological studies have focused on characterizing the community structure or identifying the bacteria in the GI tract of animals and this screening is still ongoing. However, it has to be realized that data about the community structure is not very informative in an ecological sense since the most important ecological parameter is how the different organisms are operating and interacting with each other to establish and maintain a well-functioning ecosystem. In simple words, it is very important to know what each organism is doing. Given the fact that only a limited fraction of GI tract microbes can yet be cultured it is clear that determining the function of all community members is a challenging but very difficult task. Moreover, identifying the function of one type of microbe in a background of interactions within a complex ecosystem seems very intractable. Despite the difficulties, some technological progress has been made in analyzing the genetic potential within ecosystems and *in situ* activity of bacteria (Table 1). These developments have great promise for future research.

### What is the Genetic Potential within a Community?

Given the complexity of the community structure in the GI tract, the number of uncultured microbes, and the number of genes present in each member, it is evident that determining the genes which could have an important role in the GI tract is a very difficult task, especially for the

uncultured microbes. On the other hand, it is clear that the main focus of future studies will concern these challenges. The explosive increase in the number of fully sequenced genomes of different organisms, including those having a role in the GI tract and the development in DNA microarray technology during the past years have given researchers new tools. High throughput genome sequencing and DNA microarray technologies make it possible to obtain a complete insight into the genetic potential of organisms and total gene expression of organisms when exposed to a specific condition.

Despite the value of complete genome sequencing and subsequent comparative genomics, it is still very expensive and therefore prohibitive for many investigators. In addition, genome sequencing and microarray development are almost solely applied to well-studied isolates from which the complete or draft sequence of their genome has been determined. The full extent of microbial diversity can be accessed using Bacterial Artificial Chromosome (BAC) vectors to construct libraries of large genomic DNA fragments (>100 kb) from communities (termed metagenomic libraries). This approach provides direct access to large genomic fragments isolated directly from microbes in natural environments provides a method to access community DNA, which can be used to study the phylogenetic, physical and functional properties of the community genome without the necessity for cultivation. Recently, a metagenomic approach has been performed to study the viral community in human feces (Breitbart *et al.*, 2003). The authors estimated that the fecal community studied consisted of approximately 1,200 viral genotypes. BAC technology has been applied in a limited way to prokaryotic genomics (Rondon *et al.*, 1999, 2000; Beja *et al.*, 2000; Liles *et al.*, 2003). BACs can be used to clone complex loci such as biosynthetic pathways, secretion systems and pathogenicity islands, because the average insert size is usually *ca.* 100kb and because the genes for many bacterial pathways are clustered in the genome. Also because BAC inserts are large, a relatively small number of clones is required to provide complete coverage of the metagenome reducing the amount of work required to screen a BAC library. Bacterial BAC libraries can be used to detect gene expression from poorly studied, difficult to manipulate or as yet uncultivated species. Thus BAC libraries can serve to archive DNA for genomics purposes and also be used to analyze gene expression as the first step in functional genomics analysis. Since this method is not based on PCR, 16S rRNA gene genes recovered from the BAC libraries can be analyzed and compared phylogenetically with those recovered from the PCR based approach.

One limitation of the BAC cloning approach is that only 2-3% of clones contain a 16S rRNA gene (Beja *et al.*, 2000). However, the “SuperPhyloBAC” cloning vector can be used to circumvent this limitation. This modified high copy number BAC cloning vector contains an *I-CeuI* restriction site that enables cloning of large fragments from mixed microbial community DNA such that each cloned fragment contains a phylogenetically-informative end containing the entire 16S gene and its ITS region as well as *ca.* half the 23S-encoding gene.

Table 1. Overview of the current approaches to address question related to the genetic potential of ecosystems and the *in situ* activity of microbes.

Question	Approach	Target	Outcome experiment	Is cultivation required?	Is the identity of the target gene required?	Can microbes be identified directly?	Can <i>in situ</i> activity be detected?	Main Limitations
What is the physiology of the microbes?	Cultivation	Isolates	Phenotypic characterization	Yes	No	Yes	No	Not representative
	BAC vector cloning	Genomic DNA	Gene sequences	No	No	No	No	Bias in NA extraction and PCR; laborious
	RDA	Genomic DNA	Unique gene sequences	No	No	No	No	Bias in NA extraction and PCR; sensitive for false positives
	Subtractive hybridization	Genomic DNA	Unique gene sequences	No	No	No	No	Bias in NA extraction, sensitive for false positives
What is the genetic potential within a community?	Probe-based cell sorting	Genomic DNA, plasmid DNA, rRNA	Sorted cells containing certain gene sequences	No	Yes	Yes	No	Dependent on sequence data
	IVET	Promoter regions	Identification of induced promoters	Yes	Yes	Yes	Yes	Cultivation required
	RT-PCR	mRNA	Specific gene expression	No	Yes	No	Yes	Bias in NA extraction and RT-PCR
	DNA microarray	mRNA	Transcriptional fingerprints	No	Yes	No	Yes	Bias in NA extraction and NA labeling, expensive
What is the <i>in situ</i> activity of microbes?	<i>In situ</i> isotope tracking	Labeled biomarkers	Identification of substrate-utilizing microbes	Yes	No	Yes	Yes	Only suitable for simple pathways

A limitation of BAC library analysis is that the number of different genes in a community is too large to get a clear indication of the genetic potential of an ecosystem. This is due to the fact that the gene content of strains belonging to the same microbial species may already differ as much as 20% (Boucher *et al.*, 2001). However, the number of clones could be narrowed by subtracting genetic differences between microbes or ecosystems. Several methods aimed at recovery of unique genomic information discriminating one microbe from another have been developed, such as DNA microarray analysis and subtractive hybridization (SH). The latter method is of special interest as it displays the difference between two organisms rather than the similarity. SH involves hybridization of tester DNA that contains the target DNA fragments of interest with excessive driver DNA as reference followed by separation of unhybridized target from hybridized common sequences. Among subtractive hybridization techniques, representational difference analysis (RDA) and suppressive subtractive hybridization (SSH) are widely used (Lisitsyn, 1995; Diatchenko *et al.*, 1996; and Felske, 2002). Both SSH and RDA are PCR-based approaches which enrich tester specific DNA fragments without physical separation of single stranded and double-stranded DNA. In short, RDA and SSH rely on the digestion of both tester and driver DNA with restriction enzymes and subsequent ligation of the DNA fragments to adaptors containing primer target sites for PCR amplification. In RDA the complexity of both genomes is reduced by PCR amplification, removal of the adaptor sites, and addition of new adaptors to tester DNA only prior to the hybridization. On the other hand SSH has no amplification prior to hybridization, but instead different adaptors are ligated to tester and driver DNA. After tester and driver DNA preparations for RDA and SSH analysis, hybridization and specific PCR amplification steps are used to selectively amplify tester specific DNA.

SH has been successfully used to characterize differences between pathogenic and non-pathogenic strains. Genomic fragments specific for pathogenic *Escherichia coli* C1845, associated with diarrhea and urinary tract infections, food-borne pathogenic *E. coli* O157:H7, and uropathogenic *E. coli* strain 536 in comparison to non-pathogenic strains were subtracted and identified (Janke *et al.*, 2001; Blanc-Potard *et al.*, 2002; Allen *et al.*, 2003). In addition, SH has been used to characterize genetic differences between *Helicobacter pylori* (Akopyants *et al.*, 1998). Moreover, SH has been applied to compare genomes of related bacterial species *E. coli* and *Salmonella typhimurium* (Bogush *et al.*, 1999). In this study, about 60% of the fragments identified by SSH were unique for only one of the genomes. The remaining fragments represented similar sequences which however have diverged considerably during evolution in both species. Besides the characterization of animal pathogens, SH has also successfully been used to identify genome fragments which were unique for *Ruminococcus flavefaciens* FD-1 with respect to *R. flavefaciens* JM1, two closely related cellulolytic rumen bacteria (Antonopoulos *et al.*, 2004). Likewise, attempts to compare ruminal ecosystems with each other and determine their uniqueness in terms of genetic potential are underway (Galbraith *et al.*, 2004). With this technology it may be possible to retrieve “ecosystem-

specific” genome fragments. Studying the expression of distinctive genes under different conditions could be studied subsequently. At the moment we are developing a community-based RDA method in our laboratory which will enable us to identify differences between community structures specifically. The combination of this approach with flow sorting will result in retrieval of “ecosystem specific” microbes and exclude sequence analysis of genes from microbes common to both samples or systems.

The main problem with the described metagenomic approaches is the difficulty of linking specific gene sequences to the microbes from which they originate. However, a recent paper describes a cell sorting approach which might enable researchers to make this link based on cell capture with DNA-targeted probes (Zwirgmaier *et al.*, 2004). The approach is based on using polynucleotide probes for whole cell hybridization followed by capturing the cells on a microplate coated with DNA fragments which are complementary to the probes, since it is assumed that part of the polynucleotide probe will remain outside the target cells. Therefore, the combination of metagenomic analysis of ecosystems, subsequent probe development, and cell sorting looks like a very promising strategy to link novel gene sequences to uncultured bacteria.

#### *What is the In Situ Activity of Microbes?*

In the past attempts to answer the question “what is the function of a certain microbe in an ecosystem” have been performed by characterizing the physiology of microbes in pure or well-controlled mixed cultures. Although these studies have resulted in a phenotypic classification of microbes, the ability to perform a certain function in culture does not mean that these microbes perform the same function *in situ*. This is well demonstrated with environmental samples that have been incubated with isotopically (stable or radioactive) labeled substrates. These isotopes have been traced back by extracting biomarkers, such as DNA or lipids, or by combining microautoradiography and *in situ* hybridization (Radajewski *et al.*, 2003). Isotope tracking has for the first time been applied successfully to aquatic sediments and the microbes involved in methane oxidation and acetate-coupled sulfate reduction, respectively could be identified (Boschker *et al.*, 1998). A good example of the contradiction between phenotypic characterization and *in situ* isotope tracking is described by Manfield *et al.* (2002). In contrast to findings from phenotypic characterization of phenol-degrading isolates from a bioreactor, *in situ* isotope tracking revealed that phenol degradation in the microbial community was dominated by an uncultured member of the *Thauera* genus. Recently, developments have been made in combining isotope tracking with DNA microarray analysis (Adamczyk *et al.*, 2003, Polz *et al.*, 2003). This approach looks promising because it links functional aspects and phylogenetic identification. Despite its value, one should keep in mind that some microbes might be able to discriminate different isotopes (Londry and Des Marais, 2003). In addition, it will be difficult to characterize the utilization of the labeled substrates when they are complex and their utilization requires complex pathways or multiple microbes. Moreover, the use of labeled substrates is limited by their availability. Although the application of isotope tracking in GI tract ecology studies appears therefore

complicated, *in situ* utilization of, for example prebiotics, in animal model systems can probably be studied accurately using this approach.

A more specific way to determine the *in situ* activity of bacteria in an ecosystem is to measure the expression of functional genes. An excellent study by Hooper *et al.*, (2001) demonstrated the power of this approach in investigating host-microbe interactions by using DNA microarrays. In this study the global transcriptional responses of germ-free mice to colonization by *Bacteroides thetaiotaomicron* were examined. In addition the specific cellular responses were determined by laser-capture microdissection. Their results demonstrated that *B. thetaiotaomicron* was able to modulate expression of genes having a variety of physiological functions, including those for nutrient absorption, and immune responses. This approach should also be applied to study responses of the microbes when they are in contact with the host. In this way, interactions between designated partners can be studied on a molecular level in detail. Unfortunately, most of the currently available methodology for transcriptional analysis is mainly applicable to poly A-tailed mRNA, which is abundant in eukaryotes but rare in prokaryotes. The use of random hexamers is currently being used to get cDNA's representing total RNA of prokaryotes, but the bias introduced by this approach is difficult to determine. In addition, only a small fraction of total bacterial RNA consists of mRNA, and therefore the detection of specifically transcribed genes, particularly those that are poorly expressed or that have unstable messages, remains a challenge especially for environmental samples. However, some progress has been made in the detection of mRNA in environmental samples, including those from the GI tract. Deplancke *et al.* (2000) were able to specifically detect mRNA levels of adenosine-5'-phosphosulfate reductase at different locations in the mouse GI tract by using an RT-PCR approach. In addition, Fitzsimons *et al.* (2003) could detect *slpA* expression of *Lactobacillus acidophilus* in fecal samples spiked with this lactic acid bacterium. Also some quantitative assessment of gene expression in GI tract has been performed. By using real-time RT-PCR, the expression level of four different genes of *H. pylori* could be determined during its colonization of the gastric mucosa in humans and mice (Rokbi *et al.*, 2001). Similarly, the germination level of genetically engineered *Bacillus subtilis* spores in the mouse GI tract could be assessed using a competitive RT-PCR approach (Casula and Cutting, 2002).

A different approach for measuring gene expression in ecosystems is called *in vivo* expression technology (IVET). The IVET strategy allows identification of promoters that are specifically induced when bacteria are exposed to certain environmental conditions (Rainey and Preston, 2000). Although the approach has mainly been used to study gene expression of pathogens, it has also successfully been used to study colonization of *Lactobacillus reuteri* in the mouse GI tract (Walter *et al.*, 2003). In this study the expression of only three genes could be linked to *Lactobacillus* colonization, which is lower than one would expect in such a complex ecosystem. These genes showed homology to xylose isomerase (*xyIA*), peptide methionine sulfoxide reductase (*msrB*), and a gene with unknown function, respectively. Despite the fact that the application

of IVET and mRNA-based studies in GI tract ecology is still in its infancy, developments in these technologies look very promising for unraveling functions of microbes in the GI tract.

It is evident that the use and development of novel approaches, such as those described above should be continued and applied more frequently to the GI tract ecosystem. This may ultimately help us in answering one of the most important questions in microbial ecology of the GI tract: What are the GI tract microbes doing?

### Concluding Remarks

This review provides a brief history concerning the insights on GI tract microbial ecology with the main focus on the use of novel nucleic acid-based molecular techniques. The application of molecular techniques, largely based on 16S rRNA genes, in microbial ecology has led to a new specialized research area called "Molecular Microbial Ecology". It is clear that the application of these modern molecular techniques will for the first time enable researchers to obtain a complete description of the genetic diversity in the GI tract, which was not possible using the classical culture techniques. Ultimately, the use of nucleic acid-based approaches targeting 16S rRNA, functional genes, and mRNA *in situ* combined with proteomic and metabolomic approaches 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. This is the ultimate goal of the microbial ecologist. We have not discussed the application of proteomics and metabolomics in this review, since these techniques are relatively new and have not been used in ecological studies yet. However, it is already clear that these non-nucleic acids based approaches will complement genomic approaches. Genomics decodes sequence information of an organism and provides a "parts catalog" while proteomics and metabolomics attempt to elucidate the functions and relationships of the individual components and predict outcomes of the modules they form on a higher level. For example, with metabolomics it should be possible to identify signaling molecules which are excreted in the GI tract for communication between host cells and different microbes, and the effect of these signaling molecules on the bacterial or host physiology can than be studied using genomic and proteomic approaches.

Despite explosive developments in modern molecular techniques and all their associated challenges, a potential danger is that scientists may easily be tempted to perform descriptive rather than hypothesis-driven research. Microbial ecology is the study of life and not of techniques and therefore, the focus of Molecular Microbial Ecology should be more on ecology and less on molecular.

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