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A Molecular View of the Intestinal Ecosystem

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Abstract

This review describes the state of the art as well as the initial results of molecular methodologies used to study the ecology of the complex microflora of the human intestinal tract. The detection and identification of many of these organisms has largely been hampered by the incomplete knowledge of their culture conditions. Many of the molecular methodologies are rooted in the use of ribosomal RNA (rRNA) and its encoding genes to describe the relationship between the bacteria in such communities and their individual identity. This approach permits the elucidation both qualitatively as well as quantitatively of the abundance of bacterial species and how their presence interacts with diet and health. Emphasis is given to the analysis of complex communities rather than detection of individual groups of bacteria. The potential of novel advances in molecular technologies such as DNA arrays for analysis of the intestinal ecosystem are also discussed.

Introduction

For decades microbial ecology was hampered by lack of clear concepts on the identity of micro-organisms and lack of methodologies to analyse complex communities. Detection and identification were almost completely established on culture-based methods and the species concept was based on phenotypic rather than genotypic characters. Since gene expression is often influenced by environmental factors, such as substrate supply, pH, temperature and redox potential, the phenotype of an organism is less stable than its genotype. As a consequence bacterial taxonomy contains many controversies, and for a long time taxonomy was a purely applied field. Due to the introduction of molecular biology in bacteriology, taxonomy has come into a new phase. The new molecular approach has not only activated the interest in evolution and the origin of life, but also opened up the opportunity to analyse complex communities on the basis of DNA sequence diversity. By simply retrieving DNA sequences from the environment and comparing those with known sequences from the database, it became clear that most of those DNA sequences were new (Amann *et al.,* 1995). It clearly confirmed that culturing studies have essentially filtered our view of the biodiversity of microbial life.

Novel molecular technologies are being increasingly used for analysis of the complex intestinal ecosystem and contribute to a better understanding of the interaction between host and microbes in the intestinal tract (Vaughan *et al.,* 1999). In this review, an attempt is made to give an overview of these advances and to identify opportunities and pitfalls in applying these methods to the study of community structure and dynamics of intestinal bacteria. These methods are applicable to the mammalian gastrointestinal tract in general but our present focus is their impact on the analysis of the human system. In addition, novel techniques under development which may further this research are addressed.

Difficulties of Culturing Studies on the Intestinal Microflora

Biodiversity as well as overall numbers of microorganisms in the gut intestinal tract are determined by a range of factors, both intrinsic such as the GI tract location or genetic background, and extrinsic factors such as diet and health. Research has mainly concentrated on the easily accessible microflora of the faeces, and the information concerning microbial populations associated with the mucosa and upper intestinal tract sections is scarce. The richest and most complex part of the human intestinal microflora resides in the colon. Normal populations exceed 10^{11} per gram of stool, and according to culture studies, consist of a mixed culture of an estimated 300-400 species of bacteria, with some 30-40 species accounting for 99% of the population (Finegold *et al.,* 1974; Moore and Holdeman, 1974a). The initial inoculum is derived from the mother at the time of birth and, although the microflora alters as the subject ages (Mitsuoka, 1992), culturing studies indicate that it is fairly constant in composition with *Bacteroides* and *Eubacterium* spp. dominating in adults.

A large proportion of the bacteria encountered in faeces are strict anaerobes, often non-sporulating (Moore and Holdeman, 1974b). Although it is relatively easy to obtain a total viable count, enumerating individual bacterial species by culturing methods is laborious and time consuming. Drawbacks associated with culture-based techniques are exacerbated in anaerobic habitats. Selective media are not available for most of the strict anaerobes and several hundred isolates from each faecal specimen should be identified for reliable statistics. Following their individual isolation, end-product analysis of fermentation in pure cultures is essential for reliable identification. Due to slow growth, the identification of a single anaerobe will generally take up some two weeks. As a consequence, studies on population dynamics of the intestinal microflora are often limited in the number of subjects or the number of bacterial species investigated, thus limiting the statistical reliability of results (Benno *et al.,* 1989; Minelli *et al.,* 1993; Mitsuoka, 1992).

In 1992 around 5000 bacterial species had been described, but there are estimates that no more than 1%

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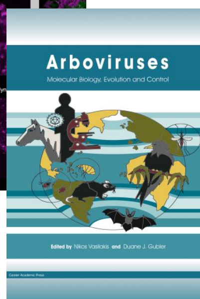

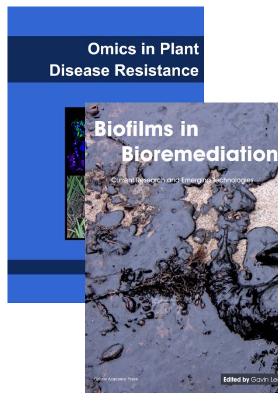

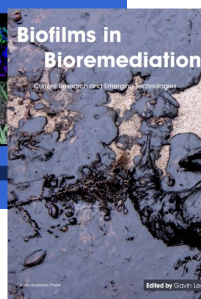

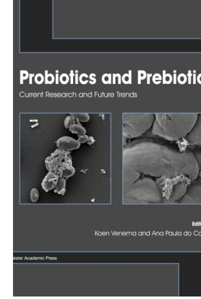

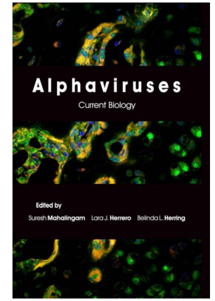

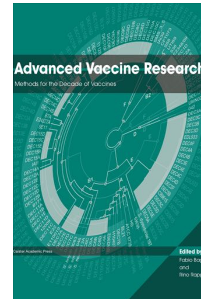

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of all bacterial species present in our biosphere has ever been isolated (Amann *et al.,* 1995; Ward *et al.,* 1992). Only those organisms whose niche could relatively easily be mimicked in the laboratory have been isolated and identified. It may be well into the new millennium before gastrointestinal members that require specific conditions, or live in interdependent consortia, will be isolated and described. Thus, despite the extensive culturing studies performed on the mammalian intestinal ecosystem using conventional techniques, it must be assumed that, as for other natural ecosystems, the fraction of well-described species is limited. Indeed, recent estimates of culturability range from 15 to 58% (Langendijk *et al.,* 1995; Suau *et al.,* 1999; Wilson and Blitchington, 1996). Although traditional cultivation methods are generally tedious, expensive, and are probably more open to misinterpretation with intestinal samples, they remain the "gold standard" for identification.

Molecular Characterisation by rRNA Analysis

The introduction of genetic-based technologies and in particular those relating to 16S rRNA typing are rapidly replacing conventional detection and enumeration methods in studies of the mammalian intestinal tract (Tannock, 1999). Several critical attributes favour the use of ribosomal sequences for classification of microorganisms in the environment. The first is their universal distribution among all cellular life forms. The second is that their essential function in all organisms translates into a very slow genetic evolution and as a result the sequences coding for rRNA are highly conserved. Furthermore, the mutation rate of rRNAs corresponds to evolutionary divergence of organisms. Identification and enumeration are basic prerequisites for ecological studies and it is now acknowledged that the most desirable classification scheme reflects natural evolutionary relationships (Woese, 1987). The primary structure of all ribosomal sequences consists of alternating conserved and variable domains which makes them very suitable for the detection and identification of microbial species and ideal targets for specific DNA probes. By aligning the appropriate 16S rRNA sequences, genus-specific and species-specific sequences can be identified. As a result, molecular methods address detection and classification at the same time. Comparative sequencing of the 16S rRNA molecule has become by far the most commonly used measure of environmental diversity. The 5S rRNA is rather small giving limited information, while the 16S rRNA, consisting of about 1,500 nucleotides (nt), provides a large amount of information for phylogenetic inference and is a reasonable size for sequencing. The 23S rRNA, generally 3,000 nt, offers substantial information but requires more sequencing, so 16S rRNA has essentially become the established reference. Some attention has also been given to the internal transcribed spacer (ITS) regions separating rRNA genes. However, as these sequences play no role in the functional ribosome they do not reach the high copy number targets of the rRNA genes themselves, and also mutate so rapidly that they may only provide taxonomic information at the subspecies to strain-level.

Inferring species identity from genetic data is a hazardous undertaking. Currently, identification of unknown organisms occurs first and foremost on the basis of well-established rRNA classification schemes. As a rule of thumb, a new species is encountered when it possesses a sequence similarity of less than 97% with any known organism in the database (Fox *et al.,* 1992; Stackebrandt and Goebel, 1994). But however straightforward the rRNA approach to phylogenetics may seem, evidence is accumulating that bacterial life just isn't that simple. The species concept is receiving renewed attention upon the arrival of complete genome sequences for prokaryotes of different descent. In particular, horizontal gene transfer appears to be an important factor in bacterial adaptation and speciation, and may to a certain extent blur the picture of the presently established evolutionary lines of descent based on rRNA data (Doolittle, 1999). Still, the state of the art is well rooted in the use of rRNA as a phylogenetic marker.

Direct Molecular Detection

Isolation of Nucleic Acids

Molecular techniques promise a more full and accurate description of the true diversity, structure and dynamics of complex microbial communities than our present culturing studies, yet suffer from experimental bias and selectivity in their own way (Table 1) (Wallner *et al.,* 1997). One of the more notorious is selective nucleic acid extraction. The type of fingerprinting technique will dictate the type of protocol used for isolation of nucleic acid. For example, ribotyping requires high quality genomic DNA with minimal shearing, while for pulse field gel electrophoresis (PFGE), intact genomes are the starting material. When investigating whole communities, a reliable method for extraction and purification of DNA and/or RNA from the sample is the one of the most critical steps, since all further analyses assume the complete and representative presence of accessible nucleic acids. Not all microbial cells are lysed with equal ease and numerous protocols have been reported for extraction of nucleic acids from faecal specimens involving enzymatic, chemical and mechanical breaking of the cells (Zoetendal *et al.,* 1999; Wang *et al.,* 1996; Klijn *et al.,* 1995). Recently, the more preferred methods involve disruption by bead beating, sometimes in combination with other treatments (Dore *et al.,* 1998; Wilson and Blitchington, 1996; Zoetendal *et al.,* 1998). Briefly, glass or zirconium beads are added to the sample in a buffered solution and shaken vigorously. A potential disadvantage of the latter is shearing of the nucleic acids which may result in low yields of DNA or RNA, and increase the formation of chimeric structures during amplification of certain genes (Kopczynski *et al.,* 1994; Wang and Wang, 1996). Optimisation of the extraction procedure that is compatible with the applied fingerprinting method will obviously give more credence to the final results. In one study, the effect of an increased bead beating time on the lysis efficiency of faecal samples and the integrity of the extracted DNA was followed by TGGE analysis of 16S rRNA amplicons (Zoetendal *et al.*, 1998). In this way the nucleic acid extraction procedure could be adapted to ensure complete disruption for a true TGGE pattern.

Sequencing of 16S rRNA Genes

Ribosomal RNA sequences can be obtained either directly from rRNA or from the encoding genes located at various positions in the genome, *i.e.*, rDNA. In practise, sequences of 16S rRNA's are determined by creating rDNA clone libraries rather then rRNA libraries and there are several

reasons for this. The reverse transcription-polymerase chain reaction (RT-PCR), which converts rRNA into rDNA, is a sensitive technique requiring high quality template. The 1500 nt size of 16S rRNA, the presence of post-transcriptionally modified ribonucleotides (McCloskey and Crain, 1998), or processing resulting in fragmentation (Monstein *et al.,* 1998), can limit the efficiency of the reverse transcriptase reaction causing premature termination of the transcription, which is already frequent when transcribing long (>400) stretches of bases.

Several programmes for determining the most similar sequence to the clone are available at various internet sites. A comprehensive sequence data set, currently greater than 10,000 small subunit rRNA entries, is available in generally accessible databases such as Genbank, EMBL, Ribosomal Database project (RDP) (Maidak *et al.,* 1999), ARB (Strunk and Ludwig, 1995), and the Antwerp database on small subunit rRNA (van de Peer *et al.,* 1999). The latter three databases contain sequences that are aligned according to their secondary structure paradigm. They are maintained by specialists generally through national funding programs and provide services such as alignment of newly submitted sequences, probe check, chimera tests and phylogenetic information (Ludwig *et al.,* 1998).

The number of culture-independent surveys performed to reveal the bacteria present in human faeces is very limited. A pioneering study involved the construction of a 16S rDNA clone library by PCR using conserved bacterial primers with DNA extracted from a human faecal sample as the template (Wilson and Blitchington, 1996). A total of 50 clones of 700-bp were sequenced and the same faecal sample was also cultured on medium suitable for growth of anaerobic bacteria. The rDNA sequences found in the cloned material were compared with those found in the colonies (same 700-bp fragment) and the results suggested that the two methods were in reasonable agreement. However, other findings in the study suggested that additional research into the true diversity of human faecal microflora was needed. For example, an estimated 41% of all cloned material was not represented by any clonal sequence obtained, and only 31% of the sequenced colony isolates corresponded to a known species in the databases. A more recent 16S rDNA clone library constructed for identification of the predominant bands encountered by TGGE (see below) of human faecal flora revealed that only two out of 15 complete rDNA clones had more than 97% identity to a sequence in the database (Zoetendal *et al.,* 1998). The most thorough direct analysis of 16S rDNA to date involved sequencing 500-bp fragments from 284 rDNA clones generated by a 10-cycle PCR of DNA from a single human faecal sample (Suau *et al.,* 1999). Three phylogenetic groups, namely *Bacteroides*, *Clostridium coccoides* and *Clostridium leptum* constituted 95% of the clones. Comparative sequence analysis again indicated that only 24% of the clones corresponded to described species in the databases. Thus, the vast majority of bacteria in the human intestinal tract have evaded scientific description and our knowledge concerning microflora diversity is still inadequate.

When bearing in mind that only some 30 species make up the bulk of the bacterial population at any one time (Finegold *et al.,* 1974; Moore and Holdeman, 1974a), it is practical to focus on specific groups of interest within the complex community. These may be the predominant or most active species, specific physiological groups or readily identifiable (genetic) clusters of phylogenetically related organisms. Several 16S rRNA fingerprinting methods can be invaluable for assessing and selecting the sequences or microbes of interest as described below.

Molecular Fingerprinting Methods for Microbial Communities

During the past decades most attention was focused on the identification of pure cultures of micro-organisms on the basis of genetic polymorphisms of DNA encoding rRNA, such as ribotyping, amplified-fragment length polymorphism (AFLP), and randomly amplified polymorphic DNA (RAPD) (O'Sullivan, 1999). Many of these methods require prior cultivation of the bacteria, and therefore, are regarded as less suitable for use in general population description. Although it is beyond the scope of this review to discuss all these techniques, they are essential for a true description of biodiversity in the intestinal tract and remarkable observations generated using these techniques will be acknowledged. Much less attention was given to the molecular characterisation of complex communities. The application of molecular techniques in ecology has given a major breakthrough in the analysis of microbial ecosystems and their function (Figure 1) (Akkermans *et al.,* 1999). In particular, research into the biodiversity and community behaviour over time of microorganisms in natural ecosystems has been awakened by the advent of fingerprinting techniques for complex communities (Muyzer,

Table 1. Potential uses and Drawbacks of Various Methods for Analysis of Complex Microbial Communities

Method	Uses	Drawbacks
Culturing	Isolation; "The ideal"	Not representative; slow
16S rDNA sequencing	Identification	Large scale cloning is laborious
DGGE/TGGE	Rapid comparative analysis; identification by band extraction; detection of specific groups	Semi-quantitative
T-RFLP	Rapid comparative analysis; very sensitive; potential for high throughput	Semi-quantitative; identification only possible with clone library
SSCP	Rapid comparative analysis	Semi-quantitative; identification only possible with clone library
FISH	Detection; enumeration; comparative analysis possible with automation	Requires probe design; laborious without automation
Dot-blot hybridisation	Detection; estimates relative abundance	Requires probe design; laborious
FISH/Flow cytometry	Enumeration; potential for high throughput	Under development

1999). Denaturing or temperature gradient gel electrophoresis (DGGE/TGGE) methods have been successfully applied to the analysis of mammalian intestinal tract samples (Zoetendal *et al.,* in press). Various new techniques, such as single strand conformation polymorphism (SSCP) (Schwierger and Tebbe, 1998) and terminal-restriction fragment length polymorphism (T-RFLP or TRFP) (Marsh, 1999) have subsequently been shown to be highly useful in the analysis of complex microbial communities. The feasibility of the TRFP approach for characterising the microbial community in faecal samples was recently demonstrated for deer faecal pellets (Clement *et al.,* 1998).

DGGE/TGGE

TGGE and DGGE are gel-electrophoretic separation procedures for double stranded DNA's of equal size but with different base-pair composition or sequence (Muyzer and Smalla, 1998). In principle, the methods are sensitive enough to separate DNA's on the basis of single point mutations (Sheffield *et al.,* 1989). Both techniques are gaining increased popularity in microbial ecology for analysing the diversity of total bacterial communities. Briefly, the 16S rRNA genes are amplified using the appropriate primer pair, one of which has a G+C "clamp" attached to the 5' end that prevents the two DNA strands from completely dissociating even under strong denaturing conditions. During electrophoresis through a polyacrylamide gel containing denaturants, migration of the molecule is essentially arrested once a domain in a PCR product reaches its melting temperature. Following staining of the DNA, a banding pattern emerges that represents the diversity of the rRNA gene sequences present in the sample. The intensity of an individual band is a semi-quantitative measure for the relative abundance of this sequence in the population.

TGGE/DGGE of 16S rRNA amplicons are exceptional tools to study the bacterial species composition of unknown samples. Since individual bands can be excised and sequenced, the identity of the bacteria present in the sample can be determined without cultivation. By inter-sample comparison, dominant shifts in population composition can be monitored and bacterial population dynamics can be studied in more detail. For example, TGGE of rRNA amplicons from intestinal samples of one individual revealed that their predominant faecal bacteria remained remarkably stable over at least a 6-month period (Zoetendal *et al.,* 1998). Furthermore, unrelated individuals had a unique TGGE pattern reflecting their differences in faecal microbial composition. Remarkably, this uniqueness of a person's microflora appears to reach right down to the strain level. Using molecular typing techniques it was observed that each person harbours at least one specific *Bifidobacterium* or *Lactobacillus* strain (Kimura *et al.,* 1997). A similar observation has been made for *Eubacterium ramulus* (Simmering *et al.,* 1999).

By constructing a clone library of one person's 16S rDNA sequences, the most dominant bands in the TGGE profile were identified and sequenced, which revealed that the majority were derived from undescribed bacterial species (Zoetendal *et al.,* 1998). Notably, three species with greatest similarities to *Ruminococcus obeum*, *Eubacterium halii* and *Fusobacterium prausnitzii* were dominant in all individuals investigated. DGGE analysis was also used to examine the contribution of non-culturable microbes in necrotizing enterocolitis (NEC) of the bowel of neonates, but no differences were detected between faecal samples from healthy infants compared to those with NEC (Millar *et al.,* 1996). More recently, DGGE has being used to monitor the bacterial succession in the faeces of several infants (Favier *et al.,* 1999). The bacterial diversity remains very low during the first few weeks after birth with some

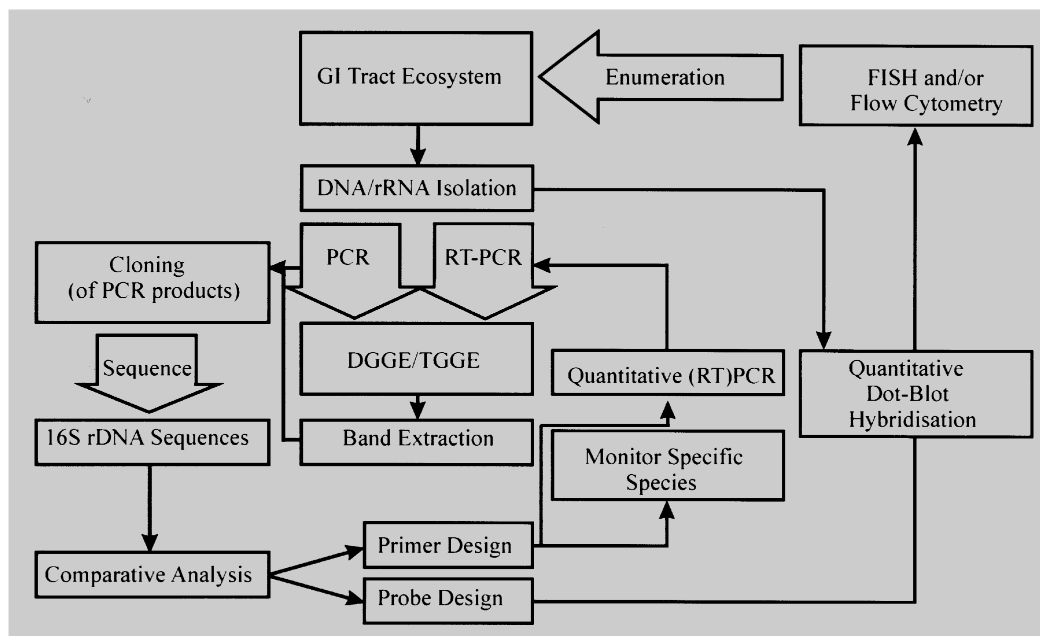

Figure 1. Flow chart of various molecular approaches to analyse the human intestinal microbial community.

species only transiently present while others managed to establish themselves. The infant's DGGE profiles became more similar to his/her relatives with time. Thus, besides diet, environmental factors such as "transmission" from the mother, and genetic factors, influence the intestinal composition (Favier *et al.,* 2000).

Monitoring of the predominant faecal microflora during administration of probiotics to children demonstrated that no extensive changes occurred (Figure 2) (Heilig *et al.,* unpublished, de Vos *et al.,* 1999). Although changes within certain individuals were occasionally observed, they did not correlate with the feeding of the probiotic. Furthermore, using primers specific for bifidobacteria and *Lactobacillus* rRNA sequences, different species could be differentiated and followed in time during probiotic feeding trials using TGGE or DGGE (Heilig *et al.*, 2000; Lubbers *et al.,* 1999).

Other applications of these techniques include identifying 16S rRNA sequence heterogeneity (Nubel *et al.,* 1996), monitoring specific physiological groups, monitoring enrichment and facilitating isolation (Muyzer, 1999), and determining PCR biases. As an alternative to comparing DGGE profiles by eye, similarity indices may be calculated by computer analysis of scanned fingerprints or using Shannon-Weaver indices (Nubel *et al.,* 1999; Ramirez-Saad, 1999; Zoetendal *et al.,* 2000). These are more subjective approachs to monitor the effects of diet or medication on the gut intestinal microflora.

Because of the potential artefacts and bias introduced by nucleic acid extraction methods and during amplification in the PCR-based fingerprinting techniques, it is necessary to trace the sequences back to the intestinal sample. Moreover, since the ecologist describes "diversity" not only by considering all species but also by their prevalence or scarcity (Stahl, 1997), techniques to provide data of specific cell numbers are required. By designing species-specific hybridisation probes to the identified bacterial sequences, the various species in a microbial community can be quantified in even greater detail. This can only be realised by whole cell *in situ* hybridisation. Ribosomal RNA extraction for quantitative dot blot hybridisation or quantitative PCR offer semi-quantitative methods for determining the abundance of certain taxa.

Quantitative (RT)PCR of 16S rRNA

Although PCR is the most sensitive technique to detect sequences that are present in very low concentration, the many factors that influence the amplification reaction can cause misleading results in quantitative PCR. For example, biased amplification due to variations in primer pairs, the melting temperature of the template DNA, exhaustion of substrates within the PCR reaction, different efficiency of amplification with different sized amplicons, and chimera formation between 16S rRNA molecules from two different microorganisms, are some of the potential problems within a PCR reaction (von Wintzingerode *et al.,* 1997). Moreover, the copy number of 16S rRNA genes per genome can vary quite considerably depending on the species. Since information on genome size and copy number of *rrn* operons is lacking for the vast majority of microbes, competitive PCR procedures with internal standards for 16S rDNA amplification will not accurately reflect bacterial cell numbers nor ratios of nucleic acids (Farrelly *et al.,* 1995), but nevertheless will give a general estimate. For example, PCR using primer pairs specific for 16S rRNA of 12 different groups of bacteria present in the human intestine indicated the predominance of *Clostridium clostridiiforme, Fusobacterium prausnitzii* and *Peptostreptococcus productus* in comparison to other species such as *Eubacterium biforme, E. coli* and various lactic acid bacteria (Wang *et al.,* 1996).

As an alternative to rDNA, quantification of rRNA that is isolated directly from the ribosomes may be used to reveal the metabolically most active members of a bacterial community. This novel approach involves competitive reverse transcription-PCR (RT-PCR) of community 16S rRNA and known concentrations of standard, and subsequent separation of the amplicons by TGGE and quantification of the band intensities (Felske *et al.,* 1998). So far this technique has been successfully used to monitor spatial changes in bacterial activity in soil but it can also be applied to the gut intestinal tract ecosystem.

Use of DNA Probes Based on rRNA Sequences

Dot Blot Hybridisation

This technique is useful to measure the amount of a specific 16S rRNA in a mixture relative to the total amount of rRNA. Briefly, total DNA and RNA are isolated from the sample, bound to a filter using a dot or slot manifold device and hybridised with labelled oligonucleotide probes. The amount of label bound to the filter is a measure of the specific rRNA target present, and the relative amount of rRNA may be estimated by dividing the amount of specific probe by the amount of labelled universal probe hybridised under the same conditions. Obviously, the relative amount of a rRNA sequence does not reflect the true abundance of the microbe since cells of different species have different ribosome contents and the number of ribosomes within one strain will vary with growth phase. Nevertheless, the relative quantity of rRNA provides a reasonable measure of the relative physiological activity of a specific population. Using this method a range of probes were used to determine the relative proportion of rRNAs of microbial groups within faecal samples from 10 healthy adults (Dore *et al.,* 1998; Sghir *et al.,* 1999). It was demonstrated that Bacteria, Archaea and Eucarya represented approximately 78, 0.3 and 2% of total rRNA, respectively. Within the Bacterial domain, rRNAs of the groups *Bacteroides*, enterobacteria, *Lactobacillus, Cl. leptum, Cl. coccoides* and *Bifidobacterium* represented about 36, 3, <1.0, 30, 20 and 4% of total Bacterial rRNA, respectively, which accounts for 92% of all bacterial rRNA in faeces.

FISH

In contrast to most molecular methods referred to in the above sections whole cell fluorescence *in situ* hybridisation (FISH) with rRNA-targeted oligonucleotide probes is quantitative on a cell by cell basis. Ultimately, enumeration of species in the intestinal tract is best addressed by this approach. Since 1995, the use of 16S rRNA targeted oligonucleotide probes for the enumeration of various phylogenetic groups of the intestinal microflora of humans has become an important part of investigations (Bos *et al.,* 1997; Franks *et al.,* 1998; Harmsen *et al.,* 1999; Kok *et al.,* 1996; Schut, 1997; Schut *et al.,* 1998; Welling *et al.,* 1997). To facilitate enumeration, FISH has been automated and combined with image analysis that is analysed by computer software (Jansen *et al.,* 1999; Wilkinson and Schut, 1998a,

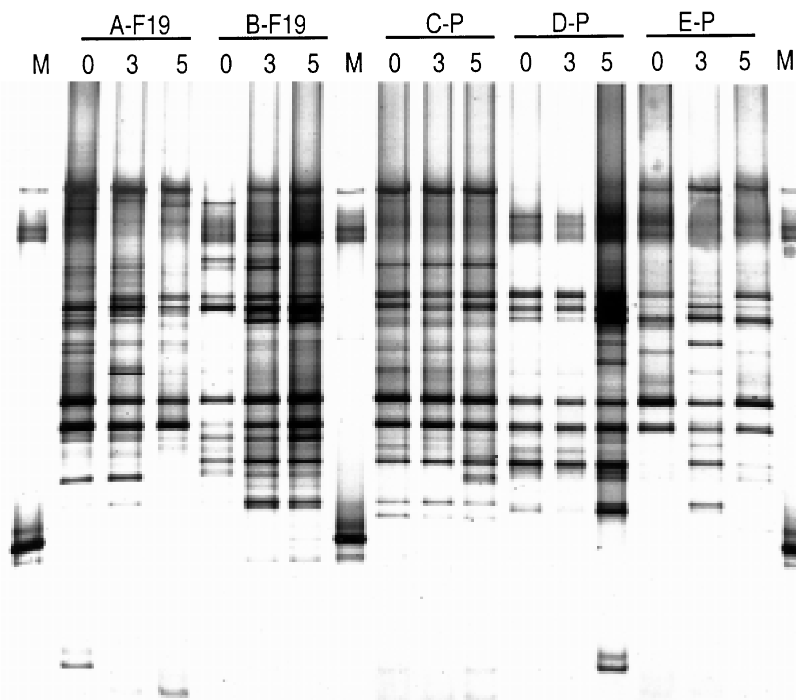

Figure 2. DGGE patterns of PCR products obtained using primers 986GCf and L1401r on total nucleic acids isolated from faecal samples. The samples of individual children A, B, C, D, and E fed with *Lactobacillus paracasei* F19 (F19) or placebo (P) were taken pre- (0 week) and post-administration (3 and 5 weeks). The DGGE pattern obtained from the *L. paracasei* F19 culture is indicated as M (Heilig, G.H.J., Vaughan, E.E., Akkermans, A.D.L., and de Vos, W.M. unpublished).

1998b). FISH enables researchers to address five ecological themes at once: i) to identify sub-populations in natural systems and to locate their niche; ii) to obtain structural insight in mixed population communities by using sets of probes; iii) to bypass cultivation problems; iv) to determine the *in situ* cellular rRNA content and "metabolic fitness"; and v) to accurately enumerate defined cell populations. The lowest level of detection is 10^6 cells per gram of faeces at present.

The first report on enumeration of bacteria in the human intestinal tract by FISH methods indicated that *Bifidobacteria* made up only 2 percent of the population (Langendijk *et al.,* 1995) instead of the usually accepted 10 percent (Finegold *et al.,* 1974). FISH performed with a *Eubacterium ramulus* specific probe indicated that it constituted an average 0.16% of the total faecal microflora and it was present in each of 20 people investigated (Simmering *et al.,* 1998). *E. ramulus* has been detected only rarely by culturing methods (Moore and Holdman, 1974). This demonstrates the power of FISH-based detection and enumeration. Using FISH probes for *Bacteroides* and *Clostridium coccoides-Eubacterium rectale,* these groups were demonstrated to constitute half of the faecal microflora while another 12% hybridised with a probe for low G+C gram-positive bacteria and *Bifidobacterium* constituted 3% of the microflora (Franks *et al.,* 1998).

Flow Cytometry

While the FISH enumeration methods (Langendijk *et al.,* 1995) rely on membrane filter/epifluorescence microscopy, flow cytometry (FCM) may be a more promising technique (Amann *et al.,* 1990). Flow cytometry has several advantages over image cytometry (microscopy) including speed and automation. One potential disadvantage is that the rapid passage of the cells through the light beam limits the time that emitted light can be collected by the photometers. While *Bifidobacterium* species in faecal samples produce sufficient signal to be detected (Figure 3) (Schut and Tan, 1999), several *Bacteroides* species are significantly smaller and their specific fluorescence is often lower than the 'auto-fluorescence' of large unlabeled cells. For the enumeration of *Bacteroides* species, therefore, the use of multiple probes per taxonomic group (multi-probe hybridisation) or the development of signal amplification procedures should be adopted. Recently, such signal amplification procedures have been developed for FISH of whole bacterial cells (Schönhuber *et al.,* 1997; Schut and Tan, 1999). This technology is based on the use of peroxidase-labelled probes in combination with precipitable fluorescent substrates (tyramide-fluorochrome complexes) that can generate 80-fold amplification of signal. A drawback of this technique is that it does not allow simultaneous multi-color staining and multiple successive hybridisations are required for identification of different bacterial taxa.

Several studies have demonstrated the use of flow cytometry in association with FISH and the general conclusion is that this combination is a very powerful tool for the rapid and automated analysis of mixed microbial communities (Davey and Kell, 1996; Porter *et al.,* 1996; Thomas *et al.,* 1997). The feasibility of this technique has

been reported for enumeration of bacterial species in the human gut microflora whereby intestinal bifidobacteria were enumerated with good precision when compared with microscopic counts (Figure 3) (Schut and Tan, 1999).

The direct detection of low numbers of particular bacteria in faecal samples is impeded by high background populations (10^{10}-10^{11} per gram) of other microorganisms. Presently, subpopulations constituting of at least 0.2-1.0% of the total population (*i.e.* exceeding 2 x 10^8 per gram) can be detected. Several species of the intestinal microflora, specifically the aerobic and facultatively anaerobic bacteria, drop well below this level. In this case, the superiority of culture techniques is apparent.

As bacteria produce very little light scattering, the range of cytological methods that can be applied is limited to those that allow analysis of fluorescence. But since fluorescence technology has vastly expanded in recent years, many metabolic parameters (*e.g.* live/dead) can presently be analysed cytometrically (Davey and Kell, 1996; McFeters *et al.,* 1995; Nebe-Von Caron and Badley, 1995; Wilkinson and Schut, 1998b).

In order to allow simultaneous detection of several taxa in one sample, multi-parameter processing of the samples is required. Available and affordable flow cytometer systems can excite up to three fluorochromes simultaneously. Several combinations of colours can thus be used to identify individual cells. One colour should be reserved for total cell staining, *i.e.* for the verification of the presence of DNA in the detected particle, and for calculation of the sample volume that has been analysed. The three remaining combinations can theoretically be applied for the identification of the cells. This system which we describe as "rainbow staining" is known to work for bacteria-sized particles. However, it has never been used in combination with FISH, and experimental evidence for its success should be obtained. The potential of flow cytometry in combination with FISH as a quantitative method for analysing the ecology of the human intestinal tract thus remains to be evaluated.

Perspectives and Conclusions

Advances in molecular technology have led to improvements in the tools available for studies in molecular ecology. Fundamental knowledge concerning molecular mechanisms is improving current methodologies, and new technologies are being developed. Several advances in probe and primer technology, and the genomics field are reported here.

Advances in Probe and Primer Technology

The denatured nucleic acids used in dot blots, cell blots or PCR reactions permit easy hybridisation with probes and primers irrespective of the hybridisation site within the target molecule. On the other hand, the accessibility of 16S rRNA in its native form to fluorescently labelled probes varies for different regions of the molecule (Frischer *et al.,* 1996; Fuchs *et al.,* 1998). It may even differ between organisms (Langendijk *et al.,* 1995). This is due to the higher order structure of both the native ribosome and the native 16S rRNA molecule. Studies that seek to disclose the enigma of *in situ* probe performance (Frischer *et al.,* 1996; Fuchs *et al.,* 1998) are invaluable to the progress of *in situ* hybridisation. It has been observed that very small (3 bp) shifts in the choice of the binding site could increase the effective fluorescence by a factor of 8.

One of the latest developments in PCR product detection is DNA probes, called molecular beacons, that fluoresce only upon hybridisation to their target sequence (Tyagi and Kramer, 1996). At present, the implementation of molecular beacons to fluorescence *in situ* hybridisation is delayed due to a lack of knowledge of the mechanism of the different aspects of FISH-staining. Molecular beacons possess enhanced specificity that results in brighter fluorescent signals. Such enhanced specificity is a general property of conformationally restrained probes (Bonnet *et al.,* 1999). This can be a useful feature to take into consideration when designing hybridisation probes, but also primers for PCR amplification. Molecular beacons allow the reliable detection of point mutations, something that cannot easily be done by using conventional linear probes, and their specificity can be adjusted more extensively. Among the greater advantages of using molecular beacons may be the redundancy of post-hybridisation washing and closed-tube, *i.e.*, cross contamination free PCR and detection.

Another advance in probe technology is the development of peptide nucleic acid (PNA) probes (Nielsen *et al.,* 1994; Nielsen, 1999). These uncharged peptide nucleic acids with a carbon-nitrogen backbone exhibit much higher target specificity as well as much higher hybridisation rates. PNA probes exhibit a sharp fall in melting temperature when encountering a mismatch, and therefore, like molecular beacons, allow for the reliable detection of point mutations. Furthermore, the chemistry permits for a higher level of hydrogen-bonding between probe and target, consequently, PNA probes can be shorter (8-15 nt) than conventional oligonucleotide probes (15-30 nt). Although primer extension is not possible with PNA, PNA-DNA chimeras have recently been produced that would support PCR applications (Uhlmann, 1998).

High-Throughput and DNA Array Technology

The emergence of genomics as a new field of scientific exploration has re-emphasised the need for rapid and cost-effective molecular biological technologies. New molecular diagnostic methods have emerged from fundamental research that use radically new approaches such as the "lab-on-a-chip" principle (Service, 1995). These microfabricated systems are intended to make complete laboratories redundant whilst facilitating fully automated massive parallel analyses using a minimum of reactants and are christened μTAS, for micro Total Analysis System (Manz *et al.,* 1991). Due to its interdisciplinary nature, much of this work occurs outside the vision of most biologists. We will address some of the recent advances made in this area.

Future molecular biological methods will use many of the technologies now common to the semiconductor industry. Microlithographic etching of silicon surfaces, for example, allows for the creation of miniaturised fluidic systems in which biomolecules are mixed, reacted, separated and detected in much the same way as in the classical laboratory but on a much smaller scale (Jacobs and Foder, 1994; Woolley *et al.,* 1996). Microfluidic systems, etched into glass or silicon, have been developed that represent pumps, multichannel dispensers, pipettes, valves, injectors and mixers (Howitz, 1999). By combining

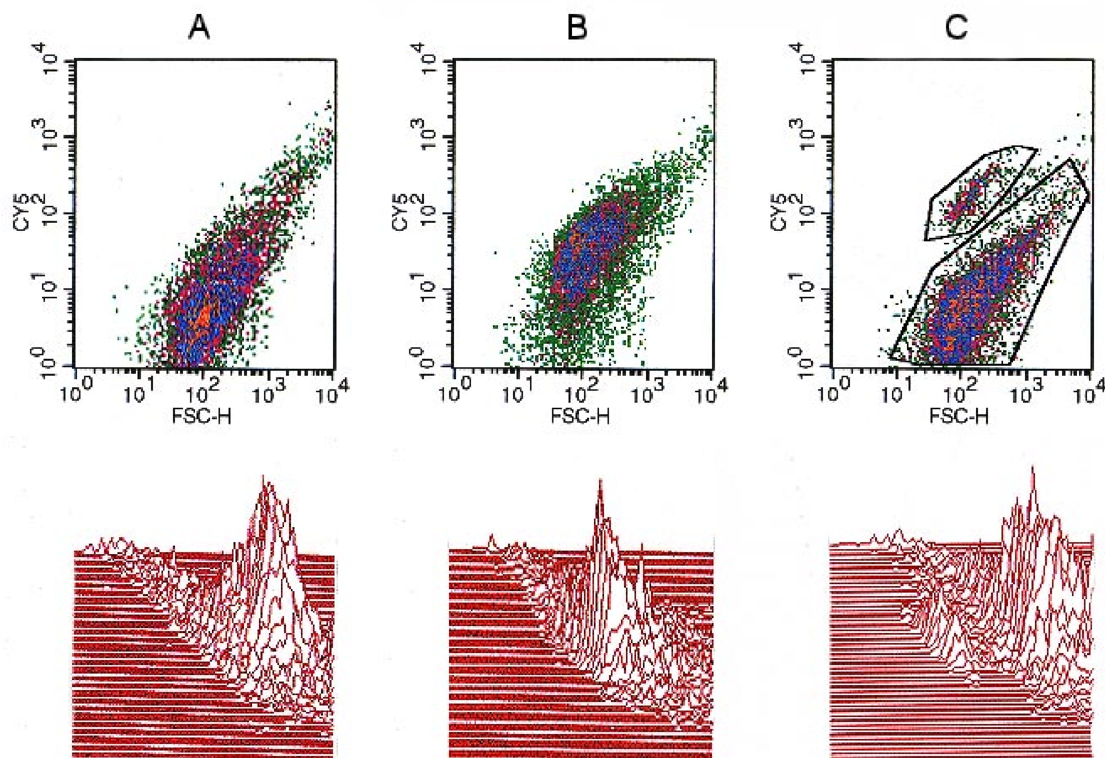

Figure 3. Flow cytograms of human faecal microflora stained by FISH with Cy5-labeled 16S rRNA-targeted oligonucleotide probes. A negative control probe (Non-Eub338, panel A) and a positive control probe (Eub 338, panel B) reveal the distribution of autofluorescence and FISH-specific fluorescence among the total population. Hybridisation with a *Bifidobacterium*-specific probe reveals a distinct sub-population with above-threshold fluorescence that can accurately be enumerated (panel C). Top panels represent plots of Cy5-fluorescence versus forward light scatter with pseudo-colours indicating (particle or cell) counts. Bottom panels represent 3D plots of the same analyses and reveal the bifidobacteria as a distinct sub-population (panel C) (Schut and Tan, 1999).

such components on one single chip (a wafer-thin quartz or silicon substrate of 3-6 inches in diameter) with a temperature-cycling microsystem, 30-cycle amplifications with subsequent detection of specific DNA can be performed in under 20 minutes (Tretyakov *et al.,* 1999). Thus, the major advantages of these microfluidic systems will be speed of analysis for tens to thousands of samples, for example sequencing or restriction enzyme mapping of DNA, and secondly, the possibility to do experiments in non-laboratory conditions.

Another development is that of DNA microarrays, also called biochips, gene chips or DNA chips. DNA microarrays are basically glass surfaces bearing arrays of numerous covalently attached DNA fragments that are available for hybridisation (Chee *et al.,* 1996; Schena *et al.,* 1996). Current applications include monitoring expression of the arrayed genes in mRNA species of living cells or detecting DNA sequence polymorphisms or mutations in genomic DNA.

Two methods currently in use for the production of arrays are post-synthesis immobilisation of DNA spots or *in situ* synthesis of oligonucleotides. The latter involves photolithography, the technology by which light is used to build oligonucleotides from their building blocks on a pre-defined location of the chip. This results in nanometer sized hybridisation spots and allows for the formation of extremely high-density arrays that can display over 100,000 oligonucleotides (Jacobs and Fodor, 1994). This technology is, however, still unaffordable for many laboratories. A myriad of post-synthesis immobilisation procedures has been developed that are more efficient when low-density arrays (*e.g.*, 1000 clones) are produced (Eggers *et al.,* 1994; Guo *et al.,* 1994; Lamture *et al.,* 1994; Joos *et al.,* 1997; O'Donnell *et al.,* 1997). Alternative technologies that allow high-density immobilisation of oligonucleotides are also being developed (Ermantraut *et al.,* 1995). At present, microscope slides are commercially available that allow direct immobilisation of oligonucleotides and hybridisation procedures are often conventional.

A novel approach to DNA sequencing, called sequencing by hybridisation (Mirzabekov, 1994), represents a breakthrough in the development of the discipline of genomics. This technology has emerged as a result of potential difficulties encountered in human genome sequencing that could take decades by conventional sequencing. By comparison, one hundred gene chips could sequence a human genome in several hours. However, the goal of sequencing by hybridisation would be to provide rapid, inexpensive automated DNA sequencing for genomes of all organisms. Reality is, of course, less than ideal and sequencing by hybridisation is still under development. Possibilities such as the combined use of high density arrays and MALDI-TOF MS machines (Matrix Assisted Laser Desorption Ionisation-Time-of-Flight Mass-

Spectrometry) may provide for alternative DNA sequencing methods in the future. By using this technology it is possible to determine the DNA sequence of an 80-bp fragment present in an individual array spot in milliseconds (Eickhoff *et al.,* 1999).

Gene chip arrays offer more exciting applications. Arrays of multiple oligonucleotides specific for certain inheritable gene disorders, arrays of cDNA obtained from whole-cell messenger RNA pools, or a large number of rRNA gene sequences can reveal within minutes the presence of genetic disease, proliferation of carcinomas, or the structure of complex microbial populations, respectively. The potential of microarray technology in microbial ecology studies was demonstrated using microchips containing oligonucleotides complementary to 16S rRNA sequences of nitrifying bacteria that could detect and identify the DNA or RNA isolated from samples containing the target bacteria (Guschin *et al.,* 1997).

Although there are still some technical challenges ahead to make the technology more accessible and affordable, chip technologies offer great advantages for the future. No important microbiological applications have yet been developed, although its use for microbial diagnostics and, in this context, the monitoring of complex microbial populations such as those of the human intestinal tract certainly deserve attention.

In conclusion, the current molecular approaches are providing considerable insight into the diversity of our complex gastrointestinal microflora. We have gone a long ways towards predicting the usual species that are encountered in the intestinal tract of the infant, child and adult. However, there are still remaining doubts about the type of microbes and their numbers in the intestinal tract. Furthermore, we have not really taken into account the succession of microflora in an individual from infancy to old age. And what of the remarkable observations made using molecular methods that each human subject has a predominant microflora that is unique and stable, and that each individual harbours long-term bacterial strains that are unique to that individual? Are we underestimating the complexity of the gastrointestinal microflora? Finally, the question must be addressed as to what activities the different microbial cells have in the intestinal tract. Interdisciplinary research efforts will correlate the presence of a particular intestinal microbe with certain activities and consequent effects on the host. It is possible that detailed knowledge of the normal intestinal microflora of each individual person will be required to appreciate the effects of medication, diet, age, lifestyle, and encounters with new types of bacteria. Only further progress in high throughput strategies can place information concerning the intestinal microbes and subsequent impact on our health within our grasp.

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