16S rRNA Hybridization Probes for the Major Groups of Intestinal Bacteria: Development and *in situ* Application

'*Whenever a man gets the idea that he is going to work out the bacteriology of the intestinal tract of any mammal, the time has come to have him quietly removed to some suitable institution*'. Jordan, *In*: Gorbach *et al.*, 1967.

Abstract

Investigations into the role of the gut microflora in pathogenesis are continuously frustrated by technical limitations. We are currently developing 16S rRNA targeted oligonucleotide probes for the enumeration of various phylogenetic groups of intestinal bacteria. Preliminary results show that bifidobacteria are grossly overestimated by cultural methods, and that members of the *Bacteroides vulgatus* cluster are underestimated. Microscopic image analysis in combination with 16S rRNA hybridization probes has proven to be a very powerful technique in ecological studies of intestinal flora.

The intestinal microflora

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 consist of a mixed culture of an estimated 100–400 species of bacteria (Moore and Holdeman, 1974). The initial inoculum is derived from the mother at the time of birth and although the climax flora alters as the subject ages (Mitsuoka, 1992) it is fairly constant in composition with *Bacteroides* and *Eubacterium* spp. dominating in adults. The stability of the gut flora can be appreciated when bearing in mind that food and drinks are in actuality minor components of the intestinal contents. Saliva (1.0 litre/day), gastric (1.5 l/d) and pancreatic (1.2 l/d) secretions, bile (1.0 l/d) and secretions of the glands in small and large intestine (2.0 l/d) form the bulk of the estimated 7 litres of fluid that enters the intestinal tract daily (Guyton, 1986). These highly concentrated endogenous secretia ensure a constant intestinal environment thus enabling the digestive enzymes to operate under controlled conditions.

The composition of the diet, and the administration of specific food sup-

plements such as fibre, has been suggested to influence the composition and activity of the flora (Benno *et al.*, 1989; Mitsuoka, 1992). However, direct evidence is difficult to obtain due to statistical inaccuracy of the cultural technique employed (Gorbach *et al.*, 1967; Finegold *et al.*, 1974), a high inter-subject variability (Minelli *et al.*, 1993), the relatively low numbers of subjects that can be investigated (Drasar *et al.*, 1986), and the difficulty in determining causative relationships from observed correlations (e.g. Benno *et al.*, 1989).

Over 95% of the bacteria encountered in faeces are non-sporulating, strict anaerobes and pre-reduced, anaerobically sterilized (PRAS) media or anaerobic chambers are essential for cultivation of these organisms (Holdeman *et al.*, 1977). Although it is relatively easy to obtain a total viable count, counting of individually identifiable bacterial species is laborious and time consuming. Viable counts of the various aerobic and facultatively anaerobic species can be obtained by using selective media. However, such media are not available for most of the strict anaerobes. Since some 30–40 species account for 99% of the flora, several hundred isolates from each sample should be identified for reliable statistics and end-product analysis of fermentation in pure cultures is essential for reliable identification. As a result, studies on population dynamics of the intestinal flora are often limited in the number of subjects or the number of species investigated and, as a consequence, the observed changes in composition are often not statistically significant.

Clinical relevance: intra-abdominal sepsis as a case

Due to the advances in medical technology and the significant elevation in live expectancy of the severely ill, an increasing number of patients in hospitals have compromised immune systems. Subjects undergoing major surgery (e.g. transplantations), chemotherapy as a treatment for cancer, and those with major trauma form the majority of such patients. Concurrently, the type of infection has changed from predominantly pathogenic to potentially pathogenic, or opportunistic. Furthermore, antibiotic resistance among nosocomial infections is becoming a world-wide threat and treatment may soon become ineffective. It is expected that alternative strategies for infection prevention will arise from knowledge on the natural physiological control of potential pathogens. However, up till now such studies have rarely been performed.

Although probably not as important on a world-wide scale as colon cancer, intra-abdominal infections are in this respect of significant interest due to the presumed involvement of normal host immunity, gut flora, and antibiotics. Almost all bacteria isolated from intra-abdominal infections are indigenous to the intestinal tract. Whether they have escaped from the intestine by 'naturally' occurring translocation or after perforation of the gut wall is the subject of debate (Sedman *et al.*, 1994), but the excessive accumulation of opportunistic microbes on the mucosal surface is believed to be very hazardous in this context (McClean *et al.*, 1994). The predominance of certain isolates does not always

correlate with the use of antibiotics (Baron *et al.*, 1992; Sawyer *et al.*, 1992). It is probably through changes in the structure of the mucous colonizing community as a whole that certain opportunists gain a foothold (Kennedy and Volz, 1985; Van der Waaij, 1989). Antibiotics greatly affect the community structure and for both *Candida* and enterococcal intra-abdominal infections antimicrobial therapy has already been identified as a major risk factor (Kujath *et al.*, 1990; Boulanger *et al.*, 1991).

So, although immediate antimicrobial treatment during peritonitis is essential, especially in the case of *Candida* infections, the increased use of antimicrobial agents in immunocompromised hosts will promote changes in the normal intestinal flora and relatively harmless organisms can suddenly give rise to serious complications (McClean *et al.*, 1994).

The human intestinal flora is responsible for a number of other health disorders. Through the production of carcinogens, some species of the intestinal flora are believed to play a role in the development of colon cancer. Crohn's disease, rheumatoid arthritis and chronic diarrhoea, are all believed to be related to intestinal bacteria or their products. Furthermore, there are indications that graft versus host reactions after transplantation are also influenced by the gut flora. The development of fundamental insight in the interaction between the healthy host and its gut flora is of primary importance to the understanding of the pathogenesis of these diseases.

The use of 16S rRNA probes for enumeration

The above clinical examples serve to illustrate the need for detailed and rapid community-level screening of the composition of the intestinal flora. Only when changes in the composition are accurately described can relationships with pathogenesis be recognized. The possibility of using 16S rRNA hybridization probes for this purpose deserves investigation.

Each living cell contains ribosomes for protein synthesis. Several tens of thousands of ribosomes are present in growing cells. Because of evolutionary changes, the sequence of the nucleotides in the rRNA molecules is unique in each bacterial species. Therefore, the bacterial ribosome is uniquely suited as a phylogenetic (evolutionary) or taxonomic marker (Olsen *et al.*, 1986; Pace *et al.*, 1986). Synthetic deoxyoligonucleotide probes can be constructed which hybridize specifically to a certain sequence of nucleotides in the rRNA molecules. The specificity of the oligonucleotide probes can be adjusted to fit any taxonomic level, from kingdom to subspecies. If a fluorescent molecule is attached to the probe, individual bacterial cells can be identified by using an epifluorescence microscope. Since the 16S rRNA sequence of over 2,000 bacterial species is currently known (Larsen *et al.*, 1993), most bacterial groups can be included in searches for taxon specific 16S rRNA sequences.

Presently, species and group-specific hybridization probes can be developed on a rational basis, the essence of which lies in the identification of a unique

sequence of approximately 20 nucleotides in the sequence of the target organism(s). Shorter stretches (10 bases) harbour the danger that the same sequence occurs at other (unforeseen) regions in one of the three ribosomal RNA's. Long sequences (50 bases) have the disadvantage that the mismatches - necessary for discrimination between target and non-target cells - do not result in a significant decrease of the dissociation temperature of the hybrid complex. This dissociation temperature can be estimated from the relationship

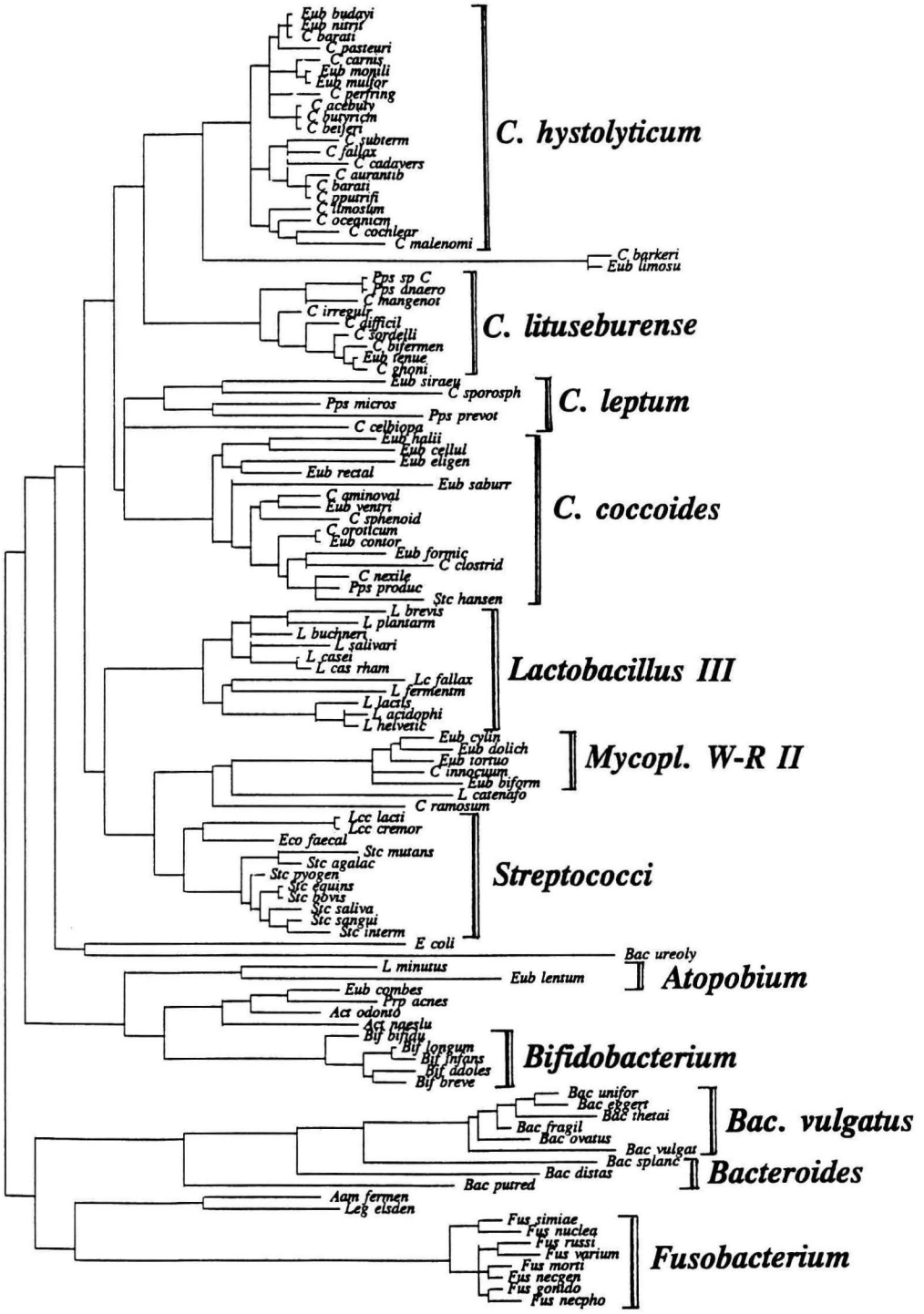
$T_d = 81.5 + 16.6 \log(Na^+) + 0.41(\%G + C)\text{-}820/\text{probelength}$
(Stahl and Amann, 1991).

The 16S rRNA contains various 'conserved' regions essential for the biochemical function of the ribosome. Such regions are used as annealing sites for primers used in PCR amplification and sequencing of the 16S (Lane, 1991). Other regions are hypervariable by nature and can serve as target regions for species and subspecies-specific hybridization probes. Sequences of intermediate specificity can also be found and these can be used to distinguish higher taxonomic groups.

During an extensive study, using cultural methods of enumeration, some 200 species of bacteria have been isolated from human faecal samples (Finegold *et al.*, 1974). Approximately 30 of these are numerically important and represent 10 distinct genera. Genus specific probes could thus prove appropriate in describing community structures at large. However, the natural classification system for bacteria, based on phenetic characteristics, is known not to comply fully with genetically based methods: not all taxonomic genera are monophyletic on the basis of their 16S rRNA sequence. *Eubacterium* spp., for example, are clustered with several *Clostridium* and *Peptostreptococcus* spp. and peptostreptococci themselves are spread over several distinct phylogenetic clusters (fig. 1). Within these phylogenetic clusters, however, conserved regions can be identified and 16S rRNA probes designed for such regions can be used to identify the majority of the population to a well defined taxonomic level. Only a dozen probes is thus required for the enumeration of the various phylogenetic groups of the gut flora (fig. 1).

Although many copies of the ribosome should ensure sufficient signal, reality is often far from ideal. The accessibility of the target region, the number of fluorochromes attached to the probe, the hybridization temperature, the metabolic status of the cells and the level of autofluorescence of non-target cells are important factors in obtaining sufficient specific fluorescent signal. Optical aid in the form of CCD-camera's is often required for small and slowly growing cells with little rRNA. Image analysis can be very helpful in discriminating between non-fading autofluorescence and rapidly fading fluorochrome

Fig. 1. (opposite page) Unrooted distance tree obtained after Kimura-2 parameter analysis and neighbour joining of 200 nucleotides of the 16S rRNA sequence of some bacterial species isolated from human faecal samples. Analysis was performed using MEGA software. The assignment of the twelve large phylogenetic clusters is supported by findings of the RDP project (Larsen *et al.*, 1993). Fluorescent oligonucleotide probes have been developed for all clusters assigned.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L fermentm
L lactis
L acidophi
L helveti
Lactobacillus III
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Eub tortuo
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Eco faecal
Stc mutans
Stc agalac
Stc pyogen
Stc equins
Stc bovis
Stc saliva
Stc sangui
Stc interm
Streptococci
E coli
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Eub lentum
Atopobium
Eub combes
Prp acnes
Act odonto
Act naeslu
Bif bifidu
Bif longum
Bif infans
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Bac thetai
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Bac ovatus
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Bac splanc
Bac distas
Bacteroides
Bac putred
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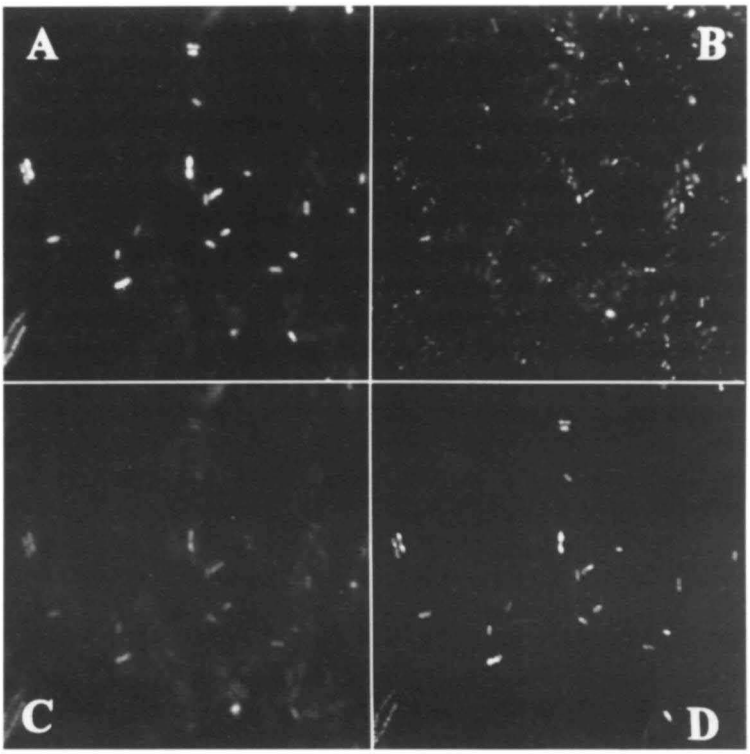

Fig. 2. Fluorescence lifetime imaging of walled mycoplasmas subgr. II in human faecal flora using 16S rRNA hybridization probes. For a period of 8 sec FITC fluorescence is recorded (panel A). Subsequently, a DAPI image is recorded under UV illumination (panel B). After 20 sec of UV illumination during which FITC fluorescence but not autofluorescence fades significantly, a second FITC image is recorded (panel C). Graphical subtraction of the image in panel C from that in panel A produces the frame in panel D with clearly distinguishable hybridized cells.

fluorescence (fig. 2). Using such fluorescence lifetime imaging techniques, only a few positively hybridized cells can be distinguished among several thousand non-target cells in a microscope field (manuscript in preparation). This is necessary to enumerate those bacterial clusters that form a minority of the population, (*e.g.* streptococci).

Whole cell in situ hybridization of faeces

We have developed a relatively simple protocol for the enumeration of hybridized cells in faecal samples (fig. 3): A known quantity of homogenized faeces is resuspended in phosphate buffered saline (PBS). For cultivation, appropriate dilutions are made and cells are inoculated on the surface of selective and non-selective agar media. Plates are incubated anaerobically at 37°C. For microscopic enumeration and probe hybridization, cells are fixed for a mini-

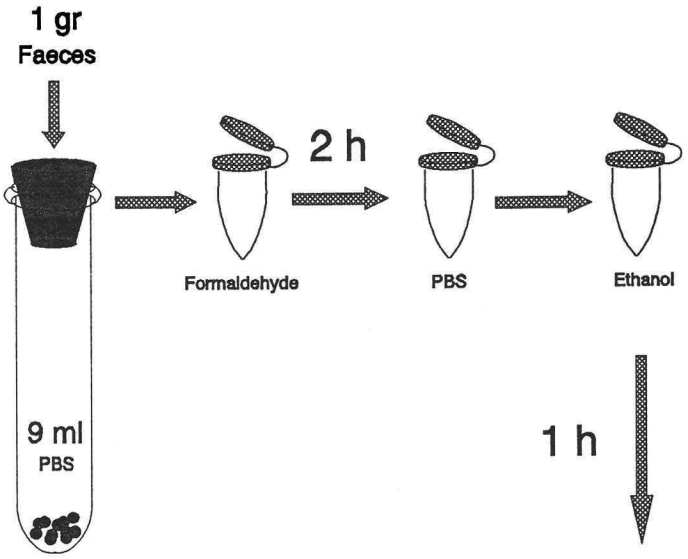

Fig. 3. Protocol for the hybridization and enumeration of faecal bacteria.

mum of 2 hrs in 4% paraformaldehyde. Debris is separated from bacterial cells by low-spin centrifugation. Cells are pelleted, washed in PBS and suspended in gradually increasing concentrations of ethanol (to 75%). After pelleting, the cells are resuspended in hybridization buffer and FITC-labelled probe is added. Hybridization is carried out at a temperature 2°C below the dissociation temperature of the probe for a minimum of 2 hrs. After hybridization, cells are stained with 4′,6-diamidino-2-phenylindole (DAPI), filtered onto a 0.2 μm pore-size polycarbonate filter and washed with hybridization buffer at hybridization temperatures. The filter is mounted on a microscope slide and viewed under an epifluorescence microscope equipped with the appropriate excitation and emission filters for FITC and DAPI. An image analysis system (Wilkinson, 1995) is used when fluorescence signals of target cells are too low to allow proper enumeration by eye.

Laboratory trials

We investigated the population of bifidobacteria in the faecal samples of ten volunteers. During this study, cultural methods were compared with the 16S hybridization method. A genus-specific *Bifidobacterium* probe targeted for position 164 (Bif164) was used (Langendijk *et al.*, submitted). The mean number of culturable bifidobacteria ($\pm$S.E.) from all samples was 2.45 ($\pm$1.40)x10^9 per gram of wet faeces. The mean number of total culturable anaerobes on BBA from all samples was 3.87 ($\pm$1.73)x10^{10} per gram of wet faeces. On average, bifidobacteria accounted for 6.9% $\pm$ 3.3% of the total culturable population. For

all ten individuals investigated, the total counts of faecal bacteria as obtained on non-selective anaerobic brucella blood agar were significantly lower than the total microscopic counts on membrane filters with DAPI as a nuclear stain.

The membrane filter technique was also used to enumerate the number of bacteria that hybridized with probe Bif164. In theory, all bifidobacteria present in faeces, including those that are nonculturable on agar media, should be detected when using this probe hybridization technique. In analogy to the discrepancy between cultural counts and DAPI total counts, we expected the cultural counts of bifidobacteria to be considerably below probe hybridization counts. By contrast, the number of culturable bifidobacteria enumerated on BIF agar (Sutter *et al.*, 1985) was not significantly different from the microscopic count using the *Bifidobacterium* probe Bif164 (av. 2.38×10^9 per g wet faeces). Based on DAPI total counts, *Bifidobacterium* spp., on average, accounted for $0.8\% \pm 0.4\%$ of the total population. This implies that nearly all bifidobacteria in human faeces are culturable, possibly as a result of their oxygen tolerance and that the contribution of bifidobacteria to the total intestinal microflora is largely overestimated when using cultural methods as sole method of enumeration. Such overestimations can approach an order of magnitude.

In a second study, we have enumerated members of the *Bacteroides vulgatus* subgroup (*Bacteroides fragilis* group). Cultural methods of enumeration result in counts half of those obtained when using a subgroup-specific hybridization probe. Currently we are developing and testing other phylogenetic group-specific probes for detection of major subpopulations of bacteria in human faeces. Included are probes specific for streptococci, the *Clostridium coccoides* subgroup, *Bacteroides distasonis* (species-specific), and subgroup II of the walled mycoplasmas (Larsen *et al.*, 1993) (see also fig. 2). Together these probes 'cover' around 50% of the total microscopic count in faecal samples.

Conclusions and prospects

Phylogenetic group-specific 16S rRNA hybridization probes can successfully be used for the enumeration of the major subpopulations of bacteria in human faeces. This method has several advantages to the classic cultural method for enumeration. The most important one is the relative ease and speed of the technique. With FITC-labelled 16S probes several samples can be processed per day. Once large-scale dynamics in composition of major subpopulations have been established species-specific probes can be used to elucidate the more detailed dynamics within such phylogenetic clusters. Before this can be achieved, the sensitivity of the technique is limited to threshold population densities of 1 positive cell per 500 negative cells, i.e. around 0.2% of the population. Several species of the intestinal flora, specifically the aerobic and facultatively anaerobic potentially pathogenic bacteria, drop well below this level. This is where the cultural technique is superior.

The use of flow cytometers, in which several tens of thousands of cells can be analyzed within minutes, will prove useful in the future. Flow cytometry in combination with 16S probe hybridization dramatically increases the number of samples that can be analyzed per study. In preliminary trials we have enumerated six phylogenetic clusters of intestinal bacteria in a single sample, some with good precision when compared with microscopic counts, in less than a day. The hybridization time being the rate limiting step. Although several problems related to signal to noise ratio still have to be overcome, 16S rRNA hybridization has already proven to be a very powerful technique in ecological studies of intestinal flora. Investigations into the dynamics of the community structure will help to fill the gaps in knowledge on the interaction between the host immunity and gut microflora. Such knowledge is crucial to the understanding of gut flora related pathogenesis.

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