Molecular Characterization of Bacterial Communities in the Human Gastrointestinal Tract

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Stellingen

1) Eeneiige tweelingen lijken ook wat betreft hun bacteriële samenstelling in de darm meer op elkaar dan op andere mensen.
Dit proefschrift

2) Fermentatieve bacteriën met relatief eenvoudige voedingsbehoeften zijn niet altijd gemakkelijk op een vaste voedingsbodem te kweken.
Dit proefschrift

3) Door de enorme toename van het aantal 16S rDNA sequenties van ongecultiveerde bacteriën in databanken, is door het bos de boom nauwelijks nog te zien.
Maidak et al. (2001) Nucleic Acids Res. 29: 173-174

4) Op basis van enkelvoudige waarnemingen kan geen uitspraak gedaan worden over PCR fouten.
Wilson and Blitchington (1996) Appl. Environ. Microbiol. 62: 2273-2278
Whitford et al. (1998) Anaerobe 4: 153-163

5) Gezien de definitie, is een reincultuur van bacteriën niet te kweken.

6) Voor de medaille-ranglijst van de Olympische Spelen verdient het de aanbeveling om het aantal behaalde medailles door het aantal inwoners van het betreffende land te delen.

7) Hoe dichter je bij Veenendaal komt, hoe vaker "Zoetendal" automatisch met twee a's wordt geschreven.

Stellingen bij het proefschrift 'Molecular Characterization of Bacterial Communities in the Human Gastrointestinal Tract'.

Erwin Zoetendal, Wageningen, 13 November 2001.

Contents

Chapter 1

General Introduction

The mammalian gastrointestinal (GI) tract can be regarded as an ecosystem, in which contributions of the microorganisms, epithelium, the immune system, and compounds that escaped digestion by the host have a major impact. This results in a very dynamic and complex ecosystem that is, however, difficult to study notably because of the complexity of the microbial community. This chapter will briefly summarize some characteristics of the GI tract and its microbes, which have lead to the aim of this thesis.

THE GI TRACT

The GI tract can be regarded as a tube extending from the mouth to the anus which is divided into several well-defined anatomical regions. The GI tract has several functions from which the digestive and absorptive functions of nutritious compounds are most well known. After intake of food, the digestion starts in the mouth and compounds that can easily be digested by the host are already absorbed before they reach the large intestine. Enzymes, such as glycosidases, lipases, peptidases, and proteinases play a key role in the digestion of food compounds (reviewed by 5).

The GI tract harbors a bacterial community consisting of approximately 10^{14} cells and therefore it outnumbers the total cells of the human body by one order of magnitude (25). The epithelium of the GI tract is covered by a mucus layer which protects the GI tract against mechanical and enzymatical damage as well as bacterial invasion. In addition, this continuous renewing mucus layer serves as an additional carbohydrate source for the bacterial community. The bacteria in the GI tract are not equally distributed. The stomach only harbors a small number of bacteria, because of the low pH (pH~2 when gastric acids are produced). The bacterial number in the small intestine is also low, because of the short transit time (~5h) of the contents. The highest bacterial number and diversity are found in the colon, because it has a relatively large transit time (~60 hours). It is estimated that this bacterial community makes up to 55% of the fecal solids (reviewed by 5).

The human colon is about 150 cm long with a surface area (undissected) of approximately 1300 cm^2. It contains on average 220 g of contents from which the moisture content is approximately 86% in the caecum, falling to 77% in the sigmoid-rectum. The pH shifts from 5.4-5.9 in the beginning of the colon to 6.6-6.9 to the end. Therefore, the physiological conditions vary from beginning to the end of the colon. In the colon water and

salts are absorbed. It is estimated that approximately 1.5 kg of material is entering the colon each day while approximately 120 g of stool are excreted each day. Because of bacterial fermentation of compounds that escaped digestion by the host the main end products found in the colon are acetate, propionate, and butyrate. These short chain fatty acids (SCFA) provide a powerful driving force for movement of water out of the colonic lumen and consequently, may constitute an important protection against diarrhea. It is estimated that epithelial cells in the colon obtain 60-70% of their energy from bacterial fermentation products (reviewed by 5).

CULTIVATION OF GI TRACT BACTERIA

The GI tract harbors a diverse bacterial community. Since the description of "*Bacterium coli commune*" in 1885 (7) many attempts have been made to isolate and characterize bacteria in the human GI tract and trying to understand their function. During the first half of the 20th century research of the bacteria in the GI tract was not so intense. An increase in interest on the GI tract bacteria appeared after several groups in the sixties started extensively studying their function in the GI tract. The major findings from this period and their importance have recently been summarized and discussed (26).

During the seventies the cultivation approaches were optimized after applying the anaerobic incubation techniques, developed by Hungate (17), to cultivate microorganisms from the human GI tract. A major increase in numbers of isolates from the GI tract has been achieved, notably by Moore, Holdeman, Finegold, and their colleagues (8, 9, 15, 16, 23). The most intensive culturing study performed has been described by Finegold and colleagues (9). In this 10 years during study fecal samples from 141 volunteers having various diets and disease states were analyzed. A high similarity in bacterial composition was found between the volunteers despite the differences in diet and disease state. These and other cultivation studies revealed many novel isolates, that the GI tract is dominated by strict anaerobic bacteria, that number of colony forming units per gram of wet weight feces reaches 10^{10} – 10^{11}, and that the dominant community is relatively stable. Even up to now still novel species are being isolated from the human GI tract. It has been estimated that up to 400 bacterial species may be present in the GI tract of healthy volunteers (6). Although many variations in composition were found in various studies, the main genera which were commonly detected include *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Fusobacterium*, *Peptostreptococcus*, and *Ruminococcus* (reviewed by 31).

Since the different parts in the GI tract are hardly accessible, the majority of studies have been performed using feces as inoculum. Only in some cases, the bacterial community at other parts in the GI tract have been studies by taking samples from biopsies and intestinal fluids, from sudden-death victims, or during surgery (2, 3, 11, 12, 18, 21, 37, reviewed by 9).

Although the culture-dependent studies gave many new insights in the distribution of the bacteria in the GI tract, they do not give a complete picture of the bacterial composition. For many ecosystems the so-called "great plate count anomaly" has been observed by comparing those counts and direct microscopic counts (4, 28). The culturability of bacteria from several habitats was found to vary between less than 1 to 15% (4). Similar comparisons have been made for bacteria from the human GI tract and it is estimated that between 10 and 50% can be obtained in culture (19, 22, 30, 35). Moore and colleagues reported that they could cultivate 93% of the total microscopic counts, although they mentioned that their microscopic counts is likely underestimated because of the staining procedure and counting clumps of cells instead of single cells (23). In addition, it has been found that the specificity of selective media is often questionable (13, 14). Although these culturability estimates are higher than observed for other ecosystems, the majority of bacteria has not been obtained in culture. Therefore, culture-independent approaches are required to study the ecology of the bacterial community in the human GI tract.

THE 16S rRNA APPROACH

The necessity of culture-independent approaches to study the ecology of microorganisms in different environments resulted in the introduction of a novel discipline called Molecular Ecology. Molecular Ecology is an emerging discipline that uses the detection of so-called biomarkers to study the ecology of microorganisms. These biomarkers can be any biological component that indicates a feature of a particular group of microorganisms. Ideally, these biomarkers are specifically detected and decomposed after cell death. Therefore, biomarkers could be cell components like proteins, lipids, DNA, RNA and even the cells themselves (24). For more than a decade, culture-independent techniques using the small subunit ribosomal RNA (SSU rRNA) sequence variability as biomarker have been added to or replaced by the conventional culturing techniques. There are various reasons to use these SSU rRNA as biomarkers (4, 36). These include (a) their presence in all cells, (b) their high degree of sequence conservation which facilitates their detection, (c) the presence of highly variable

regions in their sequences which makes them useful to discriminate at (sub)species to higher phylogenetic levels, and (d) the availability of many sequences from various organisms and cloned amplicons from a variety of ecosystems. Approximately 20,000 SSU rRNA sequences have been deposited in databases (20). Therefore, they facilitate a reliable phylogenetic characterization of cultured and uncultured microbes.

Table 1. Comparison between viable, dot blot, and FISH counts of fecal bacteria from different individuals.

	Mean counts in percentages		
Target group	**Cultivation**[a] (9)	**Dot Blot** (27)	**FISH** (10)
Bacteroides	59	36.8[b]	20[b]
Bifidobacteria	5	0.7	3
Eubacterium/Clostridium/Ruminococcus	21	14.5[c]	29[c]
Fusobacterium prausnitzii-group	<1[d]	16.3[e]	12
Coverage	10-50[f]	70[g]	64[g]

[a] Percentages for cultivation are calculated from the sum of all target groups found by Finegold and colleagues (9).

[b] The Bacto1080 and Bfra602/Bdis656 probes were used for dot blot and FISH, respectively.

[c] Data from the Erec482 probe, which covers a significant fraction of eubacteria, clostridia, and ruminococci.

[d] Percentage total fusobacteria including *F. prausnitzii*.

[e] Counts of the *Clostridium leptum*-group which include *F. prausnitzii* and phylogenetically related bacteria.

[f] Estimated culturability based on comparisons between microscopic and plate counts.

[g] Percentage of sum of all probe signals divided by the signal of the bacterial probe (Eub338).

The application of bacterial and archaeal SSU rRNA (or 16S rRNA) sequence information to study microbial communities in a culture-independent way is called the 16S rRNA approach (4). The past decade shows an explosive increase of studies in which the 16S rRNA approach was used to study various ecosystems. As a result many cloned amplicons have been sequenced and deposited in DNA databases (20). In common, all these studies confirmed the observation that the majority of bacteria in an ecosystem cannot be cultured (reviewed by 4). The past years, the application of the 16S rRNA approach to characterize and monitor bacterial communities in the human GI tract is increasing. The number of reports and review articles describing the 16S rRNA sequence diversity in the GI tract of various animals

by fluorescent in situ hybridization (FISH) and PCR-based cloning and fingerprinting techniques is increasing (reviewed by 1, 29, 32-34). These studies provided novel insights into our knowledge of the bacterial diversity in our GI tract. For example, comparison between FISH, dot blot, and viable counts published by different authors revealed that the number of bacteria related to *Fusobacterium prausnitzii* is underestimated by the viable count (Table 1).

OUTLINE OF THE THESIS

The aim of this work was to study the bacterial community in the human GI tract using 16S rRNA approaches. Using these approaches we studied the bacterial composition in the GI tract and estimated its host-specificity, temporal stability and the impact of host-related factors on the composition.

The outline of this thesis is as follows. Chapter 2 is an extensive review chapter describing the application, benefits, and drawbacks of fingerprinting approaches based on the 16S rRNA sequence variability to study the bacterial diversity in ecosystems, such as the GI tract. Chapters 3 to 5 mainly focus on the optimization and application of PCR-based 16S rRNA approaches and analysis of amplicons by temperature and denaturing gradient gel electrophoresis (TGGE and DGGE, respectively). In this way, the predominant and *Lactobacillus* group community in different GI tract samples were characterized by a combination of fingerprinting and identification of bands in the fingerprint by cloning and sequence analysis. The temporal stability, host-specificity and bacterial composition of these bacterial communities in the GI tract were determined. Chapter 6 focuses on the impact of the host genotype and the environment of the hosts on the predominant bacterial community in fecal samples from adults. The distribution of the predominant and *Lactobacillus* group bacterial community at different locations in the colon is described in Chapter 7. The impacts of the host and GI tract disorders were examined. Chapter 8 describes the FISH detection of an uncultured group of bacteria which have only been detected by PCR-based approaches in fecal samples of different individuals. Chapter 9 describes the isolation of a strain belonging to a novel genus and discusses the use of alternative cultivation approaches to isolate species from different ecosystems. Chapter 10 discusses the major findings described in the previous chapters. Chapter 11 and 12 give a summary of the thesis in English and Dutch, respectively.

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Chapter 2

Molecular Characterization of Microbial Communities Based on 16S rRNA Sequence Diversity

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INTRODUCTION

This decade has shown an impressive development in the application of molecular techniques based on 16S and 23S rRNA genes to study the microbial diversity in ecosystems. Several overviews highlight the possibilities and drawbacks of these molecular approaches in ecology (3, 42, 79, 122).

Before the rRNA approach, the composition of an ecosystem was investigated by the isolation and physiological characterisation of many microorganisms living in an ecosystem. The microbial composition of mammalian intestines, for example, has been studied extensively by plate count analysis of fecal samples, which usually contain 10^{10} - 10^{11} cfu / g (34, 43, 64, 91). One of the limitations in using these conventional microbiological methods is that easily cultivable microorganisms are detected, but not those that only grow on specific media, require unknown growth conditions, or have obligate interactions with the host or other microorganisms. Other limitations of cultivation include the selectivity of the media used, the stress imposed by cultivation procedures, and the necessity of strictly anoxic conditions. Estimates of the cultivability of GI tract microorganisms range from 10% to 50%, but may vary considerably between species or genera (53, 61, 121). As a consequence, insight into the function of the microbial community, its interactions with the host, and the influence of environmental factors on the microbial composition is very limited.

During the past decade, the rRNA approach has been used to study the microbial ecology of several ecosystems, and its application in ecological studies is still increasing. The first application of this approach in studying GI tract ecology was focused on the detection of *Bacteroides vulgatus* in fecal samples using a species-specific 16S rRNA-targeted probe (51). Recently, several populations in the GI tract have been monitored, resulting in quantification of *Bacteroides* populations by dot blot hybridization (23), and analysis of the genetic diversity of cultivable *Lactobacillus* and *Bifidobacterium* spp. (60). From the latter study, it was concluded that the microbial composition of lactic acid bacteria in the intestine varies according to each individual. Fluorescent *in-situ* hybridization (FISH) has also been used to quantify different phylogenetic groups in human fecal samples (37, 53). About two-thirds of the total bacterial community could be counted with the probes used. The polymerase chain reaction (PCR) has been used to quantify specific groups of bacteria in human feces (116), and random cloning approaches have been used to analyze the microbial diversity of feces

from single individuals (106, 121, 128). In one case, this analysis was combined with another powerful approach based on temperature gradient gel electrophoresis (TGGE) analysis of 16S rRNA and rDNA amplicons, resulting in identification of the most prominent and expressed sequences (128). In addition, individual differences and temporal changes in the predominant microbial GI tract community could easily be monitored using this approach. TGGE and other fingerprinting techniques, including denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP) analysis have been used in different ecosystems to rapidly analyze microbial communities based on sequence-specific separation of 16S rDNA amplicons (reviewed by 67, 69). This chapter describes the use, benefits and drawbacks associated with the application of these genetic fingerprinting approaches, which are based on the sequence variability of different 16S rRNA and 16S rDNA molecules, to study the microbial composition of different environments, such as the GI tract.

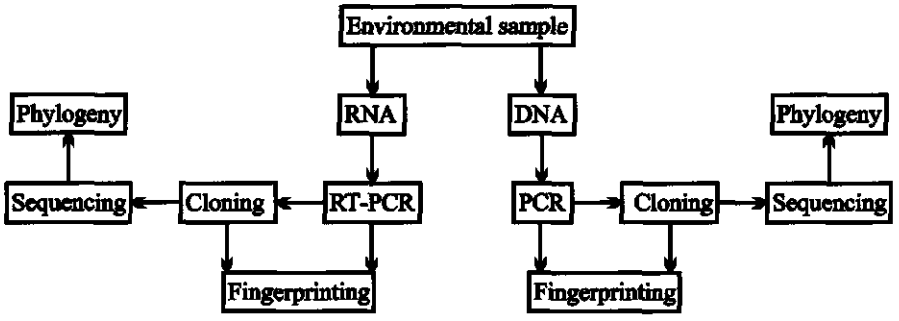


Fig. 1. Schematic outline of molecular approaches used to analyze microbial communities.

METHODOLOGY

To describe the bacterial diversity in communities, molecular approaches based on the sequence variability of the 16S rRNA gene can be used (Fig. 1). First, RNA and DNA have to be isolated simultaneously from environmental samples, and used as templates for amplification of fragments of the 16S rRNA gene by reverse transcriptase (RT)-PCR and regular PCR, respectively. Subsequently, the genetic diversity of the amplicons can be analyzed using different fingerprinting techniques. Additionally, a clone library of complete 16S rDNA and rRNA sequences can be made and divided into groups of different ribotypes

using the same fingerprinting techniques. Cloned fragments of the different ribotypes can be sequenced and analyzed phylogenetically. Comparison of the fingerprinting techniques and the cloning approaches may result in a reliable picture of the relative composition of numerically dominating microbes in a community. However, the results cannot simply be converted to total numbers of cells and the fingerprints only reflect the actual number of rRNA genes when each product is amplified equally.

EXTRACTION OF RNA AND DNA

When genetic fingerprinting techniques are used to characterize a microbial community, reliable extraction of DNA and RNA is the most critical step in the whole procedure because all further analyses are based on the extracted nucleic acids. Various nucleic acid extraction methods have been developed that can be applied to all kinds of ecosystems (1). While most of the reported isolation procedures are promoted as rapid, accurate, simple, or universal methods, a general protocol does not exist because all environments have their own characteristics and, as a consequence, require dedicated purification procedures. In general, procedures for the isolation of nucleic acids from microorganisms or environmental samples consist of three steps that will be discussed below: cell lysis, purification of nucleic acids, and isolation of nucleic acids (Fig. 2).

Cell lysis and purification of nucleic acids. One of the important steps in the extraction of nucleic acids from an environmental sample is the lysis of microbial cells. Equally efficient lysis of all cells in an ecosystem is necessary to obtain a reliable picture of the microbial community. Efficient cell lysis may be hampered by the different cell envelope composition of various microorganisms. Hence, a protocol which is suitable for one species may not necessarily be suitable for another species. Microbial cells can be lysed chemically, enzymatically or mechanically. Various Gram-negative bacteria can be lysed chemically by treatment with detergents, such as sodium dodecyl sulphate (SDS). Disruption of the cell envelope of Gram-positive bacteria by detergents needs prior treatment with enzymes such as lyzozyme, N-acetylmuramidase or other muramidases. Most of these lytic enzymes are restricted to a certain range of microorganisms, because the cell-envelope composition differs for each species (44). As a consequence, it is very difficult to develop chemical or enzymatic based lysis methods for complex communities. Therefore, procedures which include

mechanical cell lysis by French press disruption, freeze/thaw incubations, sonication, or bead beating are preferred (1, 44, 58).

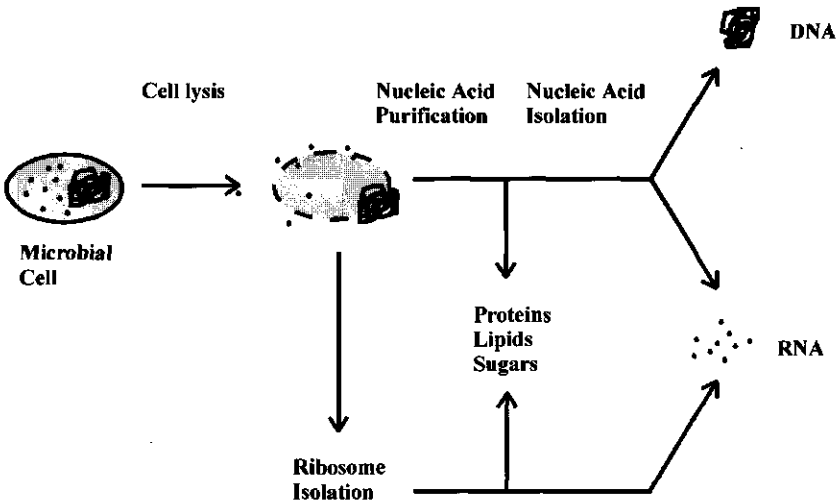


Fig. 2. Schematic representation of the procedures used to isolate DNA and RNA from a mixture of bacterial cells.

Bead beating is a widely used method to lyse bacterial communities, sometimes in combination with different chemical, enzymatic, or other mechanical lysis procedures. Bead beating has been shown to be successful in all kinds of samples, varying from soil systems to the mammalian GI tract (23, 29, 39, 85, 104, 121, 128). In this procedure, glass or zirconium beads are added to an environmental sample in a buffered solution and shaken vigorously (3000 to 5000 rpm). The beads collide during this treatment, thereby facilitating the disruption of cells between the beads. Phenol can be added to the sample to prevent enzymatic degradation of the nucleic acids during the bead beating procedure.

A disadvantage of this mechanical cell lysis is that nucleic acids are partly sheared, especially when fragile bacteria such as some Gram-negative species are involved. Sheared nucleic acids cannot be used for genetic fingerprinting methods based on intact genomic DNA, such as RAPD and RFLP. Shearing of nucleic acids may also result in a reduced recovery of bacterial DNA or RNA, or increase the formation of chimeric structures during amplification of certain genes (48). Determination of the optimal conditions for efficient cell

disruption is therefore very important. The easiest way is to examine the sample under the microscope before and after the lysis procedure (4). However, it may be difficult to distinguish between pieces of lysed cells and intact cells. Plate counting analysis of easily cultivable bacteria may give some indication of lysis efficiency when non-detrimental treatments are used during the lysis procedure (29). However, this may be difficult or cumbersome, notably when anoxic environments such as the GI tract are analyzed.

Another way to check for lysis efficiency is to determine the nucleic acid concentration before and after treatment. A disadvantage of this calculation method is that the genome size and ribosome number may vary according to cell and species, making the calculation less reliable. TGGE of 16S rDNA amplicons has been used to check the lysis efficiency of GI tract samples following different periods of bead beating (128). In this way, shearing of nucleic acids could be minimized by determining the minimal time required for maximal disruption of the cells. It was shown that at least 3 min of bead beating was necessary to lyse a *Ruminococcus*-like species in a fecal sample.

Following cell lysis, nucleic acids have to be purified, because most analytical procedures involve enzymes and require relatively pure DNA or RNA. Most proteins, carbohydrates and lipids can be removed using phenol and chloroform extractions (58). This purification procedure can be enhanced by addition of cetyltrimethylammonium bromide (CTAB), which forms complexes with the nucleic acids. It has been shown that CTAB extraction following bead beating of actinorrhisal nodules facilitated the recovery of DNA (85). High molecular size DNA can also be separated from contaminants by CsCl centrifugation. Additional steps for purification can be added to the protocol, although it should be kept in mind that each additional step results in a decreased yield of extracted nucleic acids. An alternative step is the addition of specific proteins, such as bovine serum albumine or protein gp32 to the DNA sample. These proteins have been shown to enhance the amplification efficiency of template DNA containing PCR-inhibiting compounds (50). Recently, improved DNA recovery from ancient fecal samples has been reported in which cross-links between reducing sugars and amino groups could be cleaved by adding N-phenacylthiazoliumbromide, thereby allowing for the amplification of DNA sequences (81).

Following purification, nucleic acids can be concentrated by polyethyleneglycol, isoamylalcohol or ethanol. Addition of sodium acetate facilitates the precipitation of nucleic acid fragments in ethanol. Instead of precipitation, nucleic acids can also be concentrated by binding to glass fibers or silica materials. Most commercial nucleic acid isolation kits rely on

this procedure.

When RNA is isolated, all procedures should be performed with special care. RNA is more sensitive to degradation than DNA because its ribose contains a 2′ hydroxyl group which makes RNA chemically less stable, especially under alkaline conditions. In addition, the double helix B structure found in DNA cannot be formed by RNA. Besides the chemical differences, RNase is much more stable than DNase, making removal of RNase more difficult and contamination with RNase easier. If possible, every step should be performed at 4°C or on ice, and equipment should be RNase free. Most procedures to isolate DNA and RNA are based on the principle of lysing the cells followed by direct purification of the nucleic acids. Another approach to obtain rRNA is based on the isolation of ribosomes (29, 33, Fig. 2). In this procedure, cells from soil samples are mechanically lysed, followed by the isolation of intact ribosomes by ultracentrifugation. RNA is then subsequently isolated from the ribosome collection and purified. This method, which may also be applicable to other ecosystems such as the GI tract, resulted in a high yield of purified 16S rRNA and 23S rRNA which could be used directly for RT-PCR.

A number of studies have reported the extraction of nucleic acids from fecal and rumen samples (4, 23, 104, 121, 128). Most protocols are based on bead beating cell lysis in phenol, followed by phenol/chloroform extraction. A different approach has also been described in which bacterial cells from feces are lysed by boiling in a phosphate-buffered saline (PBS) solution containing 1% Triton X100 (116). Following cell lysis, the solution was used directly as a template for PCR amplification. Amplicons derived from a variety of Gram-positive and Gram-negative species known to be present in fecal samples could be detected following amplification with specific primers. Although the number of strains tested is limited, this fast PCR approach seems to be accurate and may be useful for analyzing samples containing a low number of microorganisms.

Quantification of nucleic acids. Several methods to visualize and quantify nucleic acids have been developed. DNA fragments separated on agarose or polyacrylamide gels are usually visualized with fluorescent dyes, such as ethidium bromide (58), or by silver staining (8, 90). Staining is used to quantify the yield of nucleic acids following addition of a concentration standard. Quantification works fine when RNA and DNA are not sheared. The correlation between the concentration of nucleic acids and the signal of the stain is normally not linear and extrapolation is not possible. Instead of quantification by gel electrophoresis, DNA and

RNA can also be quantified by Southern, northern or dot blot hybridization using a universal probe for a gene of interest. A third way to measure the nucleic acid concentration is to determine the UV light absorption at 260 nm and 280 nm. Additionally, the ratio A260/A280 gives an indication of purity. DNA is relatively pure when this ratio is between 1.80 and 2.00.

Another less well-known procedure for measuring the DNA and RNA concentration is use of High-Performance Liquid Chromatography (HPLC). HPLC has been used to determine the copy number of plasmids in recombinant yeast or *Escherichia coli* cells. Chromosomal DNA, plasmid DNA, rRNA and tRNA could be separated using HPLC (19, 20). When the various methods were compared for nucleic acids isolated from marine sediments (22), it was found that the yield of DNA appeared to be similar with spectrometric and HPLC measurements, but was significantly lower when the yield was determined by the fluorescent method. This might be due to the fact that the fluorescent stain is dependent on the structure, the size and the composition of the nucleic acids. Another finding was that RNA and DNA could be separated by HPLC, so that RNA measurement was not biased by DNA and *vice versa*, which might not be the case for the other two techniques.

RT-PCR/PCR OF 16S rRNA/rDNA

In order to gain an insight into the microbial structure in different ecosystems, various methods have been developed based on the nucleic acid sequences of small subunit (SSU) rRNA or rDNA because these molecules are ideal phylogenetic and taxonomic markers (3, 123). There are various reasons to use rRNA and rDNA genes as markers, including (a) their presence in all cells; (b) their high degree of sequence conservation which facilitates their detection; (c) the presence of highly variable regions in their sequences which makes them useful to discriminate at (sub)species to higher phylogenetic levels; and (d) the presence of databases containing up to 20,000 SSU rRNA sequences (M. Wagner, personal communication) from different taxa that facilitates the phylogenetic characterization of cultured and uncultured microbes. Moreover, rDNA can be amplified by PCR *in vitro* (88).

The principle of PCR is that cycles of DNA melting, primer annealing and elongation using a thermostable polymerase are repeated, resulting in an exponential increase of amplified genes. In addition, rRNA can also be amplified, but it has first to be converted into DNA by reverse transcription. This can be done by reverse transcriptase using an oligonucleotide primer which targets the RNA template (a procedure termed RT-PCR).

Although DNA and RNA can be amplified with other techniques (14), this section will only focus on RT- and regular PCR. Some important factors which may influence the amplification procedure, notably when mixtures of DNA or RNA from different organisms are amplified, include the purity of DNA, the G+C content of the target, the secondary structure of the target, preferential amplification, and formation of chimeric structures. Several methods minimizing these factors have been reported (9, 50, 82, 114, Table 1).

Preferential amplification. Some sequences may be preferentially amplified in a mixture of different sequences from comparable genes. For 16S rDNA it has regularly been reported that variations in primer pairs result in biased amplification when using mixtures of DNA as a template (82, 86, 107, 121). Equal amplification efficiency of 16S rDNA is necessary to get an insight into the microbial composition of an ecosystem. It was suggested that the bias in amplification observed with the canonical universal primers 27F and 1492 (52) can be decreased by (a) decreasing the number of amplification cycles; (b) mixing several replicate PCR amplifications; (c) using high template concentrations; and (d) excluding degenerate primers (82). A disadvantage of high template concentrations might be a high risk of the formation of chimera, consisting of PCR fragments originating from more than one target gene. Chimera formation during the amplification of 16S rDNA from an environmental sample results in an overestimation of the biodiversity. Since the homology between different 16S rRNA genes is relatively high, chimeras are thought to arise from reannealing of different 16S rDNA genes during PCR (56).

Multiple competitive PCR and quantitative RT-PCR have been used to test the universal bacterial primers U968-GC and L1401 when used to amplify 16S rRNA from soil (32, 73). It was found that 16S rDNA clones and bacterial 16S rRNA sequences from different phylogenetic groups were not preferentially amplified, although some target sequences have some minor sequence differences at the annealing sites of the primers (32). It has been shown that there are no differences in the TGGE patterns of DNA amplified for different numbers of cycles with the same primers (128). However, it was observed that the primers which were used to amplify complete 16S rDNA were preferentially amplifying *Prevotella*-like sequences. This was specifically noted when amplified 16S rDNA was reamplified using the primer pair which amplified the V6 to V8 regions. Reamplification of amplicons using another primer pair is called nested PCR. Although primer pairs may show limited preferential amplification, this undesired bias can never be excluded. For example, if

target DNA or rRNA from an unknown, uncultured microbe is not amplified during the first PCR cycles then it will stay undetected forever.

Table 1. Overview of some general artifacts concerned with (RT-)PCR and some solutions to minimize these artifacts.

Factors causing PCR artifacts	Some bias-preventing solutions	Reference
Nucleic acid purity	Additional purification steps	-
	Use of BSA or protein gp32	50
High G+C content of template	Increase denaturing time	-
Secondary structure of template	Increase denaturing time	-
	Use DMSO	9
Preferential amplification	Decrease PCR cycles	82
	Mix replicate reactions	82
	Exclude degenerated primers	82
	Use high template concentration	82
Formation of chimeric constructs	Longer elongation time	114

Quantitative (RT-)PCR. Several studies have described the quantification of microbial 16S rDNA or rRNA amplicons by PCR and RT-PCR, respectively. It should be remembered that quantification of amplicons only reflects the relative number of ribosomes or corresponding genes in a community and not the relative frequency of a species. The number of ribosomes per cell depends on the type and activity of a species. A positive correlation between the activity of a cell and the amount of rRNA has been described (113). However, it has also been shown for two food-associated pathogens that this correlation was only found under extreme heat conditions (62). Moreover, the number of 16S rRNA genes per genome varies between species. For example, seven 16S rRNA genes were found in *E. coli* (5), five to six genes in *Streptococcus* spp. (10), and four genes in *S. pneumoniae* (6).

Besides differing copy numbers of 16S rRNA genes per genome, the genome sizes of bacteria are also different. It has been shown that differences in genome size and 16S rDNA copy number influence the ratio of amplicons when mixtures of target DNA from *E. coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Thermus thermophilus* are mixed in equal molarities (27). From this study it was concluded that the number of bacterial cells could not

be calculated exactly when both parameters are unknown.

Despite the fact that relative cell numbers cannot be extrapolated from (RT-)PCR data, changes in the structure and activity of a microbial community can be analyzed when 16S rRNA or rDNA amplicons are quantified. *E. coli* and *P. aeruginosa* 16S rRNA genes from mixtures of these bacteria with ratios of 1:100 could be quantified using the Perkin Elmer QPCR^{TM} system 5000 (11). Biotinylated PCR products were captured on streptavidin-coated paramagnetic beads after different PCR cycles, and specific PCR products were quantified by measuring the electrochemoluminescent signals from the specific reporter probes directed against the different amplicons.

Another way of quantifying 16S rRNA genes is a so-called most probable number (MPN)-PCR (108). In this method the target DNA for PCR is diluted to extinction, followed by analysis of PCR products by agarose gel electrophoresis. This approach was used to quantify the relative amounts of 16S rDNA derived from different groups of bacteria in fecal samples using different primer combinations (116). Although this form of multiplex PCR has the potential to be useful, the data have to be analyzed carefully, since the PCR conditions may not be quantitative as the primer pairs and product sizes are different.

Another approach involves the use of competitive PCR for the quantification of mRNA (115). In this method a specific standard of known concentration is added in different amounts to the target. The different sizes of the standard and target allows for differentiation and subsequent quantification following agarose or polyacrylamide gel electrophoresis.

The combination of TGGE and competitive RT-PCR resulted in the development of a new quantification method called multiple competitive RT-PCR (32). In this approach, changes in amplification conditions were minimized because the products were amplified with the same primer pairs and had the same size as the added standard. TGGE was used to separate and quantify the different products. It was found that the 20 most abundant sequences, which derived mainly from Gram-positive species of low G+C content, represented about 50% of the total microbial community in the Drentse A grassland soils.

CLONING, SEQUENCING AND PHYLOGENETIC ANALYSIS

To get an overview of the complexity of ecosystems, it is essential to classify the individuals from a population. Classification is used to clarify the relationships between different organisms. It has to be emphasized that there is no single unifying classification of organisms.

This section will focus on classification based on phylogenetic relationships between organisms.

Phylogeny is determined by scoring for the presence or absence of homologous morphological or physiological characteristics across operational taxonomic units (OTUs), which can be populations, species or strains. Both physiological and genetic characteristics can be used for phylogenetic analysis. The principle of phylogenetic analysis is the assumption that all life forms have evolved from a common origin. The common ancestor of two closely related organisms disappeared more recently than that of two more distantly related organisms. It is believed that evolution follows a pattern of successive branchings into populations in which further evolutionary changes subsequently proceed independently. Phylogeny involves the determination and analysis of these branching patterns.

Cloning, sequencing and phylogenetic analysis of 16S rDNA sequences have become powerful tools in microbial ecology, particularly since it was discovered that the majority of microorganisms in environmental samples are unknown (3). The highly conserved, but discriminative 16S rDNA molecule makes it possible to identify a species in an ecosystem without the use of unreliable culturing methods. Cloning and sequencing of 16S rDNA amplicons has become a standard procedure in molecular ecology and provides information about the genetic diversity and phylogenetic relationships between microorganisms in an ecosystem. Since up to 20,000 different SSU rRNA sequences are available in different databases, the comparison of new sequences is reliable. However, a clone library (a collection of clones from a DNA sample) has to be very large to give a reliable picture of the genetic diversity in complex ecosystems. This makes the approach expensive and time consuming. Therefore, a rapid approach for screening 16S rDNA clone libraries has recently been developed (59). Habitat-based probes were designed using subtractive hybridization. These habitat-based probes were used to screen a 16S rDNA library generated from the same habitat. It was shown that this screening method prevents sequencing many similar or identical clones of the dominant members in sediments.

Cloning. To construct a clone library, a mixture of 16S rDNA amplicons is first generated by PCR using bacterial or universal primers to amplify 16S rDNA from an environmental sample. A cloning strategy is necessary to sequence individual amplicons derived from the DNA of a complex microbial community. There are different strategies to create a clone library. DNA fragments can be cloned into a plasmid vector or a bacteriophage. Detailed

principles, possibilities and procedures for cloning DNA fragments have been described elsewhere (58, 77). Amplicons are usually cloned into a sequencing vector, which is then transformed into an *E. coli* strain. Although a great variety of different cloning vectors are available, they all show some common characteristics. One common feature is that amplicons are inserted into a gene with many restriction sites (e.g., the polylinker in that part of the *lacZ* gene coding for the α-peptide). The amplicons and the vectors have to be restricted with the same restriction enzyme(s) for cloning. Some vectors contain a 3'T-overhang at the insertion sites, and these are particularly useful for amplicons produced by certain polymerases (e.g., *Taq* polymerase) which sometimes make 3'A-overhangs (16). Amplicons of 16S rDNA are cloned into the vector with a ligase. After ligation, the vectors containing a single 16S rDNA insert are transformed into a competent *E. coli* strain. The cells are grown on selective plates and single transformants are screened for the presence of vectors containing a 16S rDNA insert by means of PCR or colony hybridization with 16S rDNA-specific probes. The vectors containing a 16S rDNA insert can be isolated after regrowing the positive transformants and can then be subjected to sequence analysis or fingerprinting.

Sequence analysis. Sequence analysis is used to provide information about the nucleotide sequence of a cloned amplicon. There are different methods to determine the sequence of a DNA fragment, but these will not be discussed here. Several programs to determine the closest relative of the DNA sequence, are available on internet sites. Mostly, these programs use homology searches provided by BLAST (2, *http://www.ncbi.nlm. nih. gov/BLAST/*) or FASTA (80, *http://biogate.mlg.co.jp/tssfree/Fasta.html*). The benefit of these programs is that the search for homology is fast and reliable, and several DNA databases can be used for comparison. When DNA sequences are compared, the alignment of sequences for highest homology is a crucial step. The alignment of sequences is performed by giving homologous or conserved parts the same nucleotide position. The variable regions in between are compared in such a way that the highest homology is found. Gaps in a nucleotide sequence are also included in the alignment, but the number of gaps should be minimized. Each position in a sequence can be one of the four nucleotide bases or a gap. This alignment of nucleotide sequences is necessary in order to construct phylogenetic trees and to develop oligonucleotide probes (52, 103).

Tree construction. It is difficult to visualize phylogenetic relationships between species from numerical values based on multiple pairwise comparisons, particularly when many different sequences are compared. An alternative way to visualize phylogenetic relationships is by the construction of a phylogenetic tree based on the identity values. The calculations for construction of phylogenetic trees can be handled in two ways: by distance matrix or from discrete character data (Table 2). In the first calculation, data based on evolutionary distances are set in a distance matrix. Most calculation methods do not weight each nucleotide mutation equally. The DNA structure plays in important role in the calculation procedures. It has been postulated that transversions are more easily recognized by the DNA repair system than are transitions because of the spherical DNA helix distortions (47). These changes are therefore considered to be less frequent and result in a lower substitution rate, which can be taken into account when calculating distance values.

Table 2. Overview of possibilities for calculating phylogenetic relationships and making a phylogenetic tree.

Tree construction	Basis for calculation	Reference
Distance matrix	Each nucleotide change equal	45
	Differing substitution rates	109
	Differing substitution rates and transition/transversion correction	47
Discrete character data	Maximum parsimony	25
	Maximum likelihood	28

Another example of differences in substitution rates is postulated for protein-coding genes. Substitution rates of the third nucleotide position in a triplet coding for an amino acid are usually higher than in the other two nucleotide positions (98).

A more complex feature is the formation of gaps. The cost of introducing a gap in an alignment is generally higher than the introduction of a base substitution. Although the introduction of gaps is necessary to align sequences, it is often omitted from distance calculations because it is difficult to verify how the gap has originated. The most frequently used distance calculation models are those developed by Jukes & Cantor (45), Kimura (47), and Tajima & Nei (109). The Jukes and Cantor model does not discriminate between different nucleotide substitutions, in contrast to that of Tajima and Nei which, however, does not

correct for nucleotide transitions or transversions, as does the model of Kimura. The model of Jukes and Cantor has probably been applied most frequently in evolutionary studies because it performed well in most studies simulating the evolution of nucleic acid sequences (112).

Phylogenetic trees can be plotted from distance matrices. Commonly used models which calculate distance trees are the unweighted pair group method using arithmetic averages (UPGMA; 101) and neighbor joining method (89). UPGMA is a clustering method which pairs the least distant sequences into a node, and subsequently pairs two nodes into a new node. The neighbour joining method uses a simplified algorithm to calculate branch lengths and tree topologies.

Discrete character data calculations are not based on evolutionary distances, but consider each character state of the nucleotide position in the sequence separately. Trees can be constructed from each nucleotide position. The data can be handled in two ways. The first way is based on the maximum parsimony principle in which the true tree is the one which requires the fewest number of mutational changes to explain the differences observed between the gene sequences (25). Only so called 'informative nucleotides' (a common nucleotide position in a set of sequence positions which favor only some of all possible trees) are used. In general, this means that a constant base in all sequences and a variable base which does not favor one tree over all others are not informative. The second way to handle discrete character data is called the maximum likelihood phylogeny. This calculation uses statistical models to calculate the probability that one sequence is converted into another sequence by mutation over time (28). More detailed explanations and comparison of the methods have been described extensively elsewhere (83, 100, 125).

It has to be realized that evolutionary events cannot be checked for and that phylogenetic trees therefore only represent a systematic ordering of genes. Furthermore, calculations based on different DNA sequences or different genes may result in completely different trees. As a consequence, it is difficult to choose which tree-constructing approach is most optimal. For 16S rDNA sequences from cultured and uncultured *Frankia* strains, it was found that trees constructed by methods based on discrete character data or distance matrices were roughly the same (125). The choice of the program might therefore depend on the speed, ease and possibilities of the different programs and on the applications of the user. A comparison of different methods can be used to demonstrate the robustness of the phylogenetic tree generated.

Phylogeny of 16S rRNA genes. Phylogeny based on 16S and 23S rRNA analysis has led to the construction of phylogenetic trees which illustrate the evolutionary relationship between different organisms. This has resulted in a division of all life into three main domains: Archaea, Bacteria and Eucarya (123, 124). The increasing number of 16S rDNA sequences of bacterial isolates has allowed phylogenetic analysis of 16S rDNA to be applied to bacterial taxonomy. The threshold for species determination is set at 70% DNA-DNA hybridization between the genomes of different strains (119). Strains showing values above this threshold are considered to be the same species and this threshold has been translated into a 16S rRNA value (102). It was estimated that strains with less than 97% 16S rDNA sequence similarity have less than 70% DNA-DNA hybridization values. This threshold can be used to determine whether two strains do not belong to the same species, but cannot be used as the only characteristic for species determination. Indeed, some *Bacillus* spp. have less than 70% DNA-DNA hybridization, but more than 99.5% 16S rRNA sequence similarity (36).

Sometimes traditional taxonomic methods can be compared to the 16S rRNA phylogeny. Some genera in the GI tract that have been characterized physiologically (e.g., bifidobacteria) form a monophyletic group in the 16S rRNA tree. However, many bacterial genera in the GI tract do not form monophyletic clusters in the 16S rRNA phylogenetic tree. In particular, the genera *Clostridium* and *Eubacterium* are mixed and divide into different distinguishable clusters (18). Other genera like *Ruminococcus* and *Butyrivibrio* are mixed in these clusters, making identification quite difficult. Additionally, it was found that the 16S rRNA sequences of two strains, identified as *Fusobacterium prausnitzii* by physiological characteristics, were not phylogenetically related to other *Fusobacterium* strains, but grouped in one of the *Clostridium* clusters (117). In such cases, physiological characteristics cannot be translated from phylogenetic characters when the species are not closely related.

The use of 16S rRNA sequence analysis in taxonomy has also resulted in proposals for renaming bacterial species. Based on their 16S rRNA sequences and their physiological characteristics, it was proposed to redesignate *Peptostreptococcus productus* and *Streptococcus hansenii* as *Ruminococcus* species (26). In conclusion, it seems that phylogenetic and physiological analysis are both necessary for a reliable identification.

Following the increase in the number of DNA sequences, databases such as EMBL and Genbank have become available on internet sites. These databases contain up to 20,000 SSU rRNA sequences and are suitable for comparing, downloading and the deposition of sequences. The most commonly used database for rRNA sequence analysis is that of the

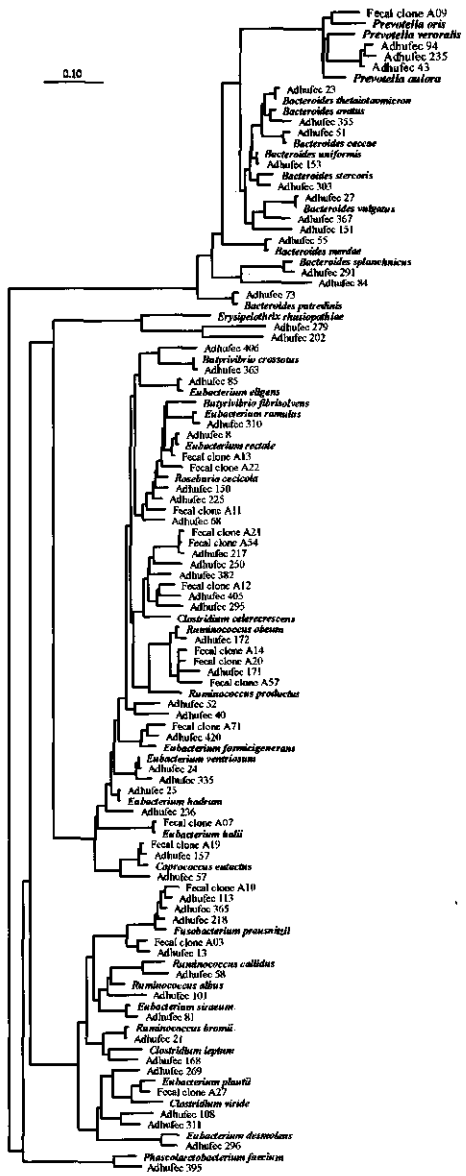


Fig. 3. Phylogenetic tree showing the phylogenetic relationships between cloned 16S rDNA from feces and the closest cultivable relatives found in 16S rDNA databases. Complete and partial 16S rDNA sequences from fecal samples were added to the phylogenetic tree of the ARB software. Sequences called fecal clone A03 – A71 and adhufec 8 – 420 were retrieved from Zoetendal *et al.* (128) and Suau *et al.* (106), respectively. These sequences and the sequences from the closest cultivable relatives were marked, and the remaining sequences were removed from the tree. Bold-marked sequences represent the closest cultivable relative found in the database; the bar indicates the calculated genetic distance between the sequences.

Ribosomal Database Project (RDP; 57, *http://www.cme.msu.edu/RDP/analysis.html*). The RDP contains an aligned database of 16S rRNA sequences, which are present in a phylogenetic tree. Another software package containing a rRNA database is the ARB software package (105). This program is comparable to the RDP, but the package needs a powerful computer for calculations. The secondary structure of the rRNA molecule is used for the similarity calculation and is visualized in the alignment program of the ARB package. This facilitates sequence analysis and is ideal when checking for sequencing errors. The phylogenetic trees in both programs are comparable.

16S rRNA databases are not only used for strain identification, but are also used to study the bacterial diversity in an ecosystem. The development of molecular methods in microbial ecology has resulted in an increasing number of sequences from cloned amplicons derived from different types of environments. Several papers on intestinal samples from different types of animals have shown that many of the cloned 16S rDNA sequences show identity below the 97% threshold to their closest cultivable relative in the DNA databases (76, 106, 120, 121, 128). This means that the species from which the sequences have been derived have not yet been cultured or, alternatively, are present in a culture collection but their 16S rDNA has not yet been sequenced. Fig 3 shows a phylogenetic tree built from 16S rDNA sequences of bacterial clones from human feces and their closest cultured relatives found in the 16S rDNA database of the ARB software package. These results reinforce the concept that most bacteria in existence have not yet been cultured (3). For the GI tract, this means that our knowledge about the role of the microbial community in the intestine is limited. Therefore, cloning and sequencing of fecal or intestinal clones is needed to determine the microbial diversity and to study the structure of the community in the GI tract.

FINGERPRINTING

Sequence analysis of 16S rDNA/rRNA clone libraries gives reliable information about the genetic diversity of an ecosystem. However, this approach is expensive, time-consuming and not suitable for monitoring complex ecosystems. It will be necessary to study complex ecosystems using alternative methods that are better suited for studying the composition and temporal variation in ecosystems, probably based on sequence differences of the nucleic acids. Fingerprinting techniques are suitable to describe the genetic diversity at different levels of a microbial community. There are many types of fingerprinting techniques which

can be useful at the community, species and even strain level. The next section describes SSCP, DGGE and TGGE fingerprinting techniques which are commonly used to study the microbial diversity of different ecosystems. These techniques are based on differences in the nucleotide sequence of amplicons of similar size and are suitable for describing ecosystems at the species level. The sequence-specific separation of PCR amplicons is an essential element, but differs between the techniques. SSCP relies on the secondary structures of the single strands, while the other techniques rely on the melting behavior of the double stranded amplicon.

SSCP. SSCP is an electrophoretic technique which has been developed for the detection of mutations in genes and has been used widely in the field of human genetics (41, 78). The principle of SSCP is that the mobility of a single-stranded DNA fragment is dependent on the secondary structure of the fragment. The secondary structure is determined by the nucleotide sequence and the physiological environment (e.g., temperature, pH and ionic strength). SSCP has been shown to be able to detect single base differences in 99% of amplicons which are up to 300 bases in size. This detection limit drops using longer fragments (41, 42). A typical SSCP profile consists of two single-stranded DNA fragments and one double-stranded fragment, although different conformations from one strand are also possible. This technique has only been used occasionally in ecological studies. SSCP of the 16S-23S rRNA spacer has been used to analyze mixtures of bacteria (92), and SSCP of different regions in the rRNA operon has been used to differentiate between root-associated fungi (17). SSCP has been used to analyze microbial communities in a few studies. For example, the V3 region of the 16S rRNA gene was used for SSCP fingerprinting of bacterial strains and environmental samples (54). The problem of bands caused by heteroduplex formation in mixed DNA samples could be solved by removing glycerol from the gel, but this removal resulted in a lower separation efficiency of the single strands. It has been reported that the bands in the profiles of the environmental samples did not correspond to bands in the profiles of those bacteria that could be cultivated. The 16S rDNA sequence of a bacterium making up about 1.5% of a community could be visualized with this technique.

Recently, a new approach for SSCP analysis was reported (96). In this study, amplicons containing the V4 to V5 region were used. One of the primers was phosphorylated at the 5'end. After amplification the phosphorylated strand could be digested selectively with lambda exonuclease. Using this technique, the number of bands per species could be

minimized and heteroduplex formation in mixed DNA samples could be prevented. Clear banding patterns could be obtained from environmental samples.

DGGE and TGGE. The separation of 16S rDNA amplicons is based either on a linear gradient of denaturants, at constant temperature in the case of DGGE (35), or on a linear temperature gradient parallel to the running direction in the case of TGGE (87), or on increasing temperature with time in the case of TTGE (also called TSGE; 127). These fingerprinting techniques are frequently used to study the microbial diversity of different ecosystems. Until end 1999, TTGE has been used occasionally in ecological studies, but there are no published articles describing its use. In DGGE, TGGE and TTGE, amplicons of the same length with different nucleotide sequences can be separated on polyacrylamide gels containing denaturants (urea and formamide). During electrophoresis the amplicons start to melt in so-called melting domains with identical melting behavior. The size of these domains may vary between 50 and 300 bp (72). In this way, the electric mobility of amplicons which contain the double helix structure and disordered single-stranded regions drops. Sequence variations within such domains causes the different melting behavior of the amplicons.

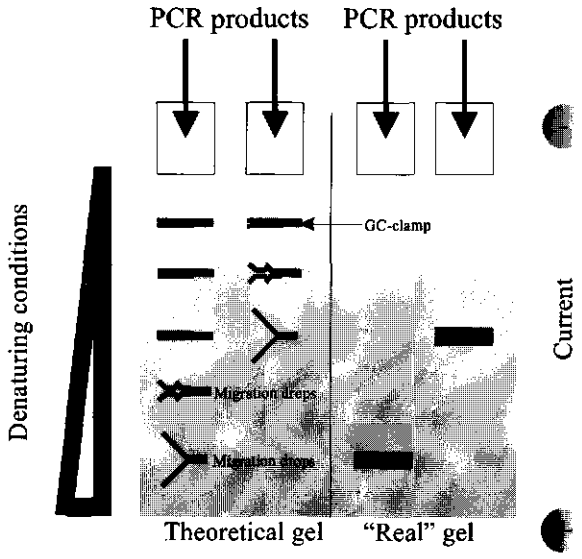


Fig. 4. Schematic representation of a polyacrylamide gel which explains the principles of DGGE and TGGE.

Attachment of a GC-clamp makes it possible that sequence variations in the most stable domains can also be separated (71, 97). This GC-clamp is a G+C rich domain which is attached to the amplicons by adding it at the 5'end of one of the primers, and which prevents complete melting of the amplicons. In principle, all single base differences at each position of the amplicons can be separated for amplicons of up to 500 bp (97). The final position of the amplicons in the gel depends on the melting behavior of the amplicons (and, therefore, the nucleotide sequence) and the running time. A simplified representation of DGGE and TGGE analysis of amplicons is shown in Fig. 4.

The introduction of DGGE into ecological studies was originally designed to separate amplified V3 regions of 16S rDNA from marine ecosystems. Amplicons derived from sulfate-reducing bacteria could be detected after blotting the DGGE profiles with a specific probe (70). Following this study, the application of these techniques to ecological studies increased enormously. Different ecosystems have been analyzed by separation of different amplified regions from 16S rDNA and 16S rRNA using these fingerprinting techniques. These techniques have not only been used for analyzing the composition and stability of different ecosystems, but have also been used for comparing DNA extraction protocols, screening of clone libraries, determining 16S rRNA sequence heterogeneities, monitoring enrichment and isolation procedures, and determining biases introduced by PCR and cloning. Recent overviews of the use of these and other methods for studying different ecosystems are available (67, 69).

To increase the separation efficiency, an optimal gradient has to be chosen. This can be done by applying the gradient perpendicular to the running direction (70). For TTGE, the optimal temperature gradient has to be calculated from known sequences. Amplicons with only one nucleotide difference can be separated when an optimal gradient is applied (71, 73). Additionally, it has been shown that a wobble base (either C or T) in the reverse primer may result in two distinct bands (49). The opposite, however, also takes place. Sometimes, 16S rDNA amplicons cannot be separated although they differ in nucleotide sequence (13, 111).

TGGE and DGGE of 16S rDNA and rRNA amplicons have been used to describe the microbial composition of several ecosystems. In these studies, different universal primer pairs have been used to describe dominant communities. An MPN (RT-)PCR can be used to check if the dominant community is visualized on TGGE (Fig. 5). In general, 16S rDNA, but also 16S rRNA, is used as a target for analyzing microbial diversity (Fig. 5). Profiles derived from 16S rRNA represent the relative number of different ribosomes in an environmental sample,

which reflects the active fraction of a community. Comparing rRNA- and rDNA-derived amplicons may give information about the activity in the microbial community of a certain group (29, 110, 128), but it has to be realized that the number of ribosomes and rRNA genes may differ per species. Several studies have shown that sequences derived from a bacterium which makes up about 1% of a microbial community can still be visualized using TGGE and DGGE (66, 70, 128), which is similar to the sensitivity of SSCP analysis. Instead of using universal primers, group- or species-specific primers can be used to focus on particular groups. The genetic diversity of uncultured ammonia-oxidizing bacteria (49) and cyanobacteria (74) has been studied using specific 16S rDNA primers for both groups. DGGE and TGGE have also been used to describe the expression of functional genes such as the [NiFe] hydrogenases from *Desulfovibrio* populations (118). The combination of D/TGGE analysis of 16S rDNA and a functional gene may be used to study relationships between the structures and functions of ecosystems.

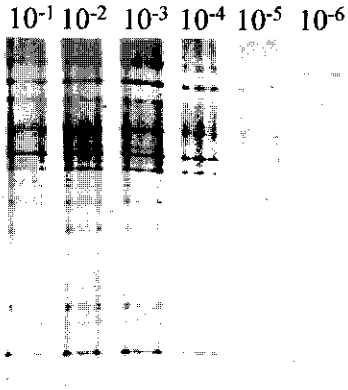


Fig. 5. TGGE profiles of amplified V6 to V8 regions of 16S rRNA from a fecal sample using the MPN RT-PCR approach. 10^{-x} represents the dilution of template RNA used for amplification.

TGGE and DGGE analysis of amplicons is semi-quantitative, i.e., an intensive band is more abundant than a weak band in a profile. When an appropriate standard template of known concentration is added to the nucleic acids extracted from a microbial community, different genes or ribosome fractions can be quantified. Bands for which the intensity is identical to the intensity of the standard can be quantified. This approach is called multiple competitive (RT-)PCR (32). Different ribosome fractions from Drentse A soils could be

quantified this way. Equal amplification of different ribosomes is necessary for quantification. In case of the Drentse A soils, the primers used did not preferentially amplify specific cloned 16S rDNA amplicons or ribosomes from strains of different phylogenetic clusters, although the primers did not match 100% to the target of any of the strains and clones tested. However, preferential amplification cannot be ruled out completely, because species can always be missed during amplification and will therefore not be analyzed.

TGGE and DGGE analysis of amplicons is a quick and reliable method for studying the dynamics of ecosystems, but the identification of single bands in the profiles is very time-consuming. Identification can be done by cutting out the bands in a profile followed by reamplification and sequence analysis. This approach has been applied successfully to ethidium bromide-stained gels (68) and silver stained gels (84, F. Schut, personal communication), but the disadvantage of this method is that a maximum of 500 bp can be used for sequence analysis. Identification can also be done by screening a clone library for dominant band positions, followed by sequence analysis. In this way, complete sequences could be retrieved, thereby making the phylogenetic analysis more reliable. This approach has been introduced by Felske *et al.* (30, 31). The identity of the bands can be checked by sequencing more clones with identical motility or by blotting the profiles and using specific probes (70, 30, 49).

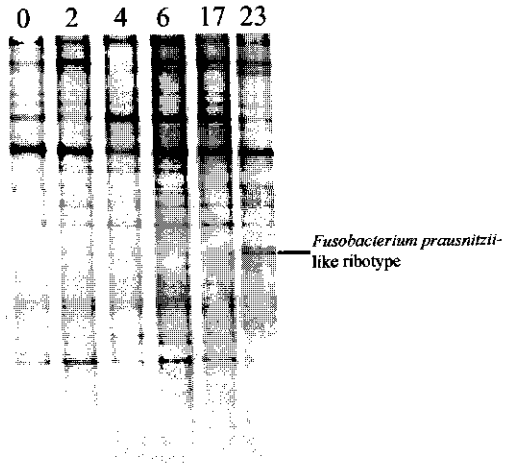
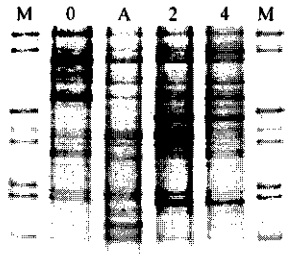


Fig. 6. DGGE profiles of the V6 to V8 regions of 16S rDNA from different fecal samples of one individual taken over a period of 23 months. A band originating from a *Fusobacterium prausnitzii*-like ribotype which increased over time is indicated.

Despite the increasing number of applications in molecular ecology, only a few studies have been performed with GI tract ecosystems. DGGE analysis of the V3 regions of 16S rDNA was used to study the role of uncultured bacteria in pre-term infants with and without necrotizing enterocolitis (NEC) (63). It was found that the number of uncultured bacteria in fecal samples from children with NEC was not more frequent than in fecal samples from children without NEC. TGGE based on the V6 to V8 regions of amplified 16S rDNA and 16S rRNA has been used to study the bacterial composition of different fecal samples (128). This study showed that each adult individual has his own fecal microbiota, which is relatively stable over time. Only a few amplicons were shared by all fecal samples. It was found that the fecal community in one person remained stable for almost 2 years (Fig. 6). A band corresponding to a cloned *Fusobacterium prausnitzii*-like ribotype increased slightly over this period. Furthermore, it was found that most dominant amplicons in an individual's profile derived from species that have not been cultured. Recently, the microbial community in the porcine GI tract has been studied using DGGE analysis of the V3 region of 16S rDNA. It was observed that unique bands were found in the fingerprints of fecal samples from pigs differing in age, and that the profiles were most similar within a single GI tract compartment and between adjacent ones (99).

A



B

	0	A	2	4
0	100.0			
A	50.4	100.0		
2	33.5	42.7	100.0	
4	62.1	37.5	73.3	100.0

Fig. 7. DGGE profiles (**A**) of the V6 – V8 regions of 16S rDNA, and (**B**) a similarity matrix expressed in Pearson correlations (x 100) of the DGGE profiles from fecal samples of one individual taken before (0), during (A), and after (2 and 4 months) treatment with doxycycline for 1 week. Lane M contains a marker consisting of cloned V6 – V8 amplicons.

Quantitative fingerprint analysis. The use of TGGE and DGGE to study complex ecosystems can be enhanced by quantifying profile similarities. Computer analysis of scanned fingerprints

can be used to calculate similarity indices between fingerprints. These indices can be used to determine the stability of microbial communities or to monitor the effect of certain conditions which may change the composition of a microbial community. The calculation of similarity indices of DGGE profiles has been used to monitor the spatial and seasonal diversity of Antarctic picoplankton assemblages (66). A similar approach was used for samples from the porcine GI tract (99). The highest similarity indices were found within a single compartment and between adjacent compartments, indicating that the microbial communities were quite similar in these compartments.

Fig. 7 illustrates how densitometric curves of DGGE profiles can be used with the Molecular Analyst software (BioRad) to quantify the effect of treatment for 1 week with the antibiotic doxycycline on the dominant microbial community in feces. A relatively stable microbial community was recovered 2 months after the treatment. The matrix illustrates these changes in a quantitative way and shows the high similarity between the fecal samples taken after the antibiotic treatment, thereby indicating the recovery of a stable microbial community. However, the community structures before and after treatment were not identical. These examples illustrate that the use of quantitative DGGE analysis is a reliable method to monitor changes in microbial communities and should be preferred above the subjective comparison made by eye.

Another method for quantifying DGGE profiles has recently been published (75). The Shannon-Weaver indices (which are the most common diversity indices and richness estimates) of DGGE profiles and two other cultivation-independent methods were used to quantify the microbial diversity and richness in different hypersaline microbial mats. A similar approach was used to study the effect of chlorobenzoates on the microbial community in soil (84). It was clearly demonstrated that the genetic diversity in the contaminated soils decreased significantly. In the case of the GI tract, the role of the host, food and antibiotics on the bacterial composition can be quantified by the methods described above. This fingerprinting analysis, in combination with multiple competitive PCR, quantitative profile comparison, or the calculation of diversity indices, can be used to determine and quantify changes in microbial composition caused by exposure to antibiotics in intestinal samples.

OLIGONUCLEOTIDE CHIP TECHNOLOGY

One of the new approaches that can be used to analyze environmental samples is the

application of oligonucleotide microchips or microarrays (reveiwed by 55, 95). Microchips consist of oligonucleotides which are immobilised in a polyacrylamide gel matrix bound to a glass slide. Labeled target DNA or RNA can be added to the microchip, and the subsequent hybridization signal can be detected and quantified using a computer-regulated camera connected to the microscope. Microarrays consist of numerous cloned or amplified DNA fragments rather than synthesized oligonucleotide probes, but the principle of microarrays is the same as that of microchips. Oligonucleotide microchips and microarrays have already been used several times for nucleotide sequencing. This sequencing by hybridisation (SBH) has been proposed as an alternative technique for genome sequencing (12, 24, 126). Microarrays have also been used to study the expression of certain genes, and also nucleotide variability between genes (15, 93).

Recently, microarrays have been used to identify unique DNA regions present in a pathogenic strain of *Pseudomonas aeruginosa* which appeared to be missing in a control strain (7). The use of microchips could eventually be relatively cheap, because the concentration of probes per chip is very low, the information per analysis is high, and the chips can be used 20 to 30 times (38). The use of microarrays could be even cheaper because cloned amplicons are produced more economically than oligonucleotide probes. The application of these microchips or microarrays to answer microbial ecological questions looks promising. Environmental samples can be screened on microchips containing hundreds or thousands of 16S rRNA targeted probes, or on microarrays containing many cloned 16S rDNA fragments. However, one of the difficulties in using these approaches is the optimization of the hybridization conditions for the different immobilized DNA fragments (46). Recently, it has been reported that nitrifying bacteria and *Bacillus* spp. could be detected and identified at the rRNA level using oligonucleotide microchips (38, 46). These approaches look promising for wider applications in microbial ecology. In the near future it might be possible to monitor expression of ribosomal and functional genes of an ecosystem with a single microchip or microarray.

CONCLUSIONS AND PERSPECTIVES

The application of TGGE, DGGE, and SSCP to studies in microbial ecology is growing and the future perspectives are promising. The combination of (RT-)PCR, cloning and fingerprinting of environmental samples may give an accurate description of a community.

Despite some pitfalls concerned with biases in nucleic acid extraction and amplification methods, these techniques have been shown to be useful for describing the microbial composition of different ecosystems. The power of these techniques is their reliability, speed and ease of use. TGGE, DGGE and SSCP are ideal for studying the temporal and spatial variation in microbial communities, both qualitatively and quantitatively. The only time-consuming aspect is the identification of specific amplicons in the profiles. The approach of combining 16S rDNA profiles and profiles of functional genes may enable the structure to be related to the function of an ecosystem. Another variant of this approach is the use of oligonucleotide microchips. Extensive data can be obtained from a single analysis and may be quantitative, although the different T_m values might be a problem in quantification.

In studying the role of the microbial community in the intestine of man and other animals, the approaches described in this chapter should be applied instead of unreliable plate counting analysis to describe the microbial composition. The fingerprinting approach has already demonstrated that the dominant microbial community in adults is quite stable with time and differs for each individual. This approach can also be used to monitor the fate of certain bacteria, such as probiotic strains in the intestine (21). The impact of these strains on the microbial community in the GI tract can be analyzed by quantifying similarities between the profiles, or calculating microbial diversity and richness indices from profiles. Changes in band positions can be identified using cloning and sequencing analysis. The introduction of internal standards to the profiles may help the changes to be monitored quantitatively. These approaches will definitely help in gaining an understanding of some aspects of the microbial community in the intestine.

It is evident that the use of PCR-based fingerprinting techniques is useful in answering ecological questions. Although the use of these techniques is still in development, their application has already been shown to be a powerful tool for determining the structure of microbial communities in different environments and monitoring changes in microbial communities without unreliable cultivation procedures.

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Chapter 3

Temperature Gradient Gel Electrophoresis Analysis of 16S rRNA from Human Fecal Samples Reveals Stable and Host-Specific Communities of Active Bacteria

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Abstract

The diversity of the predominant bacteria in the human gastrointestinal tract was studied by using 16S rRNA-based approaches. PCR amplicons of the V6 to V8 regions of fecal 16S rRNA and ribosomal DNA (rDNA) were analyzed by temperature gradient gel electrophoresis (TGGE). TGGE of fecal 16S rDNA amplicons from 16 individuals showed different profiles, with some bands in common. Fecal samples from two individuals were monitored over time and showed remarkably stable profiles over a period of at least 6 months. TGGE profiles derived from 16S rRNA and rDNA amplicons showed similar banding patterns. However, the intensities of bands with similar mobilities differed in some cases, indicating a different contribution to the total active fraction of the prominent fecal bacteria. Most 16S rRNA amplicons in the TGGE pattern of one subject were identified by cloning and sequence analysis. Forty-five of the 78 clones matched 15 bands, and 33 clones did not match any visible band in the TGGE pattern. Nested PCR of amplified 16S rDNA indicated preferential amplification of a sequence corresponding to 12 of the 33 non-matching clones with similar mobilities in TGGE. The sequences matching 15 bands in the TGGE pattern showed 91.5 to 98.7% similarity to sequences derived from different *Clostridium* clusters. Most of these were related to strains derived from the human intestine. The results indicate that the combination of cloning and TGGE analysis of 16S rDNA amplicons is a reliable approach to monitoring different microbial communities in feces.

INTRODUCTION

The human gastrointestinal tract harbors a diverse community of microorganisms which include a large number of mainly anaerobic bacteria. These have largely been studied by plate count analysis of fecal samples, which usually contain 10^{10} to 10^{11} CFU per g (9, 13, 29). One of the limitations in using conventional microbiological methods is that only easily cultivable organisms are counted. Bacteria which have obligate interactions with the host or other microorganisms, or which require unknown growth conditions, will not be selected this way. Estimates of culturability of bacteria in the gastrointestinal tract vary from 10 to 50% (16, 20, 38). Other limitations of cultivation include the selectivity of the medium used, the stress imposed by cultivation procedures, and the necessity of strictly anoxic conditions. As a consequence, insight into the interaction between the host and the microbial community, and into the influence of environmental factors on microbial composition, is still lacking.

This decade has shown an explosive development in the application of molecular techniques based on 16S and 23S rRNA to the study of microbial diversity in ecosystems (reviewed in references 1 and 32). So far, the rRNA approach has been used only incidentally to study human intestinal microbial ecology, and only specific groups of bacteria, such as *Bifidobacterium* and *Lactobacillus*, have been studied (16, 19). In addition, PCR has been used to quantify specific groups of bacteria in human feces (35), and random cloning approaches have been used to analyze the microbial diversity of feces from one individual (38). Although these studies report significant information on specific bacteria and specific individuals, the approaches are time-consuming, expensive, and unsuitable for characterizing complex microbial communities. Methods such as denaturing gradient gel electrophoresis (10) or temperature gradient gel electrophoresis (TGGE) (28) have been developed to analyze microbial communities rapidly, based on sequence specific separation of 16S rDNA amplicons (8, 22).

The aim of the present study was to use molecular approaches to describe the bacterial diversity in human fecal samples and to investigate to what extent this diversity is affected by the host. We analyzed PCR-amplified V6 to V8 regions of 16S rRNA (23) by TGGE to describe the diversity of the predominant bacteria in fecal samples. Since the ratio of 16S ribosomal DNA (rDNA) and rRNA is dependent on cellular activity (33), we compared TGGE patterns derived from 16S rRNA and rDNA amplicons. Finally, to gain insight into the phylogenetic positions of the most prominent bacteria, we prepared a 16S rDNA clone library

and sequenced clones corresponding to dominant bands in the TGGE pattern of a single individual.

MATERIALS AND METHODS

Experimental approach. To describe the bacterial diversity in the human gastrointestinal tract, we used a molecular approach based on the sequence variability of the 16S rRNA gene (Fig. 1). RNA and DNA were simultaneously isolated from fecal samples and were used as templates for amplification by reverse transcriptase PCR (RT-PCR) and regular PCR, respectively, of fragments of the 16S rRNA gene. Amplicons of the V6 to V8 regions were analyzed by TGGE. Migration distances from different gels were compared by using a marker which consisted of amplified V6 to V8 regions from nine clones with different mobilities. Additionally, a clone library of 16S rDNA amplicons *(Escherichia coli* positions 8 to 1510) from a fecal sample of one individual was prepared. The mobilities of cloned amplicons were screened by TGGE, and those corresponding to specific bands in the RNA-derived profile were sequenced.

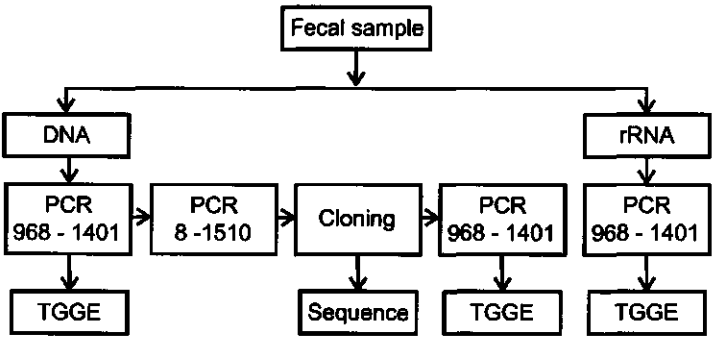


Fig. 1. Outline of the molecular approaches used to analyze the fecal bacterial communities. (RT-)PCR 968-1401 represents the (RT-)PCR with primers U968-GC and L1401, which amplify the V6 to V8 regions. PCR 8-1510 represents the PCR with primers 8f and 1510r, which amplify the complete 16S rDNA.

Recovery, preparation, and storage of fecal samples. Fresh fecal samples were collected from 16 unrelated individuals (A to P) from different geographical locations within The Netherlands and Finland, differing in dietary preferences, age (25 to 78 years), and sex (7 men and 9 women). Four fecal samples from individual A (male; 25 years old) were taken within a 6-month period at 2-month intervals, and two samples from individual B (female; 43 years old) were taken within a 7-month period. Three grams (wet weight) of fecal samples was homogenized in 50 ml of ice-cold 0.05 M potassium phosphate buffer (pH 7.0), and aliquots of 1 ml were stored at -20°C.

Parallel RNA and DNA isolation. Fecal samples (1 ml) were centrifuged at 9,000 *X g* for 5 min. The pellets were resuspended in 1 ml of a buffer containing 10 mM Tris-HCl (pH 8.0) and 150 mM NaCl (6). After the addition of 150 μl of acid phenol, consisting of phenol buffered in a solution containing 10 mM sodium acetate (pH 5.0) and 140 mM NaCl (6), 0.3 g of zirconium beads (diameter, 0.1 mm) was added. The samples were treated at 5,000 rpm for 3 min in a mini-bead beater (Biospec Products, Bartlesville, Okla.). After the addition of 150 μl of CI solution, consisting of chloroform and isoamyl alcohol in a 24:1 (vol/vol) ratio, the tubes were vortexed briefly and centrifuged for 5 min at 15,000 *X g*. The aqueous phase was split in two aliquots of 0.5 ml, one for RNA isolation and one for DNA isolation.

For RNA isolation, phenol-chloroform extractions were performed with 150 μl of acid phenol and 150 μl of CI solution. These steps were repeated until a clear interface between the aqueous and phenol-chloroform layers was obtained after centrifugation. Subsequently, an extraction with 300 μl of CI solution was performed. Finally, nucleic acids were precipitated with 2 volumes of ethanol and 1/10 volume of 3 M sodium acetate (pH 5.2) at -20°C for 30 min. After centrifugation at 15,000 *X g* for 20 min, the pellet was washed with 500 μl of 70% ethanol, air dried, and resuspended in 500 μl of a buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM NaCl, 6 mM MgCl2 , and 10 mM CaCl2 (6). Five units of RNase-free DNase (Promega, Madison, Wisc.) was added, followed by incubation at 37°C for 30 min. After phenol-chloroform extractions, RNA was precipitated as previously described and resuspended in 100 μl of 10 mM Tris-HCl (pH 8.0). The RNA solutions were checked for the presence of residual amounts of DNA by performing PCR as described in the section "RT-PCR and PCR amplification." When necessary, the DNase treatment was repeated to eliminate all DNA.

For the parallel DNA isolation, 50 μl of 3 M sodium acetate (pH 5.2) was added to the residual aliquots. Subsequently, phenol-chloroform extractions were performed with 150 μl of phenol buffered in TE, which consisted of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (18), and 150 μl of CI solution. After an additional chloroform extraction, DNA was precipitated with 2 volumes of ethanol at -20°C for 30 min. After centrifugation and washing with 70% ethanol, the pellet was resuspended in 500 μl of TE. Five units of DNase-free Rnase (Promega) was added, and the sample was incubated at 37°C for 15 min. After phenol-chloroform extractions and an extraction with chloroform only, DNA was precipitated as before and resuspended in 100 μl of TE. The amount and integrity of the nucleic acids was determined visually after electrophoresis on a 1.2% agarose gel containing ethidium bromide.

RT-PCR and PCR amplification. Primers U968-GC (5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401 (5' GCG TGT GTA CAA GAC CC) (24) were used to amplify the V6 to V8 regions of the bacterial 16S rRNA. The GC clamp in primer U968-GC creates RT-PCR products suitable for separation by TGGE (22).

RT-PCR was performed with the Geneamp Thermostable *rTth* Reverse Transcriptase RNA PCR kit (Perkin-Elmer, Norwalk, Conn.). Reverse transcriptase reaction mixtures of 10 μl contained 10 mM Tris-HCl (pH 8.3), 90 mM KCl, 1 mM MnCl2 , 200 mM each deoxynucleoside triphosphate (dNTP), 2.5 U of *rTth* DNA polymerase, 7.5 pmol of primer L1401, and 1 μl of 10-times-diluted RNA (approximately 5 ng). The mixtures were incubated at 68°C for 15 min. After this incubation, 40 μl of the PCR additive was added. The additive consisted of 5% glycerol, 10 mM Tris-HCl (pH 8.3), 100 mM KCl, 0.05% Tween 20, 0.75 mM EGTA, 3.75 mM MgCl2 , 50 mM each dNTP, and 7.5 pmol of primer U968-GC. The samples were amplified in a Geneamp PCR

system 2400 (Perkin-Elmer) by using the following program: 94°C for 1 min; 30 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 1 min; and finally, 68°C for 7 min. Aliquots of 5 μl were analyzed by electrophoresis on a 1.2% (wt/vol) agarose gel containing ethidium bromide to check the sizes and amounts of the amplicons.

PCR was performed with the *Taq* DNA polymerase kit from Life Technologies (Gaithersburg, Md.). PCR mixtures of 50 μl contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl2 , 50 mM each dNTP, 1.25 U of *Taq* polymerase, 5 pmol of the primers L1401 and U968-GC, and 1 μl of 10-times-diluted DNA (approximately 1 ng). Amplification and analysis by agarose gel electrophoresis were performed as described above for the RT-PCR, with the exception that the first incubation at 94°C was performed for 3 min instead of 1 min.

Cloning of the PCR-amplified products. PCR was performed with primers 8f [5' CAC GGA TCC AGA GTT TGA T(C/T)(A/C) TGG CTC AG] and 1510r [5' GTG AAG CTT ACG G(C/T)T ACC TTG TTA CGA CTT] (15) by using the *Taq* DNA polymerase kit from Life Technologies to amplify the bacterial 16S rDNA. PCR was performed under the following conditions: 94°C for 3 min; 30 cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 1.5 min; and finally 68°C for 7 min. The PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified PCR products were quantified by electrophoresis on a 1.2% (wt/vol) agarose gel with a DNA solution of known concentration as the concentration standard and were cloned in *E. coli* JM109 by using the Promega pGEM-T vector system. Colonies of ampicillin-resistant transformants were transferred with a sterile toothpick to 50 μl of TE and were boiled for 15 min to lyse the cells. Subsequently, PCR was performed with pGEM-T-specific primers T7 (5' AAT ACG ACT CAC TAT AGG) and SP6 (5' ATT TAG GTG ACA CTA TAG) to check the size of the inserts by using the cell lysates as the template. The plasmids containing inserts of approximately 1.6 kb in the cell lysates were used to amplify the V6 to V8 regions. The amplified V6 to V8 regions were compared to the rRNA-derived TGGE profile from the same fecal sample. Plasmids containing an insert of a clone corresponding to a dominant band were purified by the Wizard Plus miniprep DNA purification system (Promega) and were used for sequence analysis.

TGGE analysis of PCR amplicons. The Diagen (Düsseldorf, Germany) TGGE system was used for sequence-specific separation of RT-PCR products. Electrophoresis was performed in a 0.8-mm polyacrylamide gel (6% [wt/vol] acrylamide, 0.1% bisacrylamide, 8 M urea, 20% [vol/vol] formamide, 2% [vol/vol] glycerol) with 40 mM Tris-acetate (pH 8.0) as the electrophoresis buffer at a fixed voltage of 120 V (69 mA) for 18 h. A gradient from 36 to 45°C was applied parallel to the electrophoresis running direction. After the completion of electrophoresis, the gel was stained with $AgNO_3$ and developed (3).

Sequence analysis. Purified plasmid DNA (1 μl) was used for sequence analysis of the cloned 16S rDNA by using the Sequenase (T7) sequencing kit (Amersham, Slough, United Kingdom) according to the manufacturer's instructions with Infrared Dye 41 (MWG-Biotech, Ebersberg, Germany)-labeled primers 515r (5' ACC GCG GCT GCT GGC AC) (15), 338f (5' ACT CCT ACG GGA GGC AGC), and 968f (5' AAC GCG AAG AAC CTT AC) (24) as sequencing primers. The sequences were automatically analyzed on a LI-COR (Lincoln, Nebr.) DNA sequencer 4000L and corrected manually. The fraction of unidentified bases was 1% or less. The

sequences were checked for reading errors with the alignment programs of the ARB package, which are based on secondary structures of rRNA (31). Homology searches of the ARB, EMBL, and GenBank DNA databases for these partial sequences were performed with FASTA (25), and the homologies were checked with the ARB programs. The complete 16S rDNA sequences were checked for chimerical constructs by using the CHECK-CHIMERA program of the Ribosomal Database Project (17) and the ARB software package.

Nucleotide sequence accession numbers. The sequences of the fecal rDNA clones were deposited in the GenBank database. The new sequences from subject A (with their accession numbers in parentheses) are A03 (AF052408), A07 (AF052409), A09 (AF052410), A10 (AF052411), A11 (AF052412), A12 (AF052413), A13 (AF052414), A14 (AF052415), A19 (AF052416), A20 (AF052417), A21 (AF052418), A22 (AF052419), A27 (AF052420), A54 (AF052421), A57 (AF052422), and A71 (AF052423).

RESULTS

Nucleic acid extraction and reproducibility of TGGE patterns. Nucleic acids were extracted from the fecal samples by a mechanical procedure which has been shown to be effective in disrupting bacterial cells from a variety of ecosystems (6, 11, 26, 38). To optimize the efficiency of the nucleic acid extraction, the effect of increased bead-beating time on the concentration of the nucleic acids, as well as on their integrity and competence to generate a reproducible TGGE pattern, was determined. With 1 to 5 min of bead beating, no effect on the amount of nucleic acids extracted or on their integrity, as analyzed by agarose gel electrophoresis (data not shown), was observed. No nucleic acids were detected in the samples that had not been subjected to the bead-beating procedure or in the supernatant of the potassium phosphate buffer used to homogenize the fecal samples, indicating that lysis was limited. The TGGE patterns of the rRNA-based amplicons of fecal samples tested remained constant after increased bead-beating time (data not shown). However, in the rDNA-derived pattern of subject A, a new amplicon appeared only after 3 min of bead beating, suggesting the presence of a bacterium which was difficult to lyse (Fig. 2). Since further treatment did not affect the TGGE profiles, 3 min of bead beating was used in further experiments.

The effect of template concentration on the TGGE profile was studied by amplifying fecal rRNA and rDNA at different concentrations. Dilution to approximately 10 pg of DNA/μl or 1 pg of RNA/μl did not affect the TGGE profiles. However, further dilutions resulted in the disappearance of faint bands (data not shown). This indicated that the TGGE profiles were derived from 16S rRNA and 16S rDNA that were present in abundance, suggesting that they reflect the most prominent bacteria.

To determine the detection limits of the methods used, the amplified V6 to V8 regions of a cloned 16S rRNA gene were added at different concentrations to fecal V6 to V8 amplicons before TGGE analysis. Band intensities of the diluted amplicons from the clone and the fecal amplicons were compared (data not shown). This competitive-PCR approach indicated that the difference between concentrations of amplicons resulting in a dominant or a faint band was maximally 1 order of magnitude, indicating that only 90 to 99% of the amplicons can be visualized by TGGE.

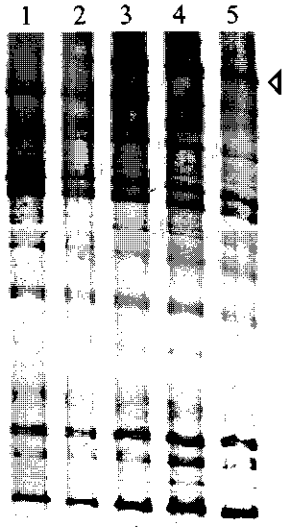


Fig. 2. Optimization of nucleic acid extraction. Lanes 1 to 5 show the TGGE patterns of amplified V6 to V8 regions of DNA extracted after 1, 2, 3, 4, and 5 min of bead beating, respectively. The arrowhead points to the dominant band in the pattern, which appeared after at least 3 min of bead beating.

The possibility of preferential amplification (27) was checked by analyzing TGGE profiles of PCR products generated after different numbers of cycles of amplification. Amplicons obtained after 25 cycles or fewer had to be concentrated. No difference was observed in the TGGE patterns of amplicons obtained after 20, 25, 30, and 35 cycles of amplification. The dominant bands of these patterns were also present in the TGGE profiles of amplicons obtained after 10 cycles, which contained large amounts of single-stranded DNA,

preventing further detailed comparison (data not shown). Since the extent of the amplification had no apparent effect on the TGGE pattern of the amplicons, we used 30 cycles of amplification in further experiments. The TGGE pattern obtained in this way shows a mixture of dominant and faint bands that were separated when the applied temperature gradient was used (Fig. 2). Careful analysis of this TGGE pattern and others revealed the presence of more than 30 prominent amplicons of different mobilities (Fig. 2).

Comparison between RNA- and DNA-derived TGGE profiles. TGGE profiles derived from fecal RNA and DNA amplicons were compared in order to determine the expression levels of the 16S rRNA genes of the most prominent bacteria, which may reflect their contributions to total activity (Fig. 3). The TGGE patterns derived from RNA and DNA from the same fecal sample were highly similar. However, some bands were more prominent in the RNA-derived profile than in the DNA-derived profile in both subjects, indicating that these bacteria could be very active metabolically. In contrast, few bands in the rRNA-derived pattern were faint, while bands at this position in the DNA-derived pattern were more prominent in both subjects. Such bands probably represent predominant bacteria in the feces that became inactive at the end of the large intestine.

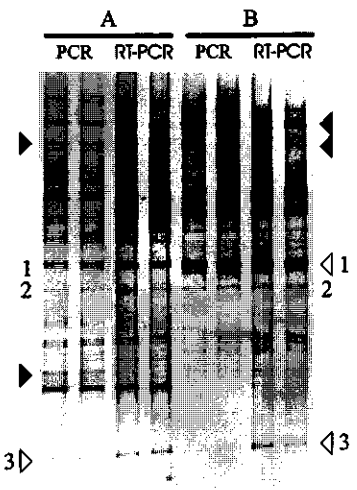


Fig. 3. Comparison between TGGE patterns of PCR and RT-PCR products of the V6 to V8 regions from simultaneous rRNA and DNA isolations of fecal samples from individuals A and B (two replicates each). Solid arrowheads indicate bands with higher intensities in DNA- than in RNA-derived patterns. Open arrowheads indicate bands of higher intensities in RNA- than in DNA-derived patterns. Numbers 1 to 3 represent dominant bands found in all TGGE profiles.

Host-dependent TGGE patterns. TGGE analysis of PCR products of fecal samples from 14 individuals (Fig. 4) was performed, and their TGGE profiles were compared to those of PCR products from individuals A and B (Fig. 3). Remarkable differences in individual banding patterns were observed. Differences were found in both the positions of specific bands and the number of bands (up to 38 in the profile of subject profile C). While most prominent bands were found at different positions, some distinct bands were observed in all fecal samples (indicated in Fig. 3 and 4). This indicates that each individual harbors a specific bacterial community in the intestine but that a few dominant bacterial species may be present in many individuals.

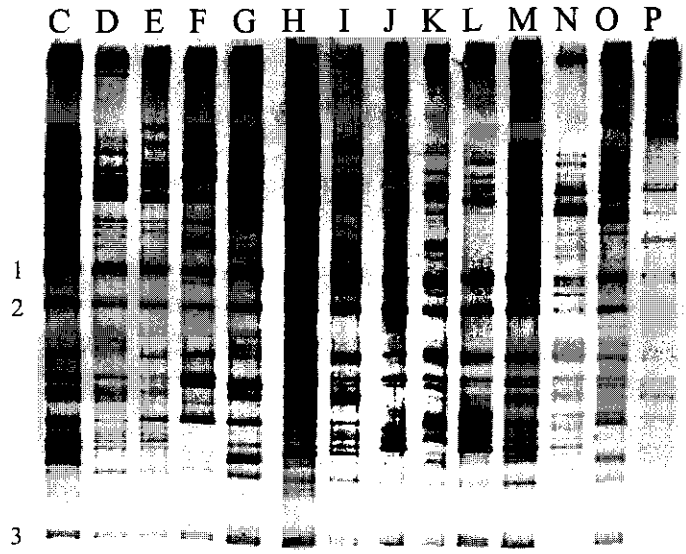


Fig. 4. TGGE of PCR products of the V6 to V8 regions of fecal samples from individuals C to Q (from Finland). Numbers 1 to 3 represent dominant bands found in all TGGE profiles.

Stability of TGGE patterns over time. Since the observed differences in banding patterns could also be influenced by temporal variation, the fecal samples of two healthy individuals, A and B, were monitored over time (Fig. 5). Four samples from individual A were taken within a period of 6 months. The two fecal samples from individual B were taken within a period of 7 months. In both individuals, the RNA-derived banding patterns were highly constant over a period of approximately half a year. Only some slight differences in the

intensities of the bands were observed in both individuals. Similar results were found in the DNA-derived patterns (data not shown). This indicates that the dominant microbial composition remains quite constant over time for these healthy individuals.

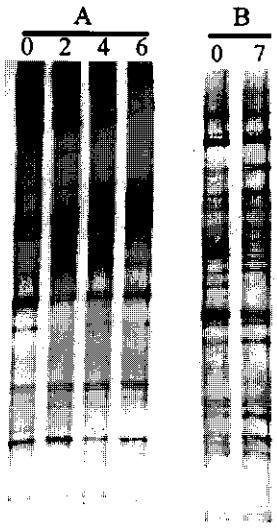
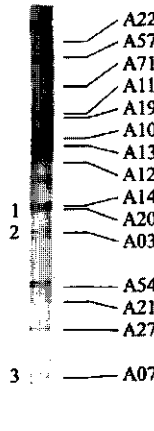


Fig. 5. (A) TGGE of RT-PCR products of the V6 to V8 regions of four fecal samples from individual A taken at different times during a period of 6 months. **(B)** TGGE of RT-PCR products of the V6 to V8 regions of two fecal samples from individual B taken after 0 and 7 months.

Phylogenetic analysis of dominant bands in a TGGE pattern. To gain insight into the phylogenetic positions of the predominant bacteria, 16S rDNA from fecal samples of individual A was amplified and cloned into *E. coli* JM109 by using pGEM-T. The V6 to V8 regions of the 16S rDNA of the cell lysates of 78 transformants were amplified, and their mobilities after TGGE were compared to the rRNA-derived pattern of individual A (Fig. 1). In this way, 45 clones were each assigned to 1 of the 15 prominent bands in the TGGE pattern, while 33 did not match any of the detectable bands (Fig. 6). Twelve of the 33 clones which did not match any band in the pattern showed similar mobilities during TGGE. The sequence showed the highest similarity (98.9%) to an unidentified rumen bacterium (GenBank accession no. AF018503) (37). The next closest relative was *Prevotella veroralis* (89.9%). Nested amplification of the V6 to V8 regions of the 16S rDNA amplicons showed the appearance of this band in the pattern (data not shown), indicating a preferential amplification of this sequence in the first PCR amplification.

For identification of the prominent bands in the rRNA-derived TGGE pattern, the plasmid DNA from the corresponding clone was purified and the nucleotide sequence of the 16S rDNA insert was determined and compared to the 16S rRNA databases. Among the sequences of the 15 prominent amplicons, only 2 showed more than 97% similarity (Fig. 6). This indicates that the majority of the sequences were derived from new, as yet undescribed bacterial species. The phylogenetic positions of these 15 sequences were located in different *Clostridium* clusters of the low-G+C gram-positive species (4).

Since their partial sequences showed low similarities to the sequences of the closest relatives in the databases, seven clones were completely sequenced, analyzed, and checked for chimerical constructs. The percentages of identity to the closest relatives were similar to those of the partial sequences.



	Clone ID	Closest Relative	Identity (%)	Number of Clones in Library	Sequence Analysis
	A22	*Roseburia cecicola*	94.1	1	P
	A57	*Ruminococcus obeum*	92.2	2	C
	A71	*Eubacterium formicigenerans*	92.3	3	C
	A11#	*Butyrivibrio fibrisolvens*	92.0	3	C
	A19	*Coprococcus eutactus*	95.3	2	C
	A10	*Fusobacterium prausnitzii*	94.7	9	P
	A13#	*Eubacterium rectale*	97.8	3	P
	A12	*Ruminococcus productus*	93.1	2	P
1	A14	*Ruminococcus obeum*	94.1	1	P
2	A20	*Ruminococcus obeum*	93.1	6	P
	A03	*Fusobacterium prausnitzii*	91.5	7	C
	A54	*Clostridium celerecrescens*	93.1	2	C
	A21	*Clostridium celerecrescens*	92.7	1	C
	A27	*Eubacterium plautii*	96.8	1	P
3	A07	*Eubacterium halii*	98.7	2	P
				45	15

Fig. 6. Identification of dominant bands in the RT-PCR pattern of the V6 to V8 regions of fecal samples from individual A. Listed are the closest relatives of the clones corresponding to the bands, their percent identity, the number of corresponding clones, and the sequence analysis. #, the V1 to V3 regions of two clones with identical mobility have been sequenced. P, partial 16S rDNA sequence; C, complete sequence. Amplicons encoded with 1, 2, and 3 were found in the TGGE patterns of all subjects.

In order to estimate whether bands in the TGGE profiles originated from one or more sequences, pairs of 16S rDNA clones which gave identical TGGE band positions in the V6 to V8 regions were further sequenced. Three pairs of clones were analyzed in this way. Two pairs corresponded to two bands in the TGGE profile of subject A (Fig. 6), and one pair

corresponded to the band which originated from the preferentially amplified sequence. Each pair of clones showed more than 99.5% identity in the V1 to V3 regions, indicating that each band harbors only one type of sequence.

DISCUSSION

In this study we have shown that TGGE analysis of the V6 to V8 amplicons of fecal 16S rRNA and rDNA is a powerful tool for analysis of complex microbial communities in fecal samples. TGGE patterns were unique for each individual. Since no major changes in banding patterns were found during amplification or dilution of the template, the differences in the patterns must reflect the differences in fecal composition among individuals. The results indicate that each individual has his or her own personal and unique microbial community, extending previous work based on the composition of *Lactobacillus* spp. and *Bifidobacterium* spp. (19) in selective plate counts (9, 13, 19, 29).

RT-PCR products and PCR products from the same fecal sample were very similar. Because eubacterial primers which show very limited efficiency in the amplification of *Archaea* and *Eucarya* have been used, the TGGE pattern reflects the predominant bacteria in fecal samples. The differences between intensities of specific bands in the 16S rDNA- and 16S rRNA-derived patterns are most probably due to differences in the activities of different groups of bacteria. The amount of rRNA per cell is dependent on the type of species and the physiological condition of the cell. In addition, differences in the copy number of the rRNA operon per species (2) also play a role in differences in intensity. Some bacteria which have reached high numbers in the intestine could have been inactivated by losing contact with the mucosa or by exposure to oxygen during the collection of the fecal samples.

In comparisons of the TGGE profiles of fecal samples with those of clones, it appeared that 42% of the clones did not match any band in the TGGE profile. Twelve clones with similar mobilities during TGGE did not correspond to any dominant band in the rRNA- and rDNA-derived profiles of individual A. The partial sequence showed the highest homology with an unidentified rumen bacterium and was grouped in the *Prevotella* cluster. However, this amplicon was found in the DNA-derived pattern after nested amplification of the V6 to V8 regions of amplified 16S rDNA. This result supports the existence of PCR and cloning biases as reported previously (27, 32, 34, 38). Another reason for the difference between the results of cloning and TGGE analysis was described by Felske et al. (7). Analysis

of a PCR amplicon by TGGE visualizes only the dominant fraction of the population, while a cloning approach randomly selects 16S rDNA amplicons. As a consequence, hundreds of bacteria which represent a numerically important part of the total community do not form visible bands in the TGGE profiles, although some of them will be selected by cloning.

The 15 clones corresponding to dominant bands in the TGGE patterns of individual A showed the highest homology with sequences derived from different *Clostridium* clusters (4). Only two partial 16S rDNA sequences showed more than 97% identity to a sequence in the databases. The other complete and partial 16S rDNA sequences did not match any of the sequences found in the ARB, GenBank, and EMBL databases. Furthermore, their similarities were below the 97% threshold for being considered the same species (30), and they could therefore be considered to be derived from new bacterial species. The identity values of clones corresponding to *Coprococcus eutactus* could be underestimated, since the sequence of this species in the databases contains a large number of unidentified nucleotides.

Two complete sequences corresponding to two bands in the TGGE pattern showed the highest homology with *Clostridium celerecrescens*. These sequences showed 97.5% identity and differed by at least 9 bases in the V6 to V8 regions. This explains the different locations of these clones in the TGGE profiles. *Ruminococcus obeum*-like and *Fusobacterium prausnitzii*-like amplicons were also found several times among the most prominent bacteria. Previously, similar observations have been reported for closely related *Bacillus benzoevorans*-like sequences in Drentse A grassland soils (8). These observations could be due to 16S rDNA sequence heterogeneity of bacterial strains (24). However, one of the three *R. obeum*-like sequences derived from a species which was difficult to lyse, indicating the presence of at least two different types of *R. obeum*-like species.

Sequences derived from *R. obeum* showed high-intensity bands in both rRNA- and rDNA-derived patterns in individual A, and they could therefore be considered numerically important in composition and in the total activity. The genera *Ruminococcus* and *Coprococcus* are anaerobic cocci, and members of this group, especially *Ruminococcus* and *Peptostreptococcus*, have been found numerically important in fecal samples (9, 21).

Clones corresponding to two dominant bands showed the highest homology with a *F. prausnitzii* sequence described previously (36). It was reported that this sequence was not clustering in the *Fusobacterium* group and that this strain was one of the most common species in human feces, based on PCR quantitation (35, 36). *F. prausnitzii*, and also *Roseburia cecicola* and *Butyrivibrio fibrisolvens*, are known to be gram-negative species but

are phylogenetically related to low-G+C gram-positive species in the *Clostridium* group described by Collins et al. (4).

The other clones traced in the TGGE profiles showed the highest homology to *Eubacterium* spp. and *Ruminococcus productus* (also known as *Peptostreptococcus productus*) (5). *Eubacterium plautii* was found as an endosymbiont of *Entamoeba histolytica* (12). The other eubacteria have been found previously in human fecal samples (9, 21).

All individual-specific patterns had some bands in common. Sequences corresponding to these bands were found in the clone library and showed the highest homologies to *R. obeum*, *Eubacterium hallii*, and *F. prausnitzii*. This indicates that these bacteria have an important function at the end of the gastrointestinal tract in all individuals. Eubacteria and ruminococci are known to have a fermentative metabolism, while fusobacteria in general have a weakly fermentative metabolism (14).

Surprisingly, *Bacteroides* spp. were not found in the TGGE profile of subject A. Since we used bacterial primers, we did not select to the benefit of *Bacteroides* spp. The TGGE profile represents only 90 to 99% of the total bacterial community. This means that bacteria which reach levels of 10^9 cells or fewer (this number is not unusual for *Bacteroides* spp.) per g of feces will not be visualized on TGGE, assuming that 1 g of feces contains 10^{11} cells.

Overall, it can be concluded that the results support the hypothesis that each healthy person has his or her own unique fecal flora and that the dominant active flora is stable over time. Since the individuals were unrelated, were of different ages, and had different dietary preferences, the reasons for this uniqueness are likely to be found in host factors. The conclusions are based on an approach which could be biased by irregular cell lysis and primer specificity, which are general drawbacks of molecular microbial ecology. Even though the quality and reproducibility of the extraction of nucleic acids and their amplification have been investigated, the possibility that some important, hitherto unknown bacteria were missed cannot be excluded. The approach reported here can be useful in studying the effects of certain diets, food components, probiotics, prebiotics, and antibiotics, as well as the genetic background of the host, on the stability and composition of the dominant microbial community.

ACKNOWLEDGMENTS

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Chapter 4

DNA Isolation Protocols Affect the Detection Limit of PCR Approaches of Bacteria in Samples from the Human Gastrointestinal Tract

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Abstract

A major concern in molecular ecological studies is the lysis efficiency of different bacteria in a complex ecosystem. We used a PCR-based 16S rDNA approach to determine the effect of two DNA isolation protocols (i.e. the bead beating and Triton-X100 method) on the detection limit of seven feces-associated bacterial species of different genera. Glycogen was used in these protocols to improve the precipitation of small concentrations of DNA in ethanol without affecting the sequential procedures. The PCR detection limit of 16S rDNA amplicons on agarose gel from the seven strains tested varied between 8.0 (±1.3) X 10^4 and 4.3 (±1.6) X 10^6 cells for the bead beating method, and between 8.0 (±1.3) X 10^4 and 5.4 (±0.7) X 10^8 cells for the Triton X-100 method. Denaturing gradient gel electrophoresis (DGGE) analysis was performed to investigate the effect of both DNA isolation protocols on the lysis efficiency of bacteria in fecal samples. A higher diversity in fecal samples was observed with the bead beating method than with the Triton-X100 method. Bands in the bead beating method-derived DGGE profiles corresponding to bands of cloned sequences of the *Clostridium coccoides-Eubacterium rectale* group and uncultured *Fusobacterium prausnitzii* were absent or had low intensity in the Triton X-100 method-derived profiles. The applicability of the bead beating method was further investigated by analyzing biopsy samples from the human colon which contain approximately 10^6 cells.

INTRODUCTION

Molecular approaches based on the sequence diversity of 16S rDNA have become very important in analyzing and monitoring bacterial diversity (4, 9, 16). These have been applied to a variety of ecosystems, including the human gastrointestinal tract (21, 22, 23, 25). In all approaches, lysis of bacterial cells is the first and one of the most crucial steps and should be equally efficient for all bacterial species and independent of the growth phase of the bacteria, the preparation of the sample, and the concentration of cells in the sample.

Determining the lysis efficiency has mainly been performed either by examining the samples microscopically or by counting the number of colony forming units before and after treatment (5, 8, 24). Since many DNA isolation protocols use toxic chemicals, the latter approach is of limited use. The main problem of microscopic examination is that cell numbers below 10^7 ml^{-1} are difficult to detect. Hence we developed a PCR-based approach to evaluate the detection limit of cells using two different DNA isolation protocols. We used the human GI tract as a model for a complex ecosystem and applied the approach to detect bacterial diversity in fecal samples and small biopsy samples from the human colon that usually contain low numbers of bacteria (2, 7).

In the study described here we optimized the recovery of low concentrations of DNA and combined flow cytometry and PCR of 16S rDNA to determine the total minimal number of living and dead cells needed to obtain a quantifiable PCR product. Although many DNA isolation procedures have been described (1), we focused on two widely used but basically different methods to estimate the difference in detection limit for different bacterial species and compared a relatively advanced method based on disruption by bead beating with a relatively simple method based on the addition of Triton X-100.

MATERIALS AND METHODS

Fecal and biopsy sample collection. Fresh fecal samples were collected and homogenized in the sampling tubes. Three grams of fecal samples were resuspended in 50 ml 0.05 M potassium phosphate buffer (pH 7.0) on ice for 5 to 10 minutes and divided in 1 ml aliquots as described previously (25). These aliquots were used for DNA isolation. Duplicate fecal and descending colonic biopsy samples (diameter ~ 3 mm) from a previous study were kindly provided (3).

Bacterial strains and culture conditions. The seven strains used for this experiment were *Bifidobacterium longum* (ATCC 15707), *Bifidobacterium breve* (ATCC 15700), *Lactobacillus casei* (ATCC 393), *Lactobacillus acidophilus* (ATCC 4356), *Clostridium clostridiiforme* (Lille-JTV), *Enterococcus faecalis* (ATCC 29212), and *Bacteroides fragilis* (Lille-TM6). Representatives of the *Bacteroides*, *Bifidobacterium* and *Clostridium coccoides-Eubacterium rectale* group (*Bacteroides fragilis*, *Bifidobacterium* spp., and *Clostridium clostridiiforme*, respectively) were chosen since these groups are dominantly present in fecal samples (12). *Enterococcus faecalis* was selected as a member of the anaerobic gram positive cocci and the *Lactobacillus* species were chosen since lactobacilli are known to be present in low numbers in fecal samples and since this genus is often used in feeding trials as probiotic. The *Lactobacillus* and *Bifidobacterium* species were grown in 10 ml MRS broth (Difco Laboratories, Detroit, MI, USA) while the other species were grown in 10 ml Wilkins-Chalgren Anaerobe Broth (Oxoid, Basingstoke, UK). All media contained 0.5 g/l L-cysteine monochloride and cultures were incubated at 37°C under anoxic conditions. Cells were harvested when they reached the stationary phase and centrifuged at 9000 X g for 5 min. After resuspending cells in 1 ml 0.05 M potassium phosphate buffer (pH 7.0), cells were extensively vortexed to minimize clumping, and ten-fold dilution series were made for flow cytometric analysis and DNA isolation.

Flow cytometric analysis. The total number of cells in different dilutions was determined by a flow cytometric count approach to quantify the total number of stationary grown cells accurately and rapidly (6, 14). The number of bacteria in the higher dilutions (lower than ~10^6 cells per ml) was determined by extrapolation from the lower dilutions, since the number of cells in these dilutions was too low for accurate counting. The bacterial count kit (Molecular probes Inc, Leiden, The Netherlands) was used according to the manufacturer's instructions in order to determine cell numbers in the sample. Samples were analyzed using a FACScalibur flow cytometer (Beckton Dickinson). A 488 nm laser was used for excitation and green fluorescence of the SYTO BC probe was collected through an FL1 (530/30 nm) detector. The system threshold was set on side scatter signals and all bacterial analyses were performed at the low rate settings (12 µl/min). The sample concentration was adjusted to keep the count lower than 1,000 events/sec to avoid coincidence. Data were collected in list mode as pulse height signals (four decades in logarithmic scale each) and 5,000 to 10,000 cells were acquired for further analysis which was performed using CellQuest software (Beckton Dickinson) and/or WinMDI version 2.8 software (http://facs.Scripps.edu/software.html).

DNA isolation. Two DNA isolation procedures were used to isolate DNA from the cultures and fecal samples. For the bead beating method, resuspended cells and fecal samples were lyzed by incubation for 1 hour at 55°C with 10 µl Proteinase K solution (20 mg/ml) and 50 µl 10% SDS solution followed by bead beating (3 min at 5,000 rpm) in 150 µl phenol (pH 7.5) using a mini bead beater (Biospec Products, Bartlesville, Oklahoma, USA). The additional incubation with Proteinase K and SDS prior to the bead beating procedure as described previously (25) did not alter the DGGE profiles. For the Triton X-100 method, cells were lyzed by adding 11% Triton X-100 (100 µl) to the cell and fecal resuspensions followed by 10 minutes incubation at 95°C (24, modified). After the cell lysis, phenol/chloroform extractions and one chloroform extraction were performed. To enhance the precipitation of low concentrations of DNA, a 20 mg/ml glycogen solution was prepared as precipitation carrier (18). After preparation this glycogen solution was cross-linked using UV light to remove

potential DNA contaminants. Before precipitation, 1 µl of the glycogen solution was added to the DNA solution followed by the addition of 96% ethanol, precipitation, washing and resuspension of the DNA in 100 µl TE (pH 8.0).

DGGE analysis of PCR-amplified products. One microliter of DNA solution of the (diluted) cultures and 10 times diluted DNA solution of the fecal samples was subsequently used as template to amplify the V6 to V8 regions of 16S rDNA using primers F-0968-GC and R-1401 (17). The amplification (35 cycles) and the analysis of 5 µl of amplicons on ethidium-stained 1.2% agarose gels was performed as described previously (25). In addition, quantification of standard DNA solution was performed by determining the absorption at 260 nm. DGGE analysis of the amplicons was performed on 8% polyacrylamide gels containing a urea plus formamide gradient from 38% to 48% (15). Electrophoresis was performed in 0.5 x TAE at 85 V at 60°C for 16 hours using the DCode or D GENE System apparatus (BioRad, Hercules, CA). After electrophoresis, gels were silver-stained (19, modified). Gels were scanned at 400 DPI and analysis of the gels was performed using the software of Molecular Analyst 1.12 (Biorad). The similarity between the DGGE profiles was determined by calculating similarity indices of the densitometric curves of the profiles compared using the pearson product-moment correlation (13). Since fecal profiles are remarkable stable in time (25), a fecal-derived clone collection with known sequences from a previous fecal sample of one of the individuals was used to identify bands in the profiles from this individual.

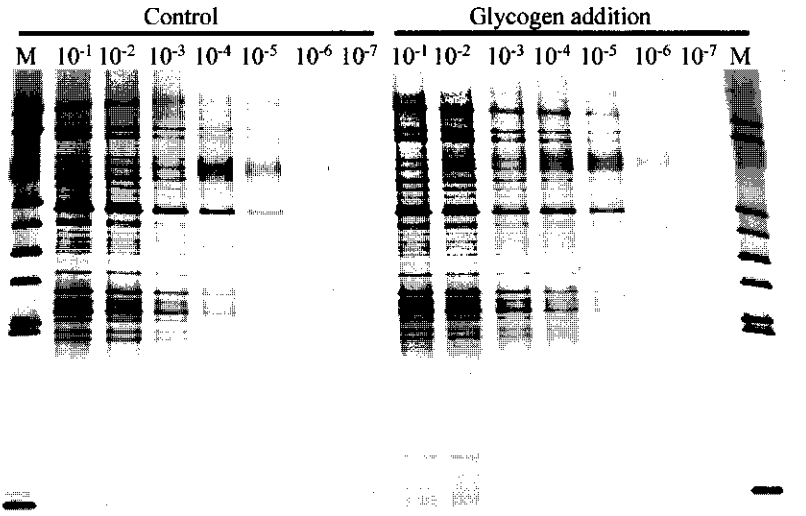

Fig. 1. Silver-stained DGGE gel showing fecal profiles of dilution series of DNA precipitated with and without the addition of glycogen. M represents a marker for DGGE analysis, 10^{-1} to 10^{-7} represent the DNA dilutions.

RESULTS AND DISCUSSION

Effect of glycogen addition. To test the effect of glycogen in enhancing the DNA precipitation, dilution series of fecal DNA solutions was made and the DNA was precipitated with ethanol in the presence or absence of glycogen. After amplifying the V6 – V8 regions of 16S rDNA the PCR fragments were analyzed by DGGE. As shown in figure 1 the complete DGGE profile could be retrieved in a 10 times more diluted DNA solution when glycogen was added before the precipitation, indicating that glycogen enhances the DNA recovery. It is clearly visible that glycogen did not affect the amplification and the silver staining procedure, and was therefore compatible with the DGGE analysis of PCR amplicons.

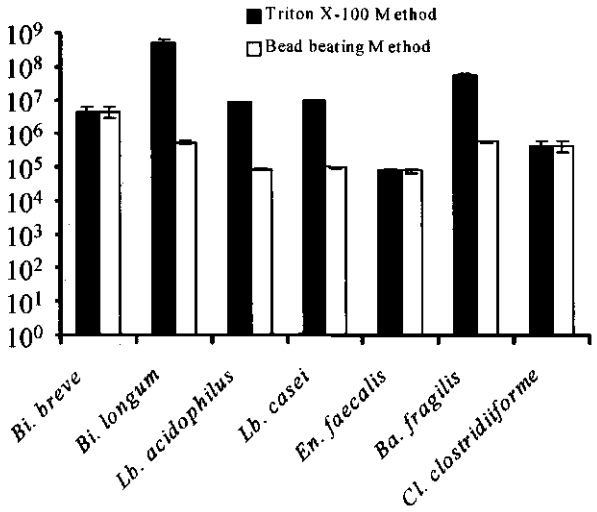

Fig. 2. Diagram showing the minimal number of cells needed to get a visible PCR product on agarose gel for different species using the two DNA isolation protocols. The mean and standard deviations of the flow cytometric counts are indicated. The black and gray bars indicate the cells lyzed with the Triton X-100 method and the bead beating method, respectively.

Detection limit of bacterial cultures. Seven feces-associated bacterial species of different genera were selected to determine the detection limit of two DNA isolation protocols that differ in the cells lysis procedure, i.e. bead beating and addition of Triton X-100. Bacterial numbers were counted by flow cytometry, which results in accurate counts of the different

species with relatively low variance. Comparison of agarose gel analysis of the PCR products from the dilution series of the Triton X-100 and the bead beating method showed an enormous difference in the detection of the different species (Fig. 2). For example at least 5.4 (±0.7) X 10^8 *Bifidobacterium longum* cells were needed initially for the Triton X-100 method in order to get a visible PCR product by agarose gel electrophoresis, while this was 8.0 (±1.3) X 10^4 cells for *Enterococcus faecalis*. This indicates that the strain-dependency of this method is high, in contrast to that of the bead beating method in which the largest difference observed was 4.3 (±1.6) X 10^6 for *Bifidobacterium breve* and 8.0 (±1.3) X 10^4 for *Enterococcus faecalis* (Fig. 2). Thus, the latter method will give a more reliable picture of the composition in a mixed culture than the Triton X-100 method. It is evident that the observed findings may be partly influenced by differences in 16S rRNA gene copy number in the genome of the different species, which may vary from 1 to more than 10 (20). However, this variation in copy number is insufficient to explain a 100-fold or higher difference between the different species as observed in this study.

Calculated PCR detection limit. The lowest number of cells detected in both methods was approximately 10^5 bacteria (Fig. 2). It was of interest to determine whether this threshold was influenced by the cell lysis or the PCR approach. Based on the minimal amount of a 450 bp DNA fragment that could be visualized (approximately 80 ng in a 5 µl sample), the number of PCR cycles (35), and the number of rDNA copies per cells (1-10), it could be calculated (11) that under ideal PCR conditions (i.e. 2 amplifications per cycle) between 5 X 10^2 and 5 X 10^3 cells were required to generate a visible PCR product. In our approach we used stringent PCR conditions and a primer that contains a GCclamp at the 5' end. Since it is known that these PCR conditions are not ideal (10), the detection limit of 10^5 cells is most likely limited by the PCR rather than by the lysis. This relatively high detection limit could probably be lowered by applying a PCR with less stringent PCR conditions and with primers which do not have a GCclamp. However, we favored this stringent PCR method since it is crucial for the DGGE analysis and therefore for the overall approach.

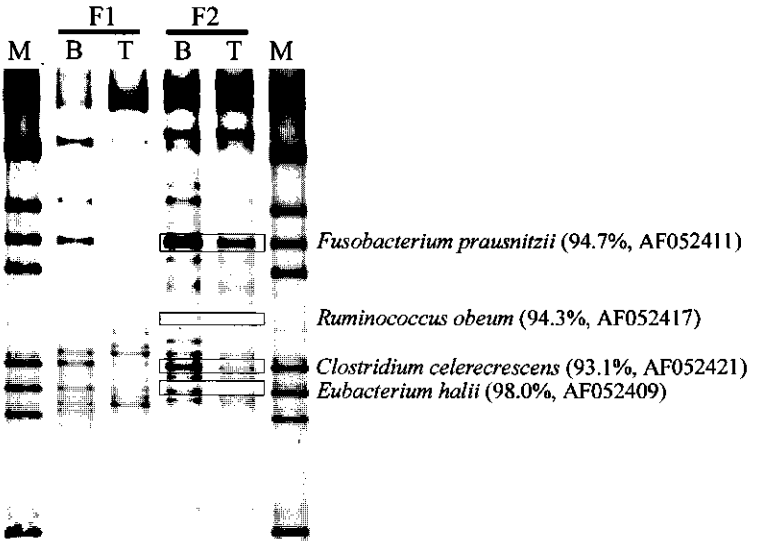

Fig. 3. Silver-stained DGGE gel showing profiles derived from fecal DNA (F1 and F2) using the bead beating (B) and Triton X-100 (T) method. M represents the marker for DGGE analysis. The boxed amplicons, which were only present in the bead beating method-derived profiles were identified by fecal clones with know sequence and identical mobility on DGGE. Accession numbers are indicated .

Analysis of fecal samples and biopsies. In order to determine the effect of the different strain-dependent detection limits of both protocols on the anticipated diversity in environmental samples, DNA was isolated from human fecal samples followed by PCR and DGGE analysis. DGGE analysis showed that the profiles generated after the Triton X-100 method were less complex than those generated after the bead beating method (Fig. 3). For each fecal sample the majority of bands were present in the profile of both methods, indicating the robustness of both DNA isolation procedures. Some amplicons missing or clearly less abundant in the Triton X-100 were identified based on their migration during DGGE (25) as *Fusobacterium prausnitzii* (94.7%), *Ruminococcus obeum* (94.3%), *Clostridium celerecrescens* (93.1%), and *Eubacterium halii* (98.0%), from which the last three belong to the *Clostridium coccoides-Eubacterium rectale* group. This indicates that the difference in detection limit of the protocols is not restricted to one specific genus or phylogenetically related group of organisms which is in line with the culture-based observations. Occasionally, a few bands in the profile generated after the Triton X-100 method were not observed in the profile generated after the

bead beating method. These bands most likely represent bacteria in low abundance that are relatively easy to lyze and therefore overestimated in the profile generated after the Triton X-100 method. The reduced complexity of the profiles after the Triton X-100 method is in line with the high strain dependency of the lysis as observed with pure cultures.

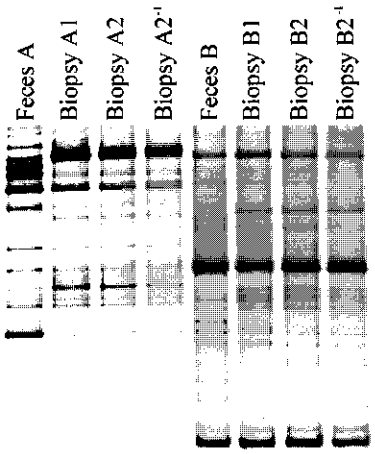

Fig. 4. Silver-stained DGGE gel showing profiles derived from DNA of feces and biopsy samples of two individuals (A and B). Biopsies A1 and A2, and B1 and B2 are duplo samples from the descending colon of individuals A and B respectively. The superscribed –1 indicates that template DNA was ten times diluted.

Since the bead beating method was found to be less strain dependent, fecal samples and descending colonic biopsy samples, which contained approximately 10^6 bacterial cells (3), were analyzed using this method. Fecal and biopsy samples from different individuals resulted in very different DGGE profiles, while duplicate biopsy samples from the same individuals gave the same profiles (similarity index: 98.9 and 98.2; Fig. 4). In individual 1 the fecal and biopsy profiles were different (similarity index: 37.0 and 37.8), while they were highly similar in individual 2 (similarity index: 93.1 and 93.4). The high similarity of the fecal and biopsy profile of the latter individual indicates that the bead beating method was not biased by the approximately 1000-fold difference in cell number. This is strengthened by the fact that 10-fold dilution of template DNA of the biopsy samples prior to PCR did not alter the profiles of both individuals (similarity index: 97.4 and 97.6 for individual A and B, respectively). Despite the fact that samples from only two individuals were analyzed, it is

evident that fecal samples do not necessarily represent the community at other locations in the human colon.

Conclusions. In this study the bias caused by the strain dependent DNA isolation procedure was investigated by combining PCR of 16S rDNA and flow cytometric counting of the cells. It is evident that the DNA isolation procedures tested have some degree of difference in lysis efficiency for the detection limit of the different species, although this was lowest in the bead beating method. It was shown that the latter method was more suitable for analyzing fecal and biopsy samples. Despite the shearing of DNA during the bead beating procedure, this method has shown to be very efficient for analyzing fecal and biopsy samples. In a previous study the optimization and application of a bead beating method for the analysis of fecal samples has already been described (25). Despite such optimization of DNA isolation protocols, one should still be critical when DNA from environmental samples is isolated. Uncultured bacteria are present in environmental samples, that may have novel properties and hence it cannot be excluded that certain bacteria will not be disrupted during a lysis treatment.

In conclusion, the present study demonstrates that the combination of flow cytometry and PCR-DGGE analysis of dilution series of pure cultures gives an estimate of the detection limit of the DNA isolation protocols. Therefore, this detection limit should be determined before DNA and PCR based approaches are used to study the bacterial diversity in an ecosystem.

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Chapter 5

Molecular Diversity of *Lactobacillus* spp., and Other Lactic Acid Bacteria in the Human Intestine as Determined by Specific Amplification of 16S Ribosomal DNA

Hans G.H.J. Heilig, Erwin G. Zoetendal, Elaine E. Vaughan, Philippe Marteau, Antoon D.L. Akkermans, and Willem M. de Vos

Submitted

Abstract

A *Lactobacillus* group-specific PCR primer S-G-Lab-0677-a-A-17 was developed to selectively amplify 16S ribosomal DNA (rDNA) from lactobacilli and related lactic acid bacteria, including members of the genera *Leuconostoc*, *Pediococcus*, and *Weissella*. Amplicons generated by PCR from a variety of gastrointestinal (GI) tract samples, including those originating from feces and cecum, resulted predominantly in *Lactobacillus*-like sequences, of which approximately 28% were most similar to the 16S rDNA of *Lactobacillus ruminis*. Moreover, four sequences of *Leuconostoc* species were retrieved that, so far, have only been detected in environments other than the GI tract, such as fermented food products. Stability in time of the GI tract bacterial community in different age groups were studied using *Lactobacillus*-specific PCR and denaturing gradient gel electrophoresis (DGGE) of the 16S rDNA amplicons. The *Lactobacillus* community in adults over a two years period showed variation in composition and stability depending on the individual, while successional change of the *Lactobacillus* community was observed during the first five months of an infant's life. Furthermore, the specific PCR and DGGE approach was used to study the retention in fecal samples of a *Lactobacillus* strain administered during a clinical trial. In conclusion, the combination of specific PCR and DGGE analysis of 16S rDNA amplicons, allows the diversity of important groups of bacteria that are present in low numbers in specific ecosystems to be characterized, such as the lactobacilli in the human GI tract.

INTRODUCTION

The human gastrointestinal (GI) tract consists of different habitats, which especially in the right and distal colon are occupied by mostly obligate anaerobic bacteria (28). The activity of these commensal bacteria in the GI tract has a major impact on the characteristics of the host. The microbiota-associated roles include protection against pathogens, development of the immune system, and positive effects on colonic health and host nutrition (6, 15). Although the diversity of the gut microbiota has been investigated extensively by anaerobic culture techniques (8, 28), it is recently receiving renewed interest due to the development and application of molecular techniques, especially those based on the 16S and 23S ribosomal RNA (rRNA) genes (45, 47, 48).

Fluorescent *in situ* hybridization (FISH) and group-specific hybridization approaches targeting rRNA in combination with advanced microscopy have indicated that the majority of the GI tract microbial community is not accounted for by cultivation (19, 39, 42, 48). Phylogenetic analysis of rRNA genes, amplified by polymerase chain reaction (PCR), has been used as a rapid and efficient strategy to investigate the biodiversity of intestinal bacteria and revealed many novel species (42, 49). Furthermore, fingerprinting methods such as denaturing or temperature gradient gel electrophoresis (DGGE or TGGE, respectively) of rRNA or rDNA amplicons, that allow the rapid evaluation of composition and activity of bacteria over time in complex ecosystems (27), have been applied to the human intestine (47, 49, 50). It was observed that each individual harbors its own unique dominant bacterial community that is relatively stable in time (49). However, all these molecular approaches, including a recent study on the bifidobacterial community in human feces (36), focus on the numerically dominant bacteria and do not address the diversity of other important species that are present in low numbers in the GI tract. These include lactic acid bacteria, such as *Lactobacillus* spp., that have been shown to constitute less than 1% of the total bacterial community (39). The genus *Lactobacillus* contains a diverse assemblage of Gram-positive, catalase-negative, non-sporulating, rod-shaped organisms and includes more than 25 species (4, 11). They inhabit a wide variety of habitats, including the GI tracts of animals and phytosphere, and are traditionally used in the manufacture of fermented foods, and more recently in functional foods (23, 45). Based on plate counts it was found that *Lactobacillus* strains are present in the GI tract of 70% of humans that consume a Western-like diet, but are not detectable in the remainder of subjects (8, 43). The number of *Lactobacillus* cells in

neonates was found to be in the range of 10^5 colony forming units per gram (CFU/g) of feces, whilst in infants of one month and older the counts ranged from 10^6 to 10^8 CFU/g of feces (20).

The primary aim of the present study was to develop a PCR primer targeting the 16S rRNA gene that is highly specific for the genus *Lactobacillus*. There have been numerous reports on the development of oligonucleotide probes or PCR primers that target specific *Lactobacillus* species (3, 5, 13, 14, 29, 30, 31), or groups of *Lactobacillus* species (40). However, designing a *Lactobacillus* genus-specific probe or primer is more challenging. The developed primer was used to analyze the diversity of members of this group in GI tract samples in space and time. Sequence analysis of the PCR amplicons generated using this primer, showed selective amplification of *Lactobacillus*-like 16S rDNA sequences, some of which have not yet been described to be present in the GI tract. Furthermore, we demonstrate that application of the primer in combination with DGGE of the 16S rDNA amplicons allows the diversity and development of these bacterial groups to be monitored and their response to the additional dietary intake of lactobacilli within the human GI tract.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Reference microorganisms, their sources and the growth media for each strain used in this study are indicated in Table 1. *Escherichia coli* transformants were grown overnight shaking at 250 rpm in Luria Bertani (LB) broth (34). *Lactobacillus* strains were grown in tubes containing 10 ml of *Lactobacillus* MRS broth (Difco Laboratories, Detroit, MI, U.S.A.), and 0.3 g l^{-1} cysteine hydrochloride. The remaining strains were grown in either LB or Wilkins West (WW) broth that contains per litre 33 g Wilkins-Chalgren Anaerobe broth (Oxoid, Basingstoke, U.K.), 2 g glucose, 4 g arginine HCl, 5 ml of 5% Tween-80, and 0.3 g cysteine hydrochloride, and the pH was adjusted to 7.0 with HCl. All bacteria were incubated at 37°C. The strict anaerobes, i.e. *Clostridium*, *Bacteroides*, and *Peptostreptococcus* species, were handled and cultured in an anaerobic glove box with a constant atmosphere of 96% N_2 and 4% H_2, and incubated in an anaerobic jar using Anaerocult A (Merck Microbiologie, Darmstadt, F.R.G.) to ensure an oxygen-free environment. Prior to inoculation of the remaining species, the tubes containing media were boiled for 20 minutes and cooled to room temperature to remove oxygen from the medium. After inoculation, which was performed on the bench, tubes were rapidly sealed with a paraffin plug to ensure anoxic conditions.

Recovery, preparation, and storage of fecal and cecal samples. Fecal samples were provided by healthy volunteers of both sexes, and of different age, in spatula-containers and were stored at –20°C until further use. Informed consent was obtained from the parents of children. One healthy full-term baby-boy, vaginally delivered and breast-fed, has participated in this study. The infant was followed in time and was breast-fed until day 17,

Table 1. Bacterial strains, their sources, and media used for their cultivation. The formation of specific PCR products with a size of 0.7 kb using primer set Bact-0011f and Lab-0677r, or 0.4 kb using primer set Lab-0159f and Uni-0515r is also indicated employing an annealing temperature of 66°C (+, present; -, absent).

Species	Source[a]	Medium[b]	PCR 0.7 kb	PCR 0.4 kb
Bacteroides fragilis	MMB S16	WW	-	ND[c]
Bacteroides thetaiotaomicron	MMB S20	WW	-	ND
Bifidobacterium lactis Bb12	VTT	WW	-	ND
Butyrivibrio fibrisolvens-like clone A11		LB	-	ND
Clostridium beyerinkii	MMB 3318	WW	-	ND
Clostridium bifermentas	NIZO B529	WW	-	ND
Clostridium celerescens-like clone A54		LB	-	ND
Coprococcus eutactus-like clone A19		LB	-	ND
Enterococcus faecalis	DSM 20478	WW	-	ND
Escherichia coli JM109	Promega	LB	-	ND
Eubacterium biforme-like clone F44		LB	ND	-
Eubacterium halii-like clone A07		LB	-	-
Eubacterium rectale-like clone A13		LB	-	-
Fusobacterium prausnitzii-like clone A03		LB	-	ND
Fusobacterium prausnitzii-like clone A10		LB	-	ND
Lactococcus lactis lactis	NIZO B17	MRS	-	ND
Lactobacillus acidophilus	VTT E96276	MRS	+	+
Lactobacillus brevis	VTT E91458	MRS	+	+
Lactobacillus buchneri	VTT E93445	MRS	+	+
Lactobacillus curvatus	VTT E90391	MRS	+	+
Lactobacillus fermentum	VTT E93489	MRS	+	+
Lactobacillus helveticus	ATTC 15009	MRS	+	+
Lactobacillus helveticus	NCIMB 8652	MRS	+	+
Lactobacillus paracasei F19	VTT	MRS	+	+
Lactobacillus plantarum	VTT E79098	MRS	+	+
Lactobacillus reuteri	VTT E92142	MRS	+	+
Lactobacillus rhamnosus LGG	ATCC 53103	MRS	+	+
Lactobacillus ruminis	VTT E91470	MRS	+	+
Lactobacillus salivarius ssp. *salicinius*	DSM 20554	MRS	+	+
Leuconostoc sp.	NIZO B179	WW	+	+
Pediococcus sp.	FM 0001	MRS	+	+
Peptostreptococcus anaerobius	MMB 2828	WW	-	ND
Roseburia cecicola-like clone A22		LB	-	ND
Ruminococcus obeum-like clone A20		LB	-	ND
Streptococcus thermophilus	NIZO B119	WW	-	ND
Vagococcus fluvialis	DSM 5731	WW	-	ND
Weissella kandleri	DSM 20593	WW	+	ND

[a]MMB, Department of Medical Microbiology, University of Groningen, Hanzeplein 1, 9713 GZ Groningen, the Netherlands. VTT, VTT culture collection, P.O. Box 1504, FIN-02044, Finland. NIZO-Netherlands Institute for Dairy Research, P.O. Box 20, 6710 BA Ede, the Netherlands. DSM-German Collection for Microorganisms, Mascheroder Weg 1, 3300 Braunschweig-Stöckheim, F.R.G. ATCC-American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. NCIMB, National Collection of Industrial, Food and Marine Bacteria, 23 St. Machar Drive, Aberdeen, AB24 3RY, Scotland, U.K. F.M. Food Microbiology, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands. [b]WW-Wilkins West. LB-Luria Bertani broth. MRS, Lactobacilli MRS broth. [c]ND not determined.

received the first formula-food after two weeks, and then received progressively more formula. Solid food was given after 3 months. Besides these non-trial affiliated volunteers, fecal samples were provided by individuals that were involved in a trial where subjects were fed with fermented products containing *Lactobacillus paracasei* F19 (23). Upon use samples were thawed on ice-water and mixed after which they were homogenized in 0.05 M ice-cold potassium phosphate buffer (pH 7.0) at a ratio of 3 g of feces (wet weight) per 50 ml (49). Aliquots of 1 ml were stored at –20°C. Cecal samples were collected as described previously (9, 22). Samples were stored at -20°C until used for bacterial DNA extraction.

DNA isolation. Bacterial DNA from fecal and cecal samples was isolated according to Zoetendal *et al.* (1998). DNA from 5 ml bacterial cultures was isolated by bead-beating, phenol-chloroform extraction, and ethanol-precipitation. Plasmid isolations from transformants were performed using QIAprep Spin Miniprep Kit (Qiagen, Hilden, FRG).

Table 2. Primers used in this study.

Primer name	Primer sequence (5'-3')	Reference
[a]S-D-Bact-0011-a-S-17	AGA GTT TGA T(C/T) (A/C) TGG CTC AG	16
[a]S-D-Bact-0124-a-S-27	GGA CGG GTG AGT AAC ACG	18
[a]S-D-Lab-0158-a-S-20	TGG AAA CAG (A/G) TG CTA ATA CC	12
[a]S-G-Lab-0159-a-S-20	GGA AAC AG(A/G) TGC TAA TAC CG	This study
[a]S-*-Univ-0515-a-A-24	ATC GTA TTA CCG CGG CTG CTG GCA	18
[a]S-G-Lab-0667-a-A-17	CAC CGC TAC ACA TGG AG	This study
[a]S-G-Lacb-0722-a-A-25	(C/T) CA CCG CTA CAC ATG (A/G) AG TTC CAC T	38
GC-clamp	CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G	27
T7	TAA TAC GAC TCA CTA TAG G	Promega
Sp6	GAT TTA GGT GAC ACT ATA G	Promega

[a]Nomenclature according to Alm *et al.* (1996)

Primer design and PCR conditions. All primers used in this study are listed in Table 2. 16S rDNA sequences of phylogenetically related species were retrieved from Genbank (www.ncbi.nlm.nih.gov) and used to perform multiple alignments using Clustal W (46). A potential target site starting at position 677 was selected and based on this site a 17-mer PCR primer was designed and designated S-G-Lab-0677-a-A-17 (Lab-0677r) according to the OPD nomenclature (1). A second primer S-G-Lab-0159-a-S-20 (Lab-0159f) was designed based on another conserved site starting at position 158 targeting *Lactobacillus* and *Enterococcus* genera (12). The specificity of the primers was screened by submitting the sequence to the Check Probe program of Ribosomal Database Project (www.cme.msu.edu/ RDP) (21), and the ARB software package (41). The genomic DNA of relevant reference strains and predominant GI tract species was used as the template in PCR experiments to validate the Lab-0677r and Lab-0159f primer specificities (Table 1).

Primers were synthesized commercially by MWG Biotech AG, Ebersberg, FRG. Bacterial primer S-D-Bact-0011-a-S-17 (Bact-0011f), formerly named 27f (18) was paired with the designed reverse primer Lab-0677r, whilst primer Lab-0159f was paired with the universal reverse primer S-*-Univ-0515-b-A-24 with a GC-clamp (Uni-0515-GCr). The GC-clamp attached at the 5'-end of one of the primers creates products suitable for separation by DGGE/TGGE. PCR was performed employing the *Taq* polymerase kit from Life Technologies (Gaithersburg, MD, USA). PCR mixtures of 50 µl contained 20 mM TRIS-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 50 mM each dNTP, 1.25 U of *Taq* polymerase, 5 pmol of each primer and approximately 250 ng of genomic DNA isolated from pure cultures. Samples were amplified in a PE Applied Biosytems GenAmp PCR System 9700 (Foster City, CA, U.S.A.), using the following program: pre-denaturation at 94°C for 5 min; 35 cycles of denaturation 94°C for 30 sec, variable annealing temperature for 20 s, extension at 68°C for 40 sec, and final extension at 68°C for 7 min.

PCR to investigate the general or *Lactobacillus*-specific GI tract bacterial community by DGGE, was performed with the following 16S rDNA primer combinations: S-D-Bact-0124-a-S-27 with a GC-clamp (Bact-0124-GCf) and Univ-515r, or Lab-0159f and Uni-0515-GCr. Nested PCR was performed with these primers on previously generated products from amplification with Bact-0011f and Lab-0677r. The cycling program consisted of 94°C for 5 min; and 35 cycles of 94°C for 30 sec, 56°C for 20 sec, 68°C for 40 min; and finally 7 min at 68°C. PCR products that were used as templates in nested PCR reactions, were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, FRG) according to the manufacturer's instructions.

DGGE analysis of PCR amplicons. PCR products generated with primers Bact-0124f-GC and Univ-0515r, or Lab-0159f and Univ-0515r-GC were separated by DGGE according to the specifications of Muyzer *et al.* (1993) using the Dcode™ system (Bio-Rad Laboratories, Hercules, Ca., USA) with the following modifications. Polyacrylamide gels (dimensions 200x200x1 mm) consisted of 8% (v/v) polyacrylamide (37.5:1 acrylamide-bisacrylamide) and 0.5xTAE (34). Denaturing acrylamide of 100% was defined as 7 M urea and 40% formamide. The gels were poured from the top using a gradient maker, and a pump (Econopump, BioRad Laboratories, Hercules, Ca., USA) set at a speed of 4.5 ml/min, and gradients of 30-60% were employed for the separation of the generated amplicons. Before polymerization of the denaturing gel (28 ml gradient volume), a 7.5 ml stacking gel without denaturing chemicals was added and the appropriate comb was subsequently inserted. Electrophoresis was performed for 16 hrs at 85 V in a 0.5xTAE buffer at a constant temperature of 60°C. Gels were stained with $AgNO_3$ according to Sanguinetti *et al.* (1994).

Cloning of the PCR-amplified products. Amplicons derived from PCR with primers Bact-0011f and Lab-0677r, and nested PCR amplicons were purified with the Qiaquick PCR purification kit according to the manufacturer's instructions. PCR products were cloned into *E. coli* JM109 by using the Promega pGEM-T vector system (Promega, Madison, Wi., USA). White colonies were transferred with a sterile toothpick to an eppendorf tube containing 20 µl of TE buffer. The cells were lyzed by heating them at 95°C for 15 min. PCR was performed on the cell-lysates using pGEM-T specific primers T7 and Sp6 to confirm the size of the inserts. The cycling program consisted of initial denaturation at 94°C for 3 min; 35 cycles of 94°C for 30 sec, 44°C for 30 sec, 68°C for 90 sec, and a final extension for 7 min at 68°C. To establish the diversity within the group of 25 selected clones of each origin, amplicons of the correct size were subjected to restriction fragment length polymorphism

(RFLP) analysis using the restriction enzyme *MspI*. Plasmids containing an unique insert of the appropriate size or corresponding to a band in the community fingerprint were purified by the QIAprep Spin Miniprep Kit (Qiagen, Hilden, FRG) and were subjected to DNA sequence analysis.

Sequence analysis. One microgram of purified pGEM plasmid was employed for sequence analysis of the cloned 16S rDNA fragments. Sequencing reactions were performed using the Sequenase (T7) sequencing kit (Amersham Life Sciences, Slough, U.K.) according to the manufacturer's specifications using IRD-800 5' end labelled primers T7 and Sp6. Sequences were automatically analyzed on a LI-COR DNA Sequencer 4000L (Lincoln, Nb, U.S.A), and corrected manually.

Phylogenetic placement. Phylogenetic analysis was performed using the ARB software package (41). Sequences were aligned and a rooted neighbor-joining tree (*E. coli* positions 31-648) was constructed using *Bacillus subtilis* as an outgroup species.

Nucleotide sequence accession numbers. Thirty-nine 0.7 kb and nine 0.4 kb sequences of the 16S rDNA determined in this study were deposited with the GenBank database and assigned the accession numbers; AF335874 to AF335918, and AF368382 to AF368390, respectively.

RESULTS

Development and evaluation of a Lactobacillus group-specific primer. Based on the alignment of the complete 16S rRNA sequences of various members of the three recognized *Lactobacillus* subgroups (4) and those of several other related lactic acid bacteria, a potential PCR primer binding site was identified starting at *E. coli* position 677 of the 16S rDNA (Table 3). Primer S-G-Lab-0677-a-A-17, complementary to this part of the 16S rRNA, was designed taking into consideration: the nucleotide mismatches with related species, the complementarity with the target at the 3'-end, possibly ending with a C, a G+C content of over 50%, and a size of approximately 20 nucleotides. Sequence comparison using the Check Probe, ARB and BLAST programs confirmed that the targeted region was conserved only among all the 16S rRNA sequences of *Lactobacillus, Leuconostoc, Pediococcus*, *Weissella*, and *Aerococcus* species present in the Ribosomal Database Project and Genbank, except for *L. maltaromicus, L. vitulinus, L. catenaformis* and *L. helveticus*. The first three of the latter lactobacilli are more related to *Clostridium* and *Carnobacterium* spp. than to the genus *Lactobacillus* (37). Their exclusion may have only limited consequences since these particular *Lactobacillus* spp. as well as members of the *Pediococcus*, *Weissella*, and *Aerococcus* species are generally not indigenous to the human GI tract. Moreover, specific PCR products were

obtained with DNA from two strains of *L. helveticus*, suggesting that the mismatches found in the database with the primer, are most likely the result of sequencing errors (see below). The terminal 3'-end of the primer was also complementary to *Eubacterium biforme* and *E. cylindroides* 16S rDNA sequences. Most importantly, the primer had one mismatch at the 3'end with 16S rDNA sequences of several other related species including some bacteria commonly found in large numbers in the GI tract, such as *Clostridium*, *Eubacterium*, *Bacteroides*, and *Bifidobacterium* spp. that should prevent annealing (Table 3).

Table 3. An alignment of primer S-G-Lab-0667-a-A-17 with target sequences of 16S rDNA (region 667 to 683 of *E. coli* numbering) from *Lactobacillus* and other species (3'-5'). The number of species found in the database is indicated in parentheses. The mismatches with the primer sequence are indicated in bold and are underlined.

S-G-Lab-0667-a-A-17 reverse primer	5'-c acc gct aca cat gga g-3'
All *Lactobacillus* spp. subgroups[a] (94)	gta agg tgg cga tgt gta cct caa g
All *Leuconostoc* spp. (18)	gta agg tgg cga tgt gta cct caa g
All *Weissella* spp. (16)	gta agg tgg cga tgt gta cct caa g
All *Pediococcus* spp. (12)	gta aag tgg cga tgt gta cct caa g
Bacteroides fragilis	gta aag tgg cga tgt g**gt** **g**ct **t**aa g
Bacteroides vulgatus	gta aag tgg cga tgt g**gt** **g**ct **t**aa g
Bifidobacterium bifidum	gta agg tgg c**a**a tgt g**gc** cct **t**aa g
Clostridium coccoides	gta aag tgg cga tgt g**at** cct **t**aa g
Clostridium perfringens	gta aag tgg cga tgt g**at** cct **t**aa g
Clostridium ramosum	gta aag tgg cga tgt gta cct **t**aa g
Enterococcus faecalis	gta aag tgg cga tgt gta cct **t**aa g
Eubacterium biforme	gta aa**a** tgg cga tgt gta cct caa g
Eubacterium rectale	gta aag tgg cga tgt gat cct **t**aa g
Lactococcus lactis	gta aag tgg cga tgt gta cct **t**aa g
Staphylococcus aureus	gta aag tgg cga tgt gta cct **t**aa g
Streptococcus thermophilus	gta aag tgg cga tgt gta cct **t**aa g
Vagococcus fluvialis	gta aag tgg cga tgt gta cct **t**aa g

[a]Includes all *Lactobacillus* species in Genbank, except *Lactobacillus maltoromicus, L. catenaformis,* and *L. vitulinus.*

The primer was experimentally tested by performing PCR on genomic DNA isolated from a range of *Lactobacillus* species, related lactic acid and other bacteria, as well as cloned 16S rDNA sequences that have been demonstrated to be present in large numbers in the GI tract (49). The optimal annealing temperature was empirically determined by raising it in steps of 2°C from 56°C to 68°C, using genomic DNA of 27 bacterial species and plasmid DNA from 9 clones (Table 1). The optimum was found to be 66°C and, following a maximum of 35 cycles, products were obtained with DNA from all tested *Lactobacillus*, *Leuconostoc,*

and *Weissella* spp., whereas the 16S rDNA of all other species was not amplified (Table 1).

Table 4. Clones with the percentage of identity to known sequences in Genbank and the RDP database, that were retrieved from intestinal samples of different origin, using primer-sets Bact-0011f and Lab-0667r (A), and Lab-0159f and Uni-0515r (B).

Fragment	Origin	Individual	Species[a]	Number of Clones	Clone (% similarity)
A	**Fecal** *Adult*	**A**	*Lactobacillus sake*	1	F45 (98)
			Eubacterium biforme	1	F44 (98)
		B	*Lactobacillus ruminis*	4	F68 (98), F70 (98), F71 (98), F73 (98)
			Eubacterium biforme	1	F67 (97)
		C	*Lactobacillus delbrueckii*	1	F93 (98)
			Lactobacillus fermentum	1	F81 (99)
			Eubacterium biforme	4	F83 (97), F85 (97), F87 (98), F96 (98)
		D	*Lactobacillus ruminis*	2	F1 (99), F11 (98)
		E	*Lactobacillus acidophilus*	2	F158 (98), F168 (98)
			Lactobacillus crispatus	2	F159 (99), F163 (99)
			Lactobacillus rhamnosus	2	F154 (99), F155 (97)
			Leuconostoc argentinum	2	F160 (98), F165 (99)
	Fecal *Infant*	**F**	*Lactobacillus casei*	1	B121 (99)
			Lactobacillus salivarius	2	B123 (99), B129 (99)
			Lactobacillus rhamnosus	3	B103 (99), B108 (99), B124 (99)
	Cecal *Adult*	**G**	*Lactobacillus ruminis*	4	S137 (99), S144 (98), S146 (99), S152 (98)
		H	*Lactobacillus gasseri*	1	S1 (99)
			Lactobacillus vaginalis	2	S6 (97), S14 (98)
		I	*Leuconostoc mesenteroides*	2	S27 (98), S38 (99)
		J	*Lactobacillus ruminis*	1	S55 (98)
Total number of clones used for phylogenetic analysis				**39**	
B	**Fecal** *Adult*	**K**	*Lactobacillus gasseri*	1	F706 (99)
			Lactobacillus casei	1	F703 (98)
			Lactobacillus paracasei	1	F723 (99)
		L	*Lactobacillus ruminis*	1	F748 (98)
			Lactobacillus ruminis	1	F754 (98)
			Lactobacillus sake	1	F749 (98)
			Lactobacillus casei	1	F747 (98)
		M	*Lactobacillus ruminis*	1	F761 (99)
			Lactobacillus ruminis	1	F763 (98)

[a]Species were named according to their closest relative.

Biodiversity of the Lactobacillus genus evaluated by Lab-0677r. The diversity of members of the *Lactobacillus* genus was investigated using primer S-G-Lab-0677-a-A-17. PCR products of 0.7 kb were generated using this primer in combination with primer Bact-0011f

on bacterial DNA isolated from fecal and other intestinal samples. Sequence analysis of unique clones from fecal origin of five healthy adults and an infant showed that sequences were amplified with significant identity to the 16S rDNA of known *Lactobacillus* spp. (Table 4). Additionally, samples taken from the cecal chyme during intestinal intubation in four healthy volunteers were used to study the diversity in this particular part of the GI tract. Ten clones were analyzed and found to contain sequences similar to *Lactobacillus* spp., showing 97-99% identity with rRNA database entries. PCR products obtained using bacterial DNA isolated from feces from an infant resulted in 6 *Lactobacillus*-like sequences (Table 4).
Among all the sequences retrieved, *L. ruminis*-like sequences were the most frequently encountered in both fecal and cecal samples. *Leuconostoc*-like sequences were found in both fecal and cecal samples, and showed similarity to *Leuconostoc mesenteroides* and *Leuconostoc argentinum*. *Eubacterium biforme*-like sequences were found in the feces of three adults (Table 4). A total of 39 sequences were obtained in this approach and these were used to construct a phylogenetic tree (Fig. 1). This phylogenetic analysis clearly showed that sequences were retrieved from all three subgroups of the genus *Lactobacillus* (37). Most representatives were positioned in the *Lactobacillus casei-Pediococcus* subgroup (I) and the majority were most similar to *L. ruminis*. Sequences retrieved from the infant feces grouped with *L. salivarius*, *L. rhamnosus*, and *L. paracasei*. Some sequences grouped within the *L. delbrueckii* subgroup (II), whilst the *Leuconostoc paramesenteroides* subgroup (III) was represented by sequences that were similar to *Leuconostoc mesenteroides* and *Leuconostoc argentinum*. Overall, the sequence-results confirmed the validity of the developed primer.

DGGE analysis of Lactobacillus amplicons. To facilitate rapid monitoring of *Lactobacillus*-specific sequences, use was made of sequence-specific separation of PCR amplicons by DGGE, that requires the presence of a stable GC-clamp attached to one of the primers. However, a low product yield was observed when either of the primers, Bact-0011f or Lab-0677r, was equipped with a GC-clamp. Circumventing the need to perform multiple PCR amplifications or concentrate samples before DGGE analysis, we opted for the use of a nested PCR approach. This involved a first round of PCR with primers Bact-0011f and Lab-0677r amplifying the *Lactobacillus*-like community, followed by a second PCR with established GC-containing universal primers. To prevent amplification of residual genomic DNA or low-yield aspecific amplicons formed in the first PCR, only samples yielding sufficient product (15 ng/µl) were used for a second round of PCR with primers Bact-0124-GCf and Uni-0515r.

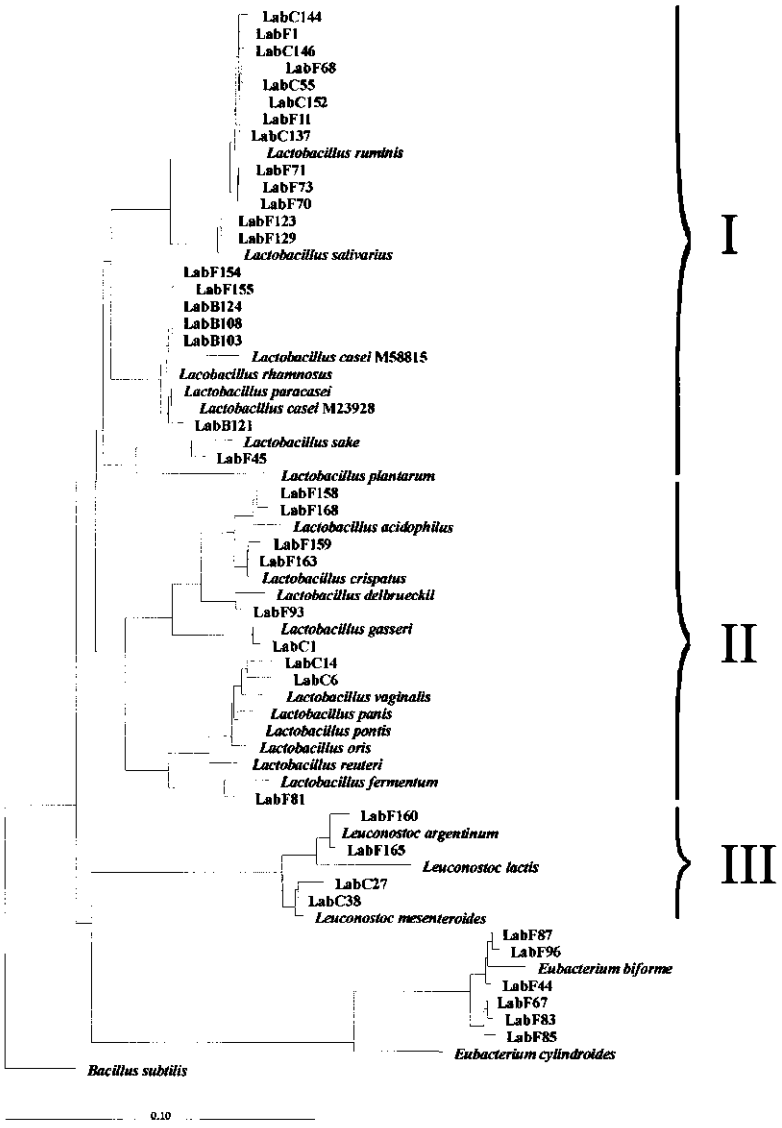

Fig. 1. Phylogenetic tree based upon the neighbor-joining method of partial 16S rDNA sequences (*E. coli* positions 31-648) from clones derived by PCR using primer Bact-0011f and specific primer Lab-0677r. Suffixed are F, feces; C, cecal chyme; B, baby. Reference sequences are included which were found to be the closest relatives of the retrieved clones. Three subgroups are indicated by: I, *L. casei-Pediococcus*-group; II, *L. delbrueckii*-group; III, *Leuconostoc paramesenteroides*-group. *Bacillus subtilis* is used as an outgroup. The scalebar represents (calculated) distance. The origin of the clones is presented in Table 4.

Succession of Lactobacillus spp. following birth. The intestinal microbial community of infants is developing and therefore highly unstable (7, 20). A study into the development of the *Lactobacillus*-like community in babies was performed by PCR with primers Bact-0011f and Lab-0677r on fecal DNA from baby F from delivery (day 1) to five months later (day 147) at regular intervals. No PCR products were obtained up to day 55, indicating that the template was either absent or the amount of template was too low to be detected. The PCR amplicons obtained from day 55 to 147 were used for the nested PCR with primers Bact-0124-GCf and Uni-0515r, and the resulting 0.4 kb fragments were separated in a 30-60% DGGE gel (Fig. 2). Two amplicons with different intensity were present from day 55 throughout the experiment and the sequences of these 0.4kb fragments were identical to clones earlier retrieved from the infant, i.e. B103 (*L. rhamnosus*) and B121 (*L. casei*) (Table 4). Successional change was observed with amplicon 3, which was identical to clone B123 (*Lactobacillus salivarius*), that appeared at day 93 and faded between day 129 and 147.

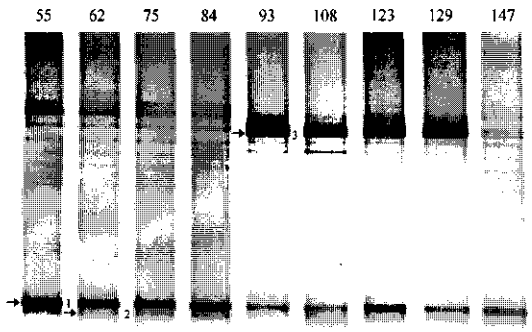

Fig. 2. Development of the *Lactobacillus*-like community over time in an infant. DGGE analysis of amplicons generated by nested PCR from fecal samples taken on day 55 up to day 147. Fragments that are indicated by arrows and numbers were identified by the Lab-clone library of the infant as described in Table 4. The origin of the fragments and the corresponding clone are: 1, *L. rhamnosus* (B103); 2, *L. casei* (B121); and 3, *L. salivarius* (B123).

Effect of consumption of lactobacilli on the Lactobacillus spp. diversity in children. The fate of orally administered *L. paracasei* F19 in a clinical trial with children was investigated using primer Lab-0677r in the nested PCR and DGGE approach as described above. Fecal samples from children between 10 and 18 months, that were administered 10^{10} CFU of *L.*

paracasei F19 twice a day for three weeks or a placebo for the same time, were analyzed in a blind fashion. Although *L. paracasei* F19 was not detected in the dominant bacterial community of these young children (47), it could be detected as a double band when the Lab-0677-specific PCR-DGGE approach was used (Fig. 3). This analysis also revealed that in some children the specific community is also unstable. This instability of the *Lactobacillus*-like community was demonstrated particularly in two individuals and appeared independent of the *L. paracasei* F19 administration. In individual I the community was changing throughout the experiment, but despite this change strain F19 could be detected during the administration period. In individual III, the double band of strain F19 or a *Lactobacillus* species with the same melting-behavior was present even before the trial started and was enhanced during the experiment. However, after the cessation of placebo-administration (Fig. 3, individual III) it disappeared. In a subject where the *Lactobacillus*-like community appeared to be stable (Fig. 3, individual II), the administration of *L. paracasei* F19 had a temporary impact on the composition but it returned to its original composition after cessation of the strain-administration.

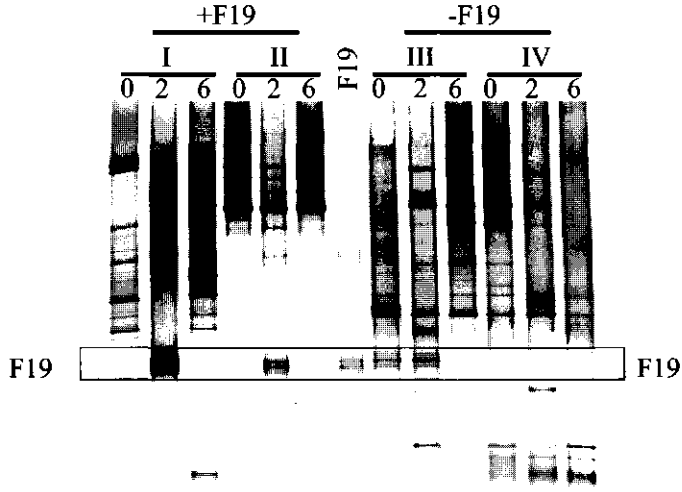

Fig. 3. Effect of orally administered *L. paracasei* F19 on the *Lactobacillus*-like community in time. DGGE analysis of amplicons generated in a nested PCR with primers Bact-0124-GCf and Uni-0515r. Children were administered *L. paracasei* F19 (I, II) or placebo (III, IV) for 4 weeks. Fecal samples were taken before the experiment (0), after 2 weeks of administration (2), and two weeks after administration had ceased (6). The PCR amplicon *for L. paracasei* F19 was concomitantly separated. The fragments in the profiles corresponding to F19 are indicated by the box.

Lactobacillus-like community development in time and space in adults. Whilst *Lactobacillus* and *Leuconostoc* sequences were readily obtained from adult fecal samples, analysis of the amplicons obtained by nested PCR revealed an overrepresentation of *Eubacterium biforme* sequences and only low amounts derived from *Lactobacillus* and *Leuconostoc* spp. (data not shown). Therefore, we introduced another more specific primer in the nested PCR strategy. For this purpose Lab-0159f, which targets the *Lactobacillus* and *Enterococcus* genera was selected in combination with Uni-0515-GCr. Although the Lab-0159f primer resulted in aspecific PCR products when used as a primary PCR primer on fecal and cecal samples (data not shown), it proved to be very selective when used in the nested PCR approach to reamplify amplicons obtained with Bact-0011f and the *Lactobacillus*-specific primer Lab-0677r, and circumvented reamplification of *Eubacterium biforme*-like sequences (Table 1). The dominant bands in DGGE were identified by either analyzing

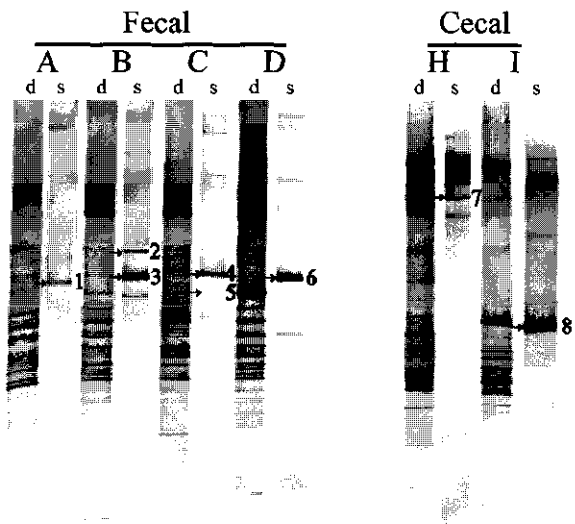

Fig. 4. DGGE analysis of the dominant (d) and *Lactobacillus*-like community (s) diversity in fecal and cecal samples from six adults (A-D, H, I), following PCR with primers Bact-0124-GCf and Uni-0515r, and nested PCR with primers Lab-0159f and Uni-0515-GCr, respectively. The dominant fragments in the *Lactobacillus*-like patterns indicated by arrows and numbers were identified by clones from the existing clone library of *Lactobacillus*-like sequences, as described in Table 4. The origin of the fragments and the corresponding clones are: 1, *L. sake* (F45); 2, *L. ruminis* (F68); 3, *L. ruminis* (F70); 4, *L. delbrueckii* (F93); 5, *L. fermentum* (F81); 6, *L. ruminis* (F1); 7, *L. gasseri* (S1); and 8, *Leuconostoc mesenteroides* (S27).

retrieved clones from the existing clone library (Table 4), or the cloning and sequencing of the nested PCR amplicons. Comparison of the dominant bacterial community with the *Lactobacillus*-like community revealed a decreased diversity for the *Lactobacillus*-like sequences where relatively few bands were present (Fig. 4). Identification of the bands resulted in the retrieval of *Lactobacillus* and *Leuconostoc*-like sequences with identities to known species ranging from 98-99%.The fecal bacterial community of several healthy adults was monitored over a period of 20 months (Fig. 5). The DGGE patterns of the dominant bacterial community of each individual were specific and stable (data not shown), which is in agreement with previous results (49). The DGGE fingerprints of the dominant *Lactobacillus*-like community obtained with the nested PCR approach with the specific Lab-0159f primer, revealed a variable stability of the *Lactobacillus*-like community among the different individuals (Fig. 5). The diversity of species increased in both individuals K and L, but individual M appeared to harbor a stable community of lactobacilli. PCR-DGGE is a semi-quantitative method, in which variation in band-intensities reflects the relative number of specific amplicons. For example, in individual K (Fig. 5) at six months the amount of sequences close to *L. gasseri* (fragment 1) decreased relative to that of the newly emerging sequences resembling *L. paracasei* (fragment 3). Identification of the dominant 0.4-kb fragments in the three adults resulted in sequences most similar (98-99%) to *L. casei*, *L. paracasei*, *L. gasseri*, *L. sake*, and *L. ruminis*. Notably, in adults L and M sequences originating from *L. ruminis*-like sequences were dominantly present throughout the observed time-frame.

DISCUSSION

This study describes the development of a *Lactobacillus*-group specific primer that is used to investigate bacterial diversity in the human GI tract. Currently, the *Lactobacillus* genus constitutes three phylogenetic clusters, the *L. delbrueckii* group, the *L. casei-Pediococcus* group and the distinct *Leuconostoc* group. It is widely acknowledged that the taxonomy of the genus is unsatisfactory, which is caused by the phylogenetically heterogeneous nature of this large assembly of microorganisms (4, 37). Additionally, the 16S rRNA sequences of this genus are highly similar to other species including *Streptococcus* and *Enterococcus* spp. Nevertheless, a *Lactobacillus*-group specific probe was developed by Sghir *et al.* (1998) that

was used in dot-blot hybridization, but has only a single mismatch with enterococci, streptococci and staphylococci. Harmsen *et al.* (1999) developed an oligonucleotide probe for fluorescent *in situ* hybridization (FISH), that targets the three subgroups and has additional specificity for some *Weissella*, *Vagococcus* and *Enterococcus* species. Attempts to use the latter two oligonucleotide probes, being the sense version of S-G-Lab-0158-a-A-20 (13) or probe S-G-Lacb-0722-a-A-25 (38) directly, as PCR primers resulted in non-specific PCR products (data not shown). In general it is not possible to use probes as specific primers since the specific bases of a probe are located centrally, whilst PCR primers harbor their specificity at the 3'-end, where initial hybridization and elongation takes place. New alignments of the 16S rRNA revealed that a reverse primer S-G-Lab-0677-a-A-17, targeting *E. coli* positions 677 to 693, would specifically encompass *Lactobacillus*, *Leuconostoc*, *Weisella*, *Pediococcus* and *Aerococcus*. Phylogenetically related genera that are usually present in intestinal samples in reasonably high numbers like *Streptococcus*, *Enterococcus*, *Vagococcus*, and *Staphylococcus* were eliminated, as well as more abundant genera such as *Bifidobacterium*, *Bacteroides*, *Eubacterium*, and *Clostridium*.

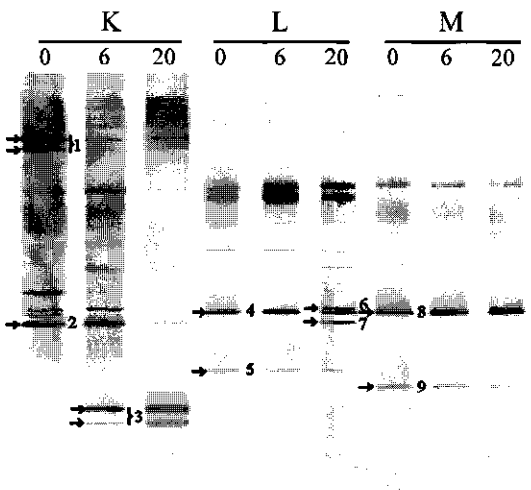

Fig. 5. Monitoring of the *Lactobacillus*-like community of adults in time. DGGE analysis of amplicons generated by nested PCR with primers Lab-0159f and Uni-0515-GCr, originating from three individuals (K, L, and M) of whom fecal samples were taken at 0, 6, and 20 months. The dominant fragments in *Lactobacillus*-like patterns indicated by arrows and numbers were sequenced and compared to known sequences in Genbank, as described in Table 4B. The origin of the fragments and the corresponding clones (see Table 4) are: 1, *L. gasseri* (F706); 2, *L. casei* (F703); 3, *L. paracasei* (F723); 4, *L. ruminis* (F748); 5, *L. ruminis* (F754); 6, *L. sake* (F749); 7, *L. casei* (F747); 8, *L. ruminis* (F761); and 9, *L. ruminis* (F763).

The specificity of primer S-G-Lab-0677-a-A-17 was established by performing PCR with this primer on bacterial DNA from pure cultures. This resulted in the formation of specific amplicons for *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weissella* species. Additionally, the effectiveness of the primer was confirmed during the investigation of the diversity of the *Lactobacillus* sequences in a variety of intestinal samples amplified with primer Lab-0677r and Bact-0011f. Notably, no PCR products were obtained from fecal samples for three out of eight adult subjects suggesting that either the numbers were below the detection level or *Lactobacillus*-like species were completely absent. Samples from five adults of different ages yielded PCR products that were used for cloning and sequencing. Specific PCR products were also readily obtained when cecal material from the proximal colon of another four healthy volunteers was used as template. Differences between cecal and fecal microbiota have been reported in humans with lactobacilli in reasonably high numbers in the cecum (22, 32).

Previous culturing studies of the human GI tract reported *Lactobacillus* species similar to those described here. For example *L. fermentum*, *L. salivarius*, and *L. casei* have been isolated from both the rectum and the oral cavity (2), whilst *L. ruminis*, *L. paracasei*, *L. crispatus*, *L. vaginalis* and *L. gasseri* were isolated from stools (24, 25, 44). However, none of the retrieved 16S rRNA sequences were identical to those of known *Lactobacillus* spp. and some showed considerable differences. Unexpectedly, a number of sequences in this study were similar to those of species that are frequently found in fermented food products. *L. delbrueckii* and *L. acidophilus* are known to be present in yoghurt, whilst *L. sake* is used in industrial meat and sourdough fermentation (10, 26). Members of the *Leuconostoc*-group are also well known for their use in fermented food products and as food-spoilage microorganisms. *Leuconostoc mesenteroides* and *Leuconostoc argentinum*-like sequences were retrieved from cecal and fecal samples. However, the retrieved intestinal sequences are not identical to those of starter bacteria, and therefore may be derived from several indigenous species rather than of transitional nature from ingested foods. Mitsuoka (1992) reported on the occasional presence of *L. ruminis* in the GI tract of humans, but *L. ruminis*-like sequences were found in six out of the thirteen individual samples in this study, indicating that it is a common GI tract species. As mentioned none of the sequences were identical and in two clones (F155, S6) the similarity to deposited sequences was only 97%. This indicates that, although the *Lactobacillus* genus is considered cultivable, the diversity appears to be still insufficiently described.

As *Eubacterium biforme* and *E. cylindroides* are considered to be common constituents of the adult GI tract (8), this coincidentally prohibits the strict specificity of primer S-G-Lab-0677-a-A-17 for the 16S rDNA of *Lactobacillus* clusters. *Eubacterium*-like sequences were found in fecal samples from three out of five adult individuals, with similarities ranging from 97-98%. The screened material from cecal origin lacked this common constituent, which supports the presence of differences in the specific bacterial communities of the proximal and distal parts of the large intestine as observed from culturing studies (32).

PCR-DGGE with nested PCR in which primers Bact-0124-GCf and Uni-0515r were used resulted in an overrepresentation of *Eubacterium biforme*-like sequences. This species is apparently present in high amounts in adult fecal samples and hence the use of general bacterial primers in the nested PCR amplified them to large proportions. This almost obscured the *Lactobacillus* and *Leuconostoc* bacterial community (data not shown) which was demonstrated to be present by the earlier cloning and sequencing results (Table 4). The use of a second more general *Lactobacillus*-targeted PCR primer Lab-0159f in a nested approach readily resulted in the retrieval of only *Lactobacillus* and *Leuconostoc*-like sequences which matched the bands found in DGGE analysis (Figs. 2 and 3, and Table 4). This supported the earlier cloning results of this study that *L. ruminis* is a dominant organism. Culture-based approaches showed that the *Lactobacillus*-like community in the GI tract at the strain level was highly fluctuating in composition and numbers over time, and was different from individual to individual (17, 24). The current culture-independent approach reveals a relatively stable *Lactobacillus* composition in the three individuals while the relative proportions could vary considerably in time depending on the individual (24). The 16S rDNA is a discriminative gene on the species level, but exact copies of sequences for different strains of the same species can undermine its power for strain detection. Thus, there is a possibility that for some individuals the stability in time of the *Lactobacillus*-like community as observed in this study may not fully represent the actual situation on the strain level.

In contrast to adults, PCR with primers Bact-0124GCf and Uni-0515r can efficiently be used in babies and infants where *Eubacterium biforme* strains are either absent or in sufficiently low numbers not to result in a distorted representation of the *Lactobacillus* community. The combination of specific PCR and DGGE could effectively demonstrate the succession of the different *Lactobacillus*-like species for the first five months of an infants' life.

Increasing consumer awareness of the link between diet and health has promoted the

introduction of lactic acid bacteria, especially lactobacilli, into functional foods such as probiotics that may exert an effect in the human GI tract (23, 33). Beneficial influences of probiotics reported include protection against gastrointestinal infections and inflammatory bowel disease, anti-allergic effects and other immune-related effects (23). The effect of consumption of probiotics on the composition of the dominant microbiota was investigated for several probiotic feeding trials using DGGE which indicated that no extensive changes occurred (47). This present study clearly demonstrates that specific PCR using S-G-Lab-0677-a-A-17 followed by DGGE allows monitoring of the presence of a *L. paracasei* strain in clinical trials. In conclusion, the strategy described here that combines specific *Lactobacillus* PCR with DGGE is widely applicable and elevates this significant group of bacteria, that is often present in low numbers within an ecosystem, from total community obscurity.

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Chapter 6

The Host Genotype Affects the Bacterial Community in the Human Gastrointestinal Tract

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Abstract

The gastrointestinal (GI) tract is one of the most complex ecosystems consisting of microbial and host cells. The host genotype, the physiology of the host, and environmental factors are suggested to affect the composition and function of the bacterial community in the intestine. However, the relative impact of these factors is unknown. In this study, we used a culture-independent approach to analyze the bacterial composition in the GI tract. Denaturing gradient gel electrophoresis (DGGE) profiles of fecal bacterial 16S rDNA amplicons from adult humans with varying degrees of genetic relatedness were compared by determining the similarity indices of the profiles compared. The similarity between fecal DGGE profiles of monozygotic twins were significantly higher than those for unrelated individuals (t_s=2.73, $P_{1\text{-}tail}$=0.0063, d.f.=21). In addition, a positive relationship ($F_{1,30}$=8.63, P=0.0063) between the similarity indices and the genetic relatedness of the hosts was observed. In contrast, fecal DGGE profiles of marital partners, which are living in the same environment and which have comparable feeding habits, showed low similarity which was not significantly different from that of unrelated individuals (t_s=1.03, $P_{1\text{-}tail}$=0.1561, d.f.=27). Our data indicate that factors related to the host genotype have an important effect on determining the bacterial composition in the GI tract.

INTRODUCTION

While prokaryotes are the most abundant form of life on our planet (1), it is also well known that microbes are present in vast amounts in the animal gastrointestinal (GI) tract where they often exceed the number of host cells (2). Therefore, they live in very close contact with each other and with the epithelial cells of the host. The succession of this complex microbial community starts after birth when empty niches become colonized by the first invading fast-growing microbes. Successive shifts of different microbial populations finally result in a climax community (3, 4). Although most of the interactions in the GI tract are still unknown, several studies indicate that signaling between host and microbes is very important in this ecosystem (5, 6). Recently, the molecular details of the communication between *Bacteroides thetaiotaomicron* and its murine host have been elucidated (7-9). Furthermore, it has been reported for various animal systems that the presence of methanogens in the intestine is a phylogenetic character that obeys 'Dollo's rule', i.e. traits that are lost in the course of evolution do not appear in any of the descendants of the common ancestor that lost these traits (10, 11). On the other hand, a recent study with humans and rats has indicated that shared and unique environmental conditions are important in the ecology of methanogens (12). These rather contradicting observations indicate the complexity of the ecology of methanogens in the GI tract. It is at least remarkable that only a fraction of humans harbors significant numbers of intestinal methanogens (13). Moreover, it has been established that each individual harbors specific strains of *Helicobacter pylori* or *Bifidobacterium* and *Lactobacillus* spp. (14, 15). All these observations argue strongly for the genetic predisposition of the host determining the composition in the GI tract.

A serious problem that limits the global analysis of the whole GI tract community is the inability to isolate and characterize all microbes. Viable plate count techniques only reveal a minor fraction of the GI tract community (16-18). Hence, analysis of the contribution of the host in determining the microbial composition in the GI tract requires the application of culture-independent techniques. These are mainly based on the sequence variability of 16S rRNA genes that have shown to be useful phylogenetic markers (19-21). Recently, it has been shown that the dominant bacterial community in feces remains stable in time (22, 23). In addition, temperature or denaturing gradient gel electrophoresis (TGGE or DGGE) of the PCR-amplified sequences of fecal 16S rRNA and rDNA indicated that the bacterial composition was host-specific (22).

To study whether factors related to the genetic predisposition of the host rather than environmental factors determine a host-specific composition in the GI tract, we analyzed fecal samples from human individuals with different degrees of genetic relatedness, varying from monozygotic twins to marital partners, and four non-human primate species. Although fecal samples do not reflect the bacterial composition in all parts of the GI tract, the majority of bacteria leaves the GI tract via the fecal route and therefore an observed effect on the fecal composition reflects an GI tract-related effect. The V6 to V8 regions of the 16S rDNA were amplified using fecal DNA as template and the amplicons were analyzed by DGGE (24, 25). Similarity indices between the DGGE profiles were calculated and statistical analyses were performed.

MATERIALS AND METHODS

Fecal sample collection. Fecal samples from fifty adult human volunteers (21 to 56 years) with varying genetic relationships (from mono- and dizygotic twins to genetically unrelated individuals) were collected after defecation in sterile plastic bags or collection tubes, and processed or transported to the lab as fast as possible, or stored at -20°C until use (freezing did not affect the procedures). In addition, fresh fecal samples were collected from four other primates (gorilla, chimpanzee, macaque, and orangutan) and transported immediately from the zoo to the lab. Fecal samples from genetically related individuals were analyzed only if they were living at separate locations. Individuals older than 60 years were not included, since it has been suggested that the physiological conditions of aging people affects the microbial community (26). In addition, two fecal samples were taken in a four months period from each of four unrelated human adults in order to assess the temporal stability of the fecal community.

DNA isolation, PCR, and DGGE analysis. The DNA isolation from fecal samples was performed as described previously (22). One microliter of the fecal DNA solution was 10 times diluted and subsequently used as template to amplify the V6 to V8 regions of 16S rDNA using primers F-0968-GC and R-1401 (27). DGGE analysis of the amplicons was performed on 8% polyacrylamide gels containing a urea plus formamide gradient from 38% to 48% (100% denaturing solution contains 7 M urea and 40% (vol/vol) formamide). Electrophoresis was performed in 0.5 x TAE at 85 V at 60°C for 16 hours using the DCode or D GENE System apparatus (BioRad, Hercules, CA). After electrophoresis, gels were silver-stained according to the protocol of Sanguinetti and colleagues (28) with some minor modifications.

Calculating similarity indices and statistical analysis. DGGE gels were scanned at 400 dpi and the software of Molecular Analyst 1.12 (Biorad) was used for comparing the DGGE profiles. Similarity indices of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearsons product-moment correlation coefficient (29). This procedure was performed three times. In this way,

comparisons were made between fecal samples originating from human individuals with different degrees of genetic relatedness, with different ages, living in similar or different environments, four non-human primate species. In addition, fecal samples taken in a four months period were compared to access the temporal stability of the host-specific bacterial community. To obtain independent comparisons for statistical analysis, a random set of comparisons was selected from each gel in which each DGGE profile occurred only once. Regression analysis and student's t-tests were performed for statistical analysis of the data.

RESULTS

Comparison between fecal samples from monozygotic twins, marital partners, unrelated individuals, and non-human primates. To study whether factors related to the genetic predisposition of the host rather than environmental factors determine a host-specific composition in the GI tract, fecal samples from human individuals with identical genetic relatedness (monozygotic twins), with similar environmental conditions (marital partners), and unrelated individuals were analyzed. Amplicons of the variable regions V6 to V8 were analyzed by DGGE, resulting in complex profiles which represent the host-specific dominant bacterial communities in which each band in a profile represents at least one unique bacterial 16S rDNA sequence (Fig. 1).

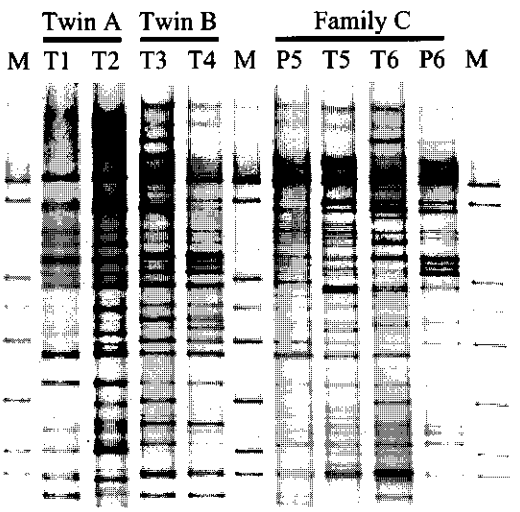

Fig. 1. DGGE profiles of the V6 to V8 regions of 16S rDNA from fecal samples of monozygotic twins (T1 and T2, T3 and T4, T5 and T6) and marital partners (T5 and P5, T6 and P6). Twin A, Twin B, and Family C indicate the three different families. M indicates the marker for DGGE analysis.

The DGGE profiles of fecal 16S rDNA amplicons from monozygotic twins from three different families showed higher similarity within twin couples than between twin couples (Table 1). In addition, the similarity between the marital partners within family C was relatively low compared to the similarity within each twin couple.

Table 1. Similarity matrix calculated from the DGGE profiles of figure 1. T indicates a member of a monozygotic twin and P indicates the marital partner of the twin member. T1 and T2; T3 and T4; and T5, T6, P5, and P6 are members of respectively Twin A, Twin B, and Family C. Similarity indices of monozygotic twins are highlighted and underlined, similarity indices of marital partners are highlighted only.

	T1	**T2**	**T3**	**T4**	**P5**	**T5**	**T6**	**P6**
T1	100							
T2	**81.2**	100						
T3	36.1	44.9	100					
T4	47.4	45.1	**74.7**	100				
P5	13.4	11.8	18.9	23.3	100			
T5	30.0	35.5	58.3	59.4	**17.2**	100		
T6	32.4	34.5	62.1	45.5	16.7	**62.8**	100	
P6	50.2	50.5	60.8	75.7	19.0	52.1	**45.2**	100

In general, the similarity indices of all monozygotic twins (N=6) were significantly higher than those of genetically unrelated individuals (t_s=2.73, $P_{1\text{-}tail}$=0.0063, d.f.=21), despite some occasional observations of high similarity indices for genetically unrelated individuals (Fig. 2.). On the other hand, a comparison between the fecal communities of the marital partners did not show significant higher similarities than those for unrelated persons (t_s=1.03, $P_{1\text{-}tail}$=0.1561, d.f.=27) notwithstanding the fact that the partners lived in the same environment and had in general comparable feeding habits (based on questionnaires). This may indicate that factors related to the host genotype or to the sex difference of the individuals compared have a significant influence on the bacterial community in the human GI tract. However, no significant difference was observed between the similarity indices of unrelated persons of the same sex and those of different sexes (t_s=0.41, $P_{2\text{-}tail}$=0.69, d.f.=15). This strongly argues for factors related to the host genotype to have an important effect on the

GI tract composition. In addition, the similarity between the bacterial communities of genetically unrelated individuals was significantly higher than that of humans compared with other primates that we consider as the baseline level (t_S=3.99, $P_{1\text{-}tail}$=0.0004, d.f.=19).

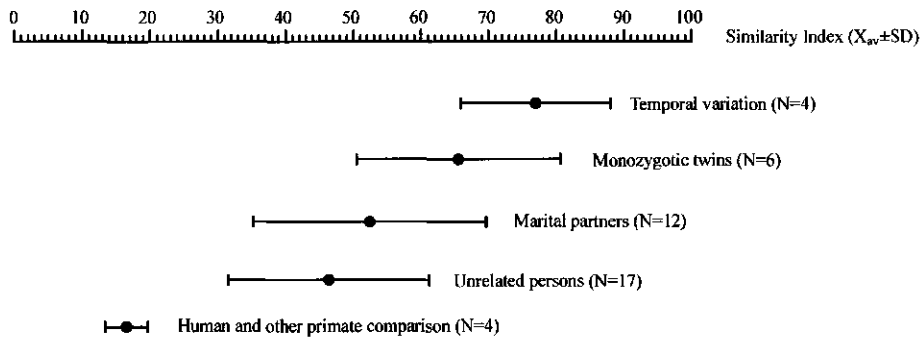

Fig. 2. Plot of the similarity indices from the human and primate, unrelated individuals, monozygotic twins, marital partners, and temporal variation comparisons. The mean (bullet) ± standard deviation (black bar) are plotted.

DGGE analysis of feces from individuals with varying degrees of genetic relatedness. To study the impact of the genetic predisposition of adult humans in more detail, similarity indices were calculated for comparisons between DGGE profiles of fecal samples from individuals with varying degrees of genetic relatedness (Fig. 3). The similarity between the DGGE profiles of fecal samples appears to show a positive linear relationship with the genetic relatedness (r) between those individuals ($F_{1,30}$=8.63, P=0.0063), despite the high variation within the similarity indices of individuals with the same genetic relatedness. For example, the SD of the similarity indices for r = 0 (genetically unrelated) and r = 0.5 (brother and sisters, parents and children) are 14.7 and 16.4 respectively. These high variations could be caused by a variety of factors, such as age, diet, condition of the host, and experimental errors. The variation caused by replicate DNA isolation, PCR amplification, and DGGE analysis was found to be small and did not exceed a SD of 3.1.

The positive relationship between the similarity indices of fecal DGGE profiles and the genetic relatedness between the hosts compared indicates that factors related to the genetic predisposition of the host have a significant influence on the bacterial composition in feces. However, since the mean age difference between the individuals decreases with an increasing

genetic relatedness (from $r = 0.25$ to $r = 1$) the effect of the age difference on the similarity index was determined by comparing the DGGE profiles belonging to the group of brothers and sisters with $r = 0.5$ (including dizygotic twins). No positive or negative relation was observed for the similarity indices and the corresponding age differences (up to 14 years difference). This indicates that the age difference between the hosts did not have a significant effect on the observed differences between the hosts.

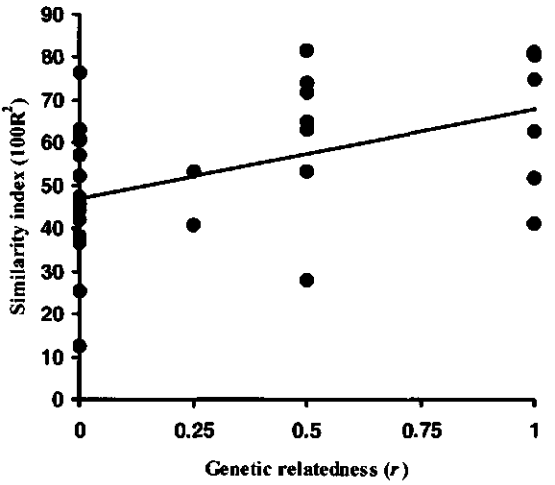

Fig. 3. Diagram showing the positive regression between the similarity indices of fecal DGGE profiles and the genetic relatedness between the individuals compared. The comparisons for each value of genetic relatedness and the linear regression that best fits the data are plotted. Comparisons were made for genetically unrelated individuals (r=0); Aunts/uncles and nephews/nieces (r=0.25); parents and children, brothers and sisters, dizygotic twins (r=0.5), and monozygotic twins (r=1).

DISCUSSION

The GI tract is a complex ecosystems consisting of microbial and host cells. The microbial community plays an important metabolic role in the GI tract by converting dietary components that escaped digestion by the host, and polymers excreted by the host into readily accessible nutrients and other compounds, such as vitamins (3). In addition, the microbial community participates in protecting the host against pathogens (30). Interactions between the host and the microbial community are therefore of considerable importance, very complex

and just starting to be understood (5, 7-9). In the current study, we describe a culture-independent analysis of the dominant bacterial composition in the GI tract based on the 16S rRNA sequence variability of bacteria. DGGE and related electrophoretic analyses of 16S rDNA amplicons have shown to be powerful in studying the ecology of bacteria in different ecosystems including the GI tract (reviewed by 31, 32). In a previous study, we have shown that the diversity and stability of bacterial communities in the human GI tract could easily be analyzed using such an approach (22). In addition, the determination of similarity indices for DGGE profiles has shown to be a suitable tool to make the comparisons objectively (33, 34). In the current study, we determined the similarity indices of fecal DGGE profiles from hosts with varying degrees of genetic relatedness. It was observed that the host genotype has a significant effect on determining the dominant bacterial composition in the GI tract. The effect of the environment seems to be of less importance as indicated by the similarity indices of the bacterial communities between monozygotic twins and marital partners.

A strong positive correlation between the similarity indices and the genetic relatedness of the hosts was found, suggesting that either the host genotype or the colonization history, presumably via the fecal oral route from mother to child, has a significant effect on the bacterial composition in the GI tract. Another possible factor influencing the fecal comparison could be the age difference between the hosts compared. As reported previously, the fecal communities might be affected by the physiological conditions of aging people (26). Therefore, individuals older than the arbitrary 60 years were excluded from this study. Since no significant relationship was found for the age differences between brothers and sisters with $r = 0.5$ and the corresponding similarity indices, it is not likely that our findings can be explained by the effect of age differences. Furthermore, the combination of previous findings that the bacterial community in adults is stable in time (22, 23) and the fact that all genetic relatives in this study are already living separately for a long period of time (more than 5 years, some more than 15 years) also argues for a strong effect of the host genotype.

Our findings may explain why the fecal composition is host-specific and stable in time in adults, and the presence or absence of certain strains in the GI tract as described previously (10, 11, 13-15, 22). Currently, we can only speculate if immunological properties of the host, specific receptors for GI tract bacteria, or other communication systems between the host and the microbial community are responsible for the observed findings. Recently, the molecular details of the communication between *Bacteroides thetaiotaomicron* and its gnotobiotic murine host have been elucidated (7-9). Unfortunately, such studies can only be performed

under well-controlled laboratory conditions and cannot be used to study the communication between humans and the GI tract community. Nevertheless, it is evident that the composition of the GI tract community is not only affected by the colonization history, the physiological (aging) effects in the GI tract and environmental factors, but also by the host genotype. Therefore, the effects of the consumption of for example probiotics or prebiotics may be difficult to determine in different individuals as a consequence of the variation in the bacterial community in the GI tract. To correct for these host-specific effects, the volunteers in a feeding trial should include individuals which are genetically closely related.

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Chapter 7

The Attached Bacterial Community from the Human Gastrointestinal Tract is Uniformly Distributed Along the Colon and Differs From the Fecal Community

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Submitted

Abstract

***Background*-The human gastrointestinal tract harbors a complex community of bacterial cells in the mucosa, lumen, and feces. Since most attention has been focused on bacteria present in feces, knowledge about the mucosa-associated bacterial community at different parts in the colon is limited to a few studies based on culturing approaches.**

***Aim*-To analyze the bacterial composition in feces and biopsies from the ascending, transverse, and descending colon using molecular approaches based on 16S rRNA sequence diversity.**

***Subjects*- The group of individuals from which fecal and colonic biopsy samples were taken include six healthy individuals (43-70 years) and four individuals diagnosed with ulcerative colitis or polyposis (30-79 years).**

***Methods*-DNA was isolated from biopsy and fecal samples. Denaturing gradient gel electrophoresis (DGGE) analysis of the PCR-amplified V6 to V8 regions of 16S rDNA, which reflects the predominant community, and *Lactobacillus* group-specific 16S rDNA amplicons were analyzed. Similarity indices were calculated to compare the DGGE profiles.**

***Results*-Flow cytometric analysis indicated that $10^5 - 10^6$ bacteria were present in the biopsy samples. DGGE analysis and similarity index comparisons demonstrated that the predominant mucosa-associated bacterial community was host-specific, equally distributed along the colon but significantly different from the fecal community ($p<0.01$). The *Lactobacillus* group-specific profiles were less complex than the profiles reflecting the predominant community. In six of the ten individuals the *Lactobacillus* group community in the biopsies was similar to the fecal community while in three individuals some minor differences between the biopsies could be detected. No significant differences were observed between the healthy and diseased individuals.**

***Conclusions*-The observed host-specific DGGE profiles of the mucosa-associated bacterial community in the colon support the hypothesis that host-related factors are involved in the determination of the GI tract microbial community.**

INTRODUCTION

The human gastrointestinal (GI) tract harbors a diverse microbial community which mainly consists of obligate and facultative anaerobic bacteria. These bacteria have an important metabolic and protective function in the GI tract (1). The complex interactions between the host and the bacterial community are of considerable importance, but just starting to be understood (2-4). Most knowledge about the bacterial community in the human GI tract has been obtained by selective cultivation of microbes from fecal samples. Recently, culture-independent approaches using the sequence variability of the 16S ribosomal RNA genes have shown that most of the predominant bacteria in human fecal samples have not been obtained in culture yet, which indicates that our knowledge about these predominant members is very limited (5-7). In addition, denaturing and temperature gradient gel electrophoresis (DGGE/TGGE) analysis of fecal 16S rDNA and rRNA amplicons has shown to be powerful approaches in determining and monitoring the bacterial community in feces (6, 8). Such studies revealed that the predominant bacterial community in mammalian feces is stable in time, host-specific, affected by aging, and not altered after consumption of certain probiotic strains (6, 8-12). Furthermore, DGGE has been used to compare bacterial communities in fecal samples from infants with and without necrotizing enterocolitis (NEC), although no NEC-associated differences were observed (13).

Our present knowledge of the bacterial diversity associated with the human GI tract is mainly based on analysis of fecal samples and in a few cases samples have been characterized that originated from different parts of the intestine. Most of these analyses with contents from sudden-death victims (14) or with biopsy samples from living individuals involved a culturing approach and focused on the attachment of certain probiotic strains (15-17), presence of sulfate reducers (18-19) and/or on bacterial population level in diseased persons (20). Since biopsy samples are very small in size and therefore more easily exposed to oxygen during sampling, the number of viable strict anaerobes might be reduced easily. Not surprisingly, relatively high levels of facultative anaerobes were reported to be present in intestinal biopsies. So far, no studies exist in which the bacterial composition of biopsy samples has been analyzed at species level. Molecular approaches based on the sequence variability of 16S rRNA genes could be instrumental to analyze the bacterial composition in intestinal biopsy samples. Recently, such an approach was used to study the bacterial diversity within the human subgingival crevice (21). In another recent study, temporal temperature gradient gel

electrophoresis analysis of 16S rDNA fragments was successfully used to compare the bacterial composition in gastric biopsy samples and showed that *Helicobacter* is detectable in healthy individuals and those suffering from gastritis (22).

The aim of our research was to determine whether the bacterial composition in colonic biopsy samples was significantly different from that in fecal samples and to investigate whether composition differences could be detected at different locations in the colon. We used DGGE approaches to characterize the 16S rDNA sequence variability of predominant bacterial composition and that of the *Lactobacillus*-like species using general and specific PCR primers (6, Chapter 5). The compositional variability was studied in feces and colonic biopsy samples from the ascending, transverse, and descending colon of ten volunteers. Since it appeared after diagnosis that four persons suffered from a colonic disorder, we also investigated if significant differences could be found between these persons and those without a diagnosed colonic disease.

MATERIALS AND METHODS

Experimental approach. To describe the bacterial diversity in fecal and biopsy samples a 16S rRNA approach was used. DNA was isolated from these samples derived from the same individual and the V6 to V8 regions were PCR-amplified using general primers and analyzed using DGGE. After scanning of the gels, similarity indices of DGGE profiles were compared and statistically analyzed. In addition, a specific PCR was performed to amplify the V2 to V4 region of the *Lactobacillus* group that subsequently were separated by DGGE. To quantify the number of bacteria per biopsy a flow cytometric approach was used.

Volunteers. Fecal and biopsy samples from ten adult human volunteers were collected as fresh as possible. The ten volunteers donating biopsy- and fecal samples were patients coming to a routine diagnostic colonoscopy. The procedure normally includes taking of biopsies, and so the study did not cause any extra risk, pain or discomfort to the participants. Informed consent was obtained from each volunteer before the sampling. The group consisted of five men and five women (table 1). With the exception of various gastrointestinal symptoms, for which they came to the examination, they considered themselves healthy. They did not follow any special dietary regime, and none had recently received any antibiotic treatment.

Colonoscopy, fecal sample collection, and treatment of samples. The colonic evacuation before the colonoscopy was performed using a laxative (Colonsteri, Orion Oy, Finland) according to the instructions of the manufacturer. The instrument used for the actual colonoscopy and sampling of biopsies was Pentax EC-3801 L. Biopsies (~ 0.5 mg) were obtained from the ascending (A), transverse (T), and descending (D) parts of the colon (two parallels per location). One of the parallel samples was stored in 0.05 M potassium phosphate buffer (pH 7.0), and the other in phosphate-buffered saline (PBS; per litre: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g

KH_2PO_4; pH7.2) with 4% paraformaldehyde. Fecal samples were obtained before the colonic evacuation. They were stored in the home freezers of the volunteers and collected immediately prior to the colonoscopy. Both, fecal and biopsy samples were subsequently deeply frozen at –70°C, shipped in dry ice and if appropriate, stored at –70°C, and thawed in ice-water prior to further analysis.

Table 1 Age, sex, diagnosed illness, and bacterial counts of the biopsies (A, T, D) of the volunteers in this study. Indicated are P (polyposis), UC (ulcerative colitis), *UC (remission of UC), SI (Similarity Index), n.d (not determined), "-" (comparison).

Patient ID	Age (years)	Sex	Diagnosed illness	Cell counts (A)	Cell counts (T)	Cell counts (D)	SI (F-D)	SI (A-D)
1	59	F	none	n.d.	2.0×10^5	1.4×10^5	83.1	97.6
2	70	M	none	n.d.	n.d.	n.d.	91.3	98.1
3	79	M	P	n.d.	n.d.	n.d.	67.1	95.9
4	36	F	UC	8.6×10^4	1.1×10^5	1.1×10^5	81.9	56.5
5	43	M	UC	6.4×10^5	6.4×10^5	1.3×10^6	22.9	91.2
6	63	M	none	n.d.	n.d.	n.d.	25.8	98.0
7	45	F	none	5.7×10^5	3.8×10^6	1.2×10^6	24.7	n.d.
8	30	F	UC*	6.5×10^5	6.9×10^6	2.5×10^6	40.7	95.8
9	43	M	none	1.7×10^5	1.2×10^5	2.0×10^5	13.6	81.6
10	51	F	none	2.9×10^5	9.0×10^5	7.7×10^5	30.0	82.0

Bacterial counts in biopsies. The paraformaldehyde-fixed biopsies were washed twice with PBS and resuspended in 50% Ethanol-PBS. After incubation for at least one hour at -20°C, the biopsies were sonicated in an ultrasonic water bath for 2 min to separate the bacterial cells from the biopsy material. This treatment has shown to be optimal to separate viable cells from each other without damaging them (23). After centrifuging at 700 *X g* for 1 min to remove host cells and debris, the supernatant was centrifuged at 9,000 *X g* for 5 min to pellet the bacteria. The bacteria were resuspended in 490 µl PBS (pH 8.4) and incubated with 5 µl propidium iodide (PI; 1 mg/ml) at 37°C for 20 min to count the total number of cells. Before flow cytometric counts, 5 µl of 0.7 µm-yellow green (YG) beads with know concentration (Polysciences, Inc) was added according to the manufacturer's instructions in order to determine cell numbers. Samples were analyzed by a FACScalibur flow cytometer (Becton Dickinson). Illumination of the samples was done with an argon ion laser (488 nm) and fluorescence of the YG beads and PI were collected in the FL1 (530/30 nm) and FL3 (>600 nm long pass) detectors, respectively. The system threshold was set on forward scatter signals and all bacterial analyses were performed at the low rate settings (12 µl/min). The sample concentration was adjusted to keep the count lower than 1,000 events/sec to avoid coincidence. Data were collected in list mode as pulse height signals (four decades in logarithmic scale each) and 5,000 to10,000 cells were acquired for further analysis which was performed using CellQuest software (Beckton Dickinson) and/or WinMDI version 2.8 software (http//:facs.Scripps.edu/software.html).

DNA isolation, PCR, and DGGE analysis. Before DNA isolation, fecal samples were resuspended in 0.05 M potassium phosphate. DNA was isolated from the fecal and unfixed biopsy samples using the bead beating method as described previously (Chapter 4). In short, samples were incubated at 55°C after addition of 10% SDS (50 µl) and 20 mg/ml proteinase K (10 µl) followed by addition of 150 µl phenol (pH 7.5) and mechanical disruption at 5,000 rpm for 3 minutes. Phenol/chloroform extractions and one chloroform extraction were performed to remove impurities. Before ethanol precipitation at –20°C was performed, 1 µl of glycogen solution (20 mg/ml) was added. After washing of the pellets, DNA was resuspended in 100 µl TE.

DNA isolated from biopsies and fecal samples (<10 ng) was subsequently used as template to amplify the V6 to V8 regions of 16S rDNA using primers F-0968-GC and R-1401 (24). The amplification (35 cycles) and the analysis of 5 µl of amplicons on ethidium-stained 1.2% agarose gels was performed as described previously [6]. DGGE analysis of the amplicons was performed on 8% polyacrylamide gels containing a urea/formamide gradient from 38% to 48% (a 100% urea/formamide solution consists of 7 M urea and 40% (vol/vol) formamide). Electrophoresis was performed in 0.5 x TAE at 85 V at 60°C for 16 hours using the DCode or D GENE System apparatus (BioRad, Hercules, CA). Subsequently, gels were silver-stained according to the protocol of Sanguinetti and colleagues (25) with some minor modifications. Gels were scanned at 400 DPI and analyzed using the software of Molecular Analyst 1.12 (Biorad). The similarity between the DGGE profiles was determined by calculating similarity indices of the densitometric curves of the profiles compared using the pearson product-moment correlation (8, 26). UPGMA, Ward's, and Neighbor joining algorithms were performed and corresponding dendrogramss showing the relationships between the DGGE profiles were constructed. Scanning and analysis of the gels was performed three times.

Amplification of 16S rDNA fragments from the *Lactobacillus*-group population was performed using a nested-PCR approach. First, the complete 16S rDNA was amplified using the canonical primers 8f and 1510r (27). After purification using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany), the *Lactobacillus*-group specific PCR was performed using primers GC124f and Lab-0677r followed by DGGE analysis on 8% polyacrylamide gels containing a urea/formamide gradient from 30% to 60% (Chapter 5). For cloning and sequence analysis, the *Lactobacillus*-group amplicons were purified, cloned, and sequenced as described previously (Chapter 5). Sequences were deposited in the GenBank database and are encoded with accession numbers AY027791 and AY027792.

Statistical analysis. Paired and student's t-tests were used for statistical analysis of comparisons between the cell numbers and between similarity indices from the scanned DGGE profiles, respectively.

RESULTS

Bacterial numbers in the biopsy samples. The bacteria in biopsy samples of approximately 0.5 mg were counted by a flow cytometric approach in order to quantify them in a culture-independent way. Since the bacteria were released from the biopsy material by a mild treatment and since it is difficult to determine how many cells were still attached after

sonication, the total count of bacteria was determined as the minimal number per biopsy. PI-stained bacterial cells could accurately be counted when beads with known concentration were added as illustrated in figure 1. The different biopsies revealed bacterial cell concentrations that varied between 8.6 X 10^4 and 6.9 X 10^6 cells depending on the location in the colon and the individual (table 1) with a mean count of 1.1 X 10^6 bacteria per biopsy. The detection limit for accurate counting was found to be 3.7 (± 1.4) X 10^4 cells per sample. The number of bacteria in biopsies from the ascending colon seems to be slightly lower than from the other locations, although no significant differences in bacterial numbers at these locations (lowest $P_{2\text{-}tail}$ was 0.075) were found as examined by paired t-test analyses.

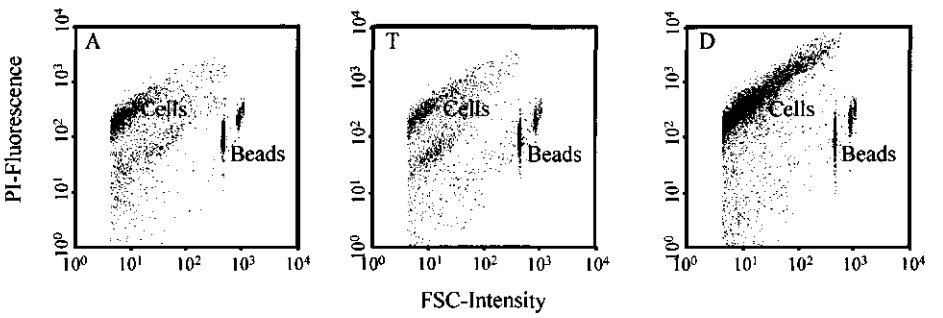

Fig. 1. Flow cytometric dot blots showing the discrimination between the PI-stained cells and the yellow green beads. The different locations of one individual with colitis ulcerosa (5) is illustrated. A, T, and D represent biopsies from the ascending, transverse, and descending colon, respectively.

Spatial distribution of the predominant bacterial community. Following DNA isolation from the fecal and biopsy samples of the ten different individuals (table 1), PCR was performed to amplify the V6 to V8 regions of 16S rDNA. Amplicons could be detected in all samples with the exception of the biopsy samples from the ascending and transverse colon of individual 7. DGGE analysis of the fecal and biopsy samples showed an enormous difference in diversity of the amplicons in the profiles from the different individuals (as illustrated in fig 2). Dilution of biopsy DNA (10 times) did not result in a change in the profile, indicating that the number of cells per biopsy was sufficient to obtain reliable and reproducible DGGE profiles. Profiles of feces and biopsy samples of the predominant bacterial community were compared to determine whether these communities are significantly different from each other. Since the biopsy samples were taken after evacuation of the colon it is very plausible that the bacteria detected in these biopsies are attached to the colonic wall and therefore in close contact with

the host cells. Remarkably, the predominant community in biopsy samples from all locations in the colon gave very similar profiles in each individual, despite the difference in diversity and diagnosed illness of the individuals (see figure 2 as illustration). In contrast, the faecal profiles were in most cases different from those obtained with the biopsy samples, indicating that it is very unlikely that fecal contamination took place during colonoscopy.

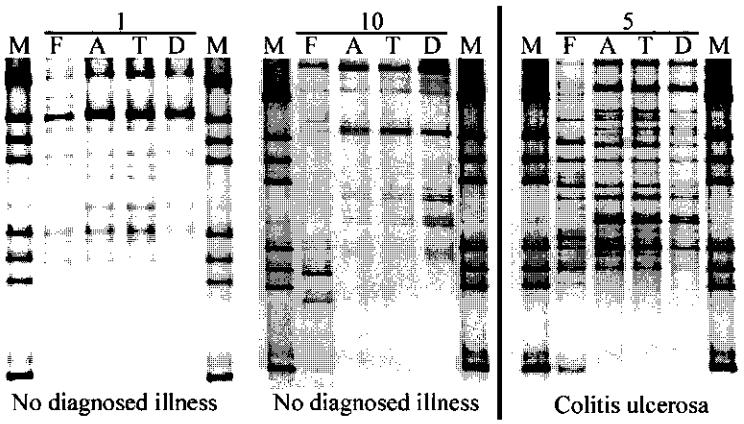

Fig. 2. Silver-stained DGGE gel showing profiles, which represent the predominant community of feces (F) and biopsies (A, T, D) of individuals 1, 5, and 10. M represents the marker for DGGE analysis. The diagnosed illness of the individuals is indicated.

To determine whether communities from feces and biopsies were significantly different in single individuals, similarity indices of the DGGE profiles were calculated. It was observed that within comparisons between a fecal sample and one of the biopsy samples the individual variation was relatively high compared to the comparisons between different biopsy samples from the same individual. For example, the similarity indices for comparisons between feces and descending biopsies varied from 13.6 to 91.3, while similarities indices between 56.5 and 98.1 were found when descending biopsies were compared to ascending biopsies (table 1). Overall, indices for the similarity indices of comparisons between all biopsy samples from the same individual were very high (91.6 ± 9.6), close to those calculated for the reproducibility of the procedures (93.4 ± 3.6). To rule out that the diagnosed illness of some of the individuals had an influence on the observed findings, the mean and standard variation of each similarity index within the healthy individuals and those with diagnosed illness were compared separately. Student's t-test revealed that there was no

significant difference between the similarity indices of both groups for each comparison, since the lowest $P_{2\text{-}tail}$ observed was 0.065 (d.f.=7) for the similarity indices for comparison between ascending and transverse colonic biopsies. This indicates that the physiological state of the individual had no significant effect on the spatial difference in composition in general.

The similarity indices between a fecal sample and one biopsy sample were compared with those between the residual biopsy samples in order to obtain independent comparisons for statistical analysis (figure 3). All combinations of comparisons showed that the bacterial composition in fecal samples was significantly different from that in the biopsy samples. The highest $P_{2\text{-}tail}$ was 0.0012 (d.f.=16) for comparison between the similarity indices of feces and transverse biopsies with ascending and descending biopsies.

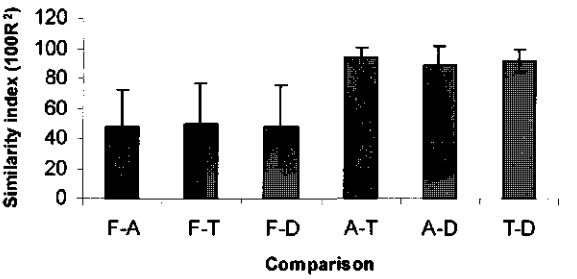

Fig. 3. Diagram showing the comparisons between fecal and biopsy samples. F represents feces, A, T, D represent biopsies from the different locations, - represents the comparison. The mean and standard deviations of the similarity indices are indicated.

Spatial distribution of the lactobacillus community. A nested PCR approach was used to specifically amplify the V2 to V4 regions of the 16S rDNA of the *Lactobacillus* group community since no amplicons were retrieved using a direct specific PCR approach. In contrast to the DGGE profiles of the predominant bacterial community the *Lactobacillus* group specific profiles were lower in diversity as illustrated in figure 4. Because of this low diversity similarity indices for the DGGE profiles cannot be determined. In contrast to the predominant bacterial community, the *Lactobacillus* community in fecal and biopsy samples were very similar in six of the ten individuals. In these individuals, only one amplicon was dominating (see for example for individuals 1 and 5 in figure 4). In the other individuals one of the fecal amplicons was the only predominant one in the biopsy samples or vice versa (such as in individual 10 in figure 4). Furthermore, in three of the ten individuals some minor

differences in the *Lactobacillus* group composition between the biopsies were found. These small differences could not be explained by the physiological condition, age or gender of the host since they were found in individual 2 (healthy), 8 (remission of ulcerative colitis), and 10 (healthy).

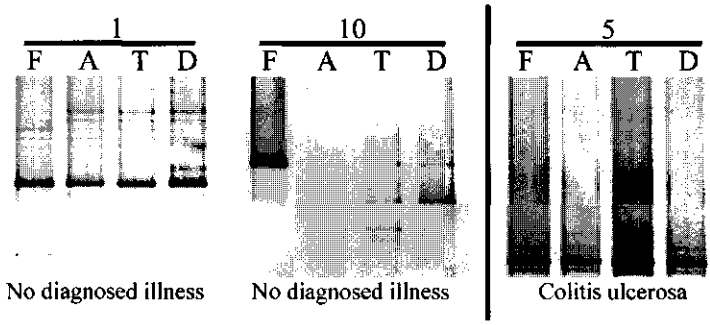

Fig. 4. Silver-stained DGGE gel showing profiles, which represent the *Lactobacillus* group community of feces (F) and biopsies (A, T, D) of individuals 1, 5, and 10. M represents the marker for DGGE analysis. The physiological conditions of the individuals are indicated.

Comparison between healthy individuals and individuals with diagnosed illness. DGGE profiles of biopsy samples from the descending colon of individuals with and without a diagnosed illness were compared to see if the presence or absence of specific bacteria could be correlated to the illness. The descending colon was chosen since all diagnosed illnesses were at least observed in this part. The profiles of the predominant bacterial community appeared to be unique for each individual and no specific amplicon could be assigned to the presence or absence of a colonic illness (figure 5A). To analyze the predominant communities, similarity indices were calculated of the comparisons between the DGGE profiles. Repetitive comparisons between the UPGMA, Ward, and neighbor-joining algorithms were performed and dendrograms were constructed. Only two clusters were found in all dendrograms, while the position of the others branches in the dendrogram changed depending on the clustering method. The large error bars of the nodes in the UPGMA tree (figure 5B) could be seen as an indication that the corresponding branches of these nodes may vary between the different algorithms. One of the repetitive clusters consisted of four healthy individuals (i.e. individuals 1, 2, 6, and 7) and the other consisted of two individuals (i.e. 3 and 5) with a diagnosed illness. This preliminary observation suggests that there might be

differences in the predominant bacterial composition between healthy and diseased individuals, although the group of individuals in this study is too small to draw a definite conclusion.

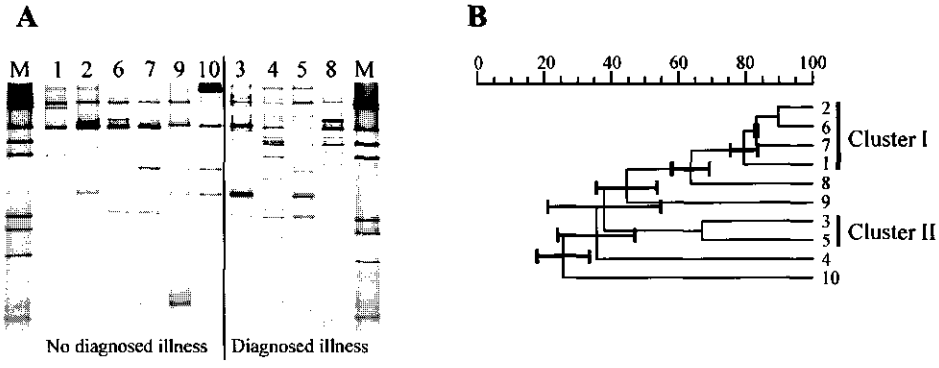

Fig. 5. (A) Silver-stained DGGE gel showing profiles, which represent the predominant community of the descending biopsy from individuals 1 to 10. M represents the marker for DGGE analysis. The physiological conditions of the individuals are indicated. **(B)** UPGMA dendrogram illustrating the correlation between the different DGGE profiles of figure 5 (A). Cluster I and II represent the repetitive clusters obtained using different algorithms. The black bars represent the error bars.

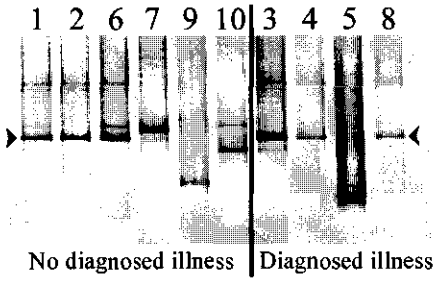

Fig. 6. Silver-stained DGGE gel showing profiles, which represent the *Lactobacillus* group community of the descending biopsy from individuals 1 to 10. M represents the marker for DGGE analysis. The physiological conditions of the individuals are indicated. The arrowheads indicate the amplicons which have been identified by cloning and sequencing.

For the *Lactobacillus*-group community also no specific differences could be found between the individuals with and without diagnosed illness (figure 6). A striking observation was the presence of an amplicon with identical DGGE band position in nine of the ten individuals. In six of them it was predominantly present. Since it appeared to be a

Lactobacillus gasseri-like species (see below) we tested whether its predominance might be a result of preferential amplification. Our nested PCR approach did not show any preference in favor of *L. gasseri* when mixtures of its DNA with that from *L. acidophilus* and *L. paracasei* were used as template DNA for PCR. Cloning and sequence analysis of the amplicon from the DGGE profiles of healthy individual 1 and individual 3 with polyposis showed that both sequences had 99% similarity with *L. gasseri*. Alignment of the two sequences showed that they only differ one base (Adenine/Thymine difference). This indicates that *L. gasseri* is likely to be a predominant *Lactobacillus* species in the biopsy samples.

DISCUSSION

In this study we have used a culture-independent approach based on the 16S rDNA sequence variability to analyze bacterial communities at different parts in the colon. Fecal and biopsy samples were taken from people with and without a diagnosed illness. Since the colon was evacuated before biopsy samples were taken, it is very likely that the bacteria in the biopsy samples are mucosa-associated. The minimum number of cells per biopsy sample as measured by flow cytometry is comparable to numbers found by cultivation of bacteria from biopsies which were obtained in a similar procedure (16). Considerable variation was found in the bacterial numbers from different biopsies. Factors that may cause this variation include the evacuation procedure, the sonication procedure, and individual differences. On the other hand, this variation in bacterial number may explain why no PCR product could be obtained from two biopsy samples.

DGGE analysis of 16S rDNA was used to determine, compare, and visualize the composition of the predominant bacterial and the *Lactobacillus*-group community. The DGGE profiles reflecting the predominant bacterial community in biopsy samples from different locations in the GI tract were highly similar to each other while they differ significantly from those of fecal samples (figure 3). Therefore, it seems that the attached bacteria are equally distributed along the complete colon and that different populations are dominating in the mucosa and the feces. Culture-dependent studies on contents from different parts of the colon (including the ascending, transverse and descending colon) of sudden-death individuals has revealed that the conditions, like for example pH and concentration of fermentation products, in these part differ considerably from each other (14). This suggests that the equal distribution of the attaching bacterial composition is very likely due to host-

bacterium interactions at the mucosa. Several studies have already suggested that the bacterial community in the GI tract has a strong effect on host and that signaling between host and bacterium is very important (2-4). In a recent study, we found a significant positive relationship between the genetic relatedness of the hosts and the similarity between their bacterial communities (8). However, it is not clear yet what the nature is of these host-related factors.

Using a nested PCR approach the *Lactobacillus*-group specific composition could be analyzed. In contrast to the predominant community, the profiles of biopsy and fecal samples were quite similar in six of the ten individuals. Furthermore, in three of the ten individuals some minor differences were found in the *Lactobacillus* group composition between the biopsies. This suggests that the changing conditions in the GI tract influence the presence or absence of certain species belonging to the *Lactobacillus* group. Another explanation could be the detection limit of these bacteria in the biopsies. Since we are focusing on a sub-population in a community, which contains approximately 10^6 bacteria, a small difference in the number of a certain species might have a large impact on its detection. Remarkably, one amplicon with highest sequence similarity (99%) to *L. gasseri*, was found in descending biopsy samples of nine of the ten individuals. Moreover, it was the most predominant one in most biopsies. Since the 16S rDNA of this species was not preferentially amplified using the nested PCR approach, *L. gasseri* may be regarded as a general mucosa-associated bacterium in humans.

Because colonic illnesses were observed during colonoscopy, samples from healthy individuals and those with a diagnosed illness were compared to each other. Since the illnesses are found especially in the descending colon, we carefully compared the samples from these regions and compared them with those from healthy individuals. No significant difference could be detected with respect to the number of bacteria per biopsy, the composition of the predominant bacterial community, and that of the *Lactobacillus* group community. This is supported by the observation that the profiles reflecting the predominant community were highly similar along the complete colon in both groups. Lactobacilli were detected in both feces and biopsies from all individuals and no significant differences in the *Lactobacillus* group populations between healthy and diseased tissue were found. Hence, no specific beneficial effect of these *Lactobacillus* species is likely to be expected. However, the number of volunteers in this study was too small to draw significant conclusions about comparisons between healthy and diseased individuals.

The molecular approach used in this study can be influenced by preferential

amplification and difference in DNA isolation efficiency of different species. DNA from biopsy samples could be diluted 10 times without changing the profiles. Furthermore, for two individuals the similarity between fecal and biopsy samples was very high, while the number of bacteria per fecal sample was at least 10^3 to 10^4 times higher. These data indicate that it is very unlikely that the procedures such as sampling, storage, and transport or preferential lysis of specific groups of bacteria have a major impact on our observations.

In conclusion, using a culture-independent approach we could clearly demonstrate that mucosa-associated bacterial communities in the colon are significantly different in composition from those in feces. A strikingly high similarity was observed between bacterial communities from different locations in the colon. This observation assumes that host-related factors are really important in the colonic ecosystem, which is in line with previous observations (2-4, 8). Small differences between healthy and diseased individuals were observed, but the number of samples analyzed was too small to allow a definite conclusion. In other studies no significant differences were observed when healthy individuals were compared to those suffering from gastritis (22) or NEC (13). However, the number of individuals in these study was also relatively low (13 and 32, respectively). Therefore, our knowledge of the exact role of GI tract bacteria in intestinal disorders remains limited. We clearly showed that sub-populations of the *Lactobacillus* group could be well characterized using a culture-independent approach. Such approaches can easily be applied to for example (sub-) populations of *Bacteroides*, sulfate-reducing bacteria, and pathogenic *Escherichia coli* strains since they have been suggested to be involved in the initiation and maintenance of ulcerative colitis, although their role is still questionable (18, 19, 28). Therefore, we suggest that systematic culture-independent approaches should be applied to study the role of varying GI tract sub-populations in the pathogenesis of colonic diseases.

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Chapter 8

Quantification of Uncultured *Ruminococcus obeum*-like Bacteria in Human Fecal Samples with Fluorescent *In Situ* Hybridization and Flow Cytometry using 16S Ribosomal RNA Targeted Probes

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Submitted

Abstract

A 16S rRNA-targeted probe was designed and validated in order to quantify the number of uncultured *Ruminococcus obeum*-like bacteria using fluorescent *in situ* hybridization (FISH). These bacteria have frequently been found in 16S rDNA clone libraries from bacterial communities in the human intestine. Thirty-two reference strains from the human intestine, including a phylogenetically related and some other *Ruminococcus* species were used as negative control and did not hybridize with the newly designed probe. Microscopic and flow cytometric analysis revealed that a group of morphologically similar bacteria in feces did hybridize with this probe. Moreover, it was found that all hybridizing cells also hybridized with a probe specific for the *Clostridium coccoides – Eubacterium rectale* group, a group that included the uncultured *R. obeum*-like bacteria. Quantification of the uncultured *R. obeum*-like bacteria and the *Clostridium coccoides – Eubacterium rectale* group by flow cytometry and microscopy revealed that these groups comprised approximately 2.5% and 16% of the total community in fecal samples, respectively. This indicates that the uncultured *R. obeum*-like bacteria are predominantly present in human feces and that they comprise a major fraction of the *Clostridium coccoides – Eubacterium rectale* group. Statistical analysis revealed no significant difference between the microscopic and flow cytometric counts and the sampling time of the feces while a significant host-specific effect on the counts was observed. Our data demonstrates that the combination of FISH and flow cytometry is a useful approach for studying the ecology of uncultured bacteria in the human gastrointestinal tract.

INTRODUCTION

The human gastrointestinal (GI) tract harbors a diverse microbial community which has an important metabolic and protective function in the GI tract (reviewed by 17). Recent studies indicate that interactions between the host and the bacterial community are of considerable importance, but very complex and just starting to be understood (4, 9, 10, 24). Most knowledge about the bacterial community in the human GI tract has been obtained by selective cultivation of microbes from fecal samples. The past 5 years, culture-independent approaches using the sequence variability of the 16S rRNA genes have demonstrated that most of the predominant bacteria in human fecal samples have not yet been obtained in culture, illustrating the limitation in our knowledge of these predominant members (15, 22, 23). In addition, denaturing and temperature gradient gel electrophoretic (DGGE/TGGE) analysis of fecal 16S rDNA and rRNA amplicons has demonstrated to a be powerful culture-independent approach in determining and monitoring the bacterial community in feces (23, 24). Such studies revealed that the predominant bacterial community in human feces is relatively stable in time, host-specific, and not significantly altered following consumption of certain probiotic strains (18, 19, 23).

Although the application of 16S rDNA-directed DGGE, cloning, and sequencing has provided new insights into the bacterial composition of the human GI tract, these approaches are all based on the use of PCR amplification methods and hence cannot be accurately converted to real numbers of bacteria. Fluorescent *in situ* hybridization (FISH) using 16S rRNA targeted oligonucleotide probes has demonstrated to be very powerful in detecting and quantifying uncultured bacteria in environmental samples (reviewed by 3). FISH analysis of fecal populations has demonstrated that these were found to be relatively stable in time (5). Various genera, like *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Lactobacillus, Collinsella, Eubacterium*, and *Clostridium* could be quantified accurately in feces using the FISH approach (5, 6, 7, 12). In addition, a group of *Fusobacterium prausnitzii*-like bacteria, which was predominantly found in several fecal clone libraries (15, 22, 23), was also found as a predominant member using the FISH approach (5, 16).

In several studies, a group of uncultured bacteria that have *Ruminococcus obeum* as closest cultivable relative was found to predominate fecal 16S rDNA clone libraries (15, 23). These were also found to be present in fecal TGGE profiles of dominant and active bacteria and could be an important member of the bacterial community in the human GI tract (23).

Since these cloned amplicons have less than 97% sequence similarity to *R. obeum* they represent a group of hitherto unknown species. Up to now no quantitative data have been obtained and the morphology of these bacteria remains unknown, since their presence is only derived from PCR-based data.

In many studies, FISH analysis is combined with microscopic analysis. Although less frequently used, also the combination of FISH with flow cytometry has been applied successfully in analyzing different microbial communities (2, 13, 20, 21). Major advantages of this combination are the multiparametric analysis of samples, and the relatively fast and sensitive quantification of populations, even those that make up only about 1% of the total community.

In the present study, we have developed a set of two probes to detect and quantify the group of uncultured *Ruminococcus obeum*-like bacteria using FISH in combination with direct microscopy and flow cytometry. After validation, the probe was used to quantify the number of hybridized cells in fecal samples from three individuals by microscopic and flow cytometric analysis. In addition, the *Eubacterium rectale – Clostridium coccoides* group, that includes the uncultured *R. obeum*-like bacteria, and the total number of bacteria were quantified. Variations over time, among individuals and between the approaches were statistically analyzed.

MATERIALS AND METHODS

Design and validation of the oligonucleotide probes. Sequences of closely related uncultured *R. obeum*-like bacteria from human fecal samples were obtained from two studies in which fecal 16S rDNA clone libraries were constructed and analyzed (Fig. 1.; 15, 23). The identity and accession number of these sequences are A14 (AF052415), A20 (AF052417), A57 (AF052422), adhufec171 (AF132243), and adhufec35.25 (AF153853). These sequences were aligned using the ARB software package (14) and probes targeting these sequences were designed. The newly designed probe (Robe63 probe) was screened for specificity based on comparative analysis of 16S rRNA sequences from the ARB database and ribosomal database project (RDP, 11) and newly deposited sequences from GenBank using the ARB and RDP software (Table. 1). The CHECK_PROBE analysis function of the RDP and the Probe Match function of ARB were used to screen the 16S and 23S rRNA sequence databases for target sequences for the newly developed probe.

Thirty-two reference strains from various phylogenetic groups in the GI tract, including *R. obeum*, were used as negative control to evaluate the specificity of the Robe63 probe (Table 2). The Eub338 probe (2) was used as positive control for the hybridization of the negative reference strains. Since the positive target of the Robe63 probe constitutes a 16S rDNA sequence of an uncultured group of *R. obeum*-like bacteria, no positive reference strain could be used. Therefore, bacteria from a reference fecal sample were added to the reference

strains in order to verify hybridization signals of the Robe63 probe. In addition, fecal samples were hybridized with the specific probe and the Erec482 probe (5), which targets the *Clostridium coccoides - Eubcaterium rectale* group that includes the uncultured *R. obeum*-like bacteria, to determine whether the Robe63-positive cells appeared double-labeled.

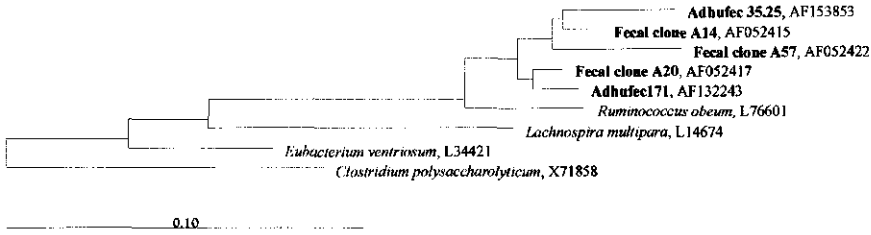

Fig. 1. Neighbor-joining tree showing the phylogenetic relationships between the 16S rDNA sequences of the uncultured *Ruminococcus obeum*-like bacteria (indicated in bold) and some closely related reference strains used in this study. The phylogenetic tree was generated from the tree-of-life of the ARB software package. Accession numbers of the 16S rDNA sequences are indicated. The bar represents 10% sequence divergence.

Table 1. Alignments of probe sequences and their target sequences having maximal two mismatches. N, R, W, and Y are the IUPAC codes for ambiguous base annotations. Dots indicate that bases are identical to the target site. Differences between the probes are represented as bold nucleotides. Mismatches of target sequences with the probe target site are underlined.

Sequence ID	Sequence
S-*-Robe-0063-a-A-20[a]	3' GC-TTG-CCC-TT**A**-AT**G**-AA**A**-TAA
S-*-Robe-0063-b-A-20[a]	3' GC-TTG-CCC-TT**T**-ATR-AA**R**-TAA
Target	5' CG AAC GGG AAW UAY UUY AUU
Fecal clones A14, A20, A57	.. ..
Adhufec35.25	.. ..
Adhufec171	NN NNN NNN NN. ... ...
Ruminococcus obeum	.. .. CY. ... ...
Rumen clone RFN27	.. .. U.A

[a] According to the ODP nomenclature (1). A 1:1 mixture of these probes is encoded as Robe63 probe.

Reference strains, culture conditions, and fixation. The thirty-two reference strains used in this study were obtained from various sources as indicated: Deutsche Sammlung von Mikroorganismen und Zellcultures [DSM; Braunschweig, Germany], American Type Culture Collection [ATCC; Rockville, Md], and Laboratory for Medical Microbiology [MMB; Groningen, The Netherlands (5)] (Table 2). The ATCC and DSM strains were cultivated as described in the respective catalogues. All MMB stains are clinical or human fecal isolates from

local and regional public health laboratories that were identified and cultivated by using standard procedures (8). Exponentially grown cells were harvested at 5,000 X g for 10 min, washed with 0.2-µm-pore-size-filtered phosphate-buffered saline (PBS; per liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4; pH7.2), and diluted 1:3 with 4% (wt/vol) paraformaldehyde in PBS. After fixation at 4°C for 16 h cells were stored in 50% ethanol-PBS until FISH analysis (2).

Table 2. Reference strains used to validate the probe hybridization conditions.

Strain	Origin	Strain	Origin
Bacteroides fragilis	(DSM 2151)	*Clostridium putrificum*	(DSM 1734)
Bacteroides distasonis	(DSM 20701)	*Clostridium sporogenes*	(DSM 795)
Bacteroides ovatus	(MMB)	*Collinsella aerofaciens*	(DSM 13713)
Bacteroides thetaiotaomicron	(MMB)	*Eubacterium cylindroides*	(MMB)
Bacteroides uniformis	(MMB)	*Eubacterium moniliforme*	(MMB)
Bacteroides vulgatus	(DSM 1447)	*Eubacterium tenue*	(DSM 20695)
Bifidobacterium dentium	(ATCC 27678)	*Eubacterium ventriosum*	(DSM 3988)
Bifidobacterium infantis	(ATCC 15697)	*Lachnospira multipara*	(DSM 3073)
Bifidobacterium longum	(MMB)	*Peptostreptococcus micros*	(DSM 20468)
Clostridium beijerinckii	(MMB)	*Ruminococcus albus*	(ATCC 27210)
Clostridium butyricum	(MMB)	*Ruminococcus bromii*	(ATCC 27255)
Clostridium carnis	(DSM 1293)	*Ruminococcus callidus*	(ATCC 27760)
Clostridium innocuum	(MMB)	*Ruminococcus obeum*	(ATCC 29174)
Clostridium nexile	(MMB)	*Streptococcus intermedius*	(DSM 20573)
Clostridium perfingens	(MMB)	*Succiniclasticum ruminis*	(DSM 9236)
Clostridium polysaccharolyticum	(DSM 1801)	*Veillonella parvula*	(DSM 20373)

Fecal sample processing. Fecal samples were collected from 3 healthy male volunteers (25-32 years) and were processed within 30 min. These volunteers provided three samples within a 4 weeks period and had not been subjected to any antibiotic treatment for the last 3 years. Fecal samples were processed as described previously (5). In short, 0.5 g of fecal sample was resuspended in 4.5 ml PBS and vortexed with addition of 5-10 glass beads for 5 min to homogenize the sample. After centrifuging at 700 *X g* for 1 min, 1 ml of supernatant was added to 3 ml of 4% paraformaldehyde in PBS and stored overnight at 4°C. After washing twice with PBS the fixed cells were stored in 50% Ethanol-PBS at –20 °C until further use (for at least 1 h). Weighted portions of the remains of the fecal samples were lyophilized to determine their dry weights.

FISH analysis of fecal samples by microscopy. For microscopic analysis, fixed cells were spotted on gelatin-coated glass slides and dried for 20 min at 45°C. Dilution series of fecal samples were performed in order to determine the optimal cell concentration for counting using the different probes. After drying of the slides, the cells were dehydrated for 2 to 3 min in a graded ethanol series with concentrations increasing from 50% to 75%

and finally 96% ethanol/H_2O. Ten microliters of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH7.5), 0.1% (wt/vol) SDS) containing 3 ng/µl of Cy3-labeled Robe63 probe or 5 ng/µl FITC-labeled Erec482 probe was added to each well, followed by incubation at 50°C for 3 h. After hybridization the slides were washed in 50 ml hybridization buffer without SDS for 10 to 20 min. For the Robe63 probe a 20% formamide (vol/vol) containing hybridization buffer and a low salt washing buffer (0.225 M NaCl, 20 mM Tris-HCl (pH7.5), 10 mM EDTA) was used. For total counts 4',6-diamidino-2-phenylindole (DAPI) was added to the wash buffer at a final concentration of 100 ng/ml. After rinsing the slides in water they were immediately air-dried and mounted in Vectashield (Vector Labs, Burlingame, CA). Digital images of the slides, viewed with a Leica (Wetzlar, Germany) DMRXA epifluorescence microscope, were taken with a Kodak Megaplus 1.4 charge-coupled device camera. These images were analyzed and fluorescent cells were counted by using Quantimet HR550 image analysis software (Leica). For each analysis 25 microscopic fields were counted.

FISH analysis of fecal samples by flow cytometry. For each hybridization 50 µl of fixed cells were centrifuged for 3 min at 9,000 *Xg* and resuspended in 20 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH8.0), 0.1% (wt/vol) SDS). After addition of 2 µl of the Cy5- and/or double (at 5' and 3' end) FITC-labeled probes (30 ng/µl and 50 ng/µl respectively) the samples were incubated for 16 hours at 50°C in the dark. The Robe63 and Erec482 probes were Cy5-labeled for counting and the Erec482 probe was FITC-labeled for validation the specificity of the Robe63 probe. After hybridization, 980 µl of prewarmed washing buffer was added and the samples were incubated at 50°C for 5 min. The hybridization buffer without SDS was used as washing buffer after hybridization with the Erec482 probe, and the low salt washing buffer consisting of 0.225 M NaCl, 20 mM Tris-HCl (pH8.0), 10 mM EDTA was used after hybridization with the Robe63 probe. The cells were centrifuged at 9,000 *X g* for 5 min and resuspended in 1 ml of ice-cold PBS (pH 8.4). To avoid loosing the signal intensity the hybridized cells were kept in the dark on ice until flow cytometric analysis. Cells hybridized with the labeled probes were compared to cells, which were incubated in hybridization buffer only, in order to optimize the settings for flow cytometric analysis. Afterwards, the unlabeled cells were incubated with 10 µl/ml of propidium iodide (PI; 1 mg/ml) at 37°C for 20 min to count the total number of bacteria. The bacterial concentration was adjusted to keep the count lower than 1,000 events/sec in order to avoid coincidence. During analysis of fecal samples, 0.7 µm-yellow green fluorescent beads (for counting PI-labeled cells) or PC fluorescent beads (for counting Cy5-labeled cells) with known concentration (Polysciences, Inc) were added according to the manufacturer's instructions in order to determine cell numbers. Samples were analyzed using a Becton and Dickinson FACScalibur flow cytometer. An air-cooled argon ion laser (488 nm) and a red-diode laser (635 nm) were used for excitation and the green, red, and far red signals of the bacteria and the beads were collected in the FL1 (530/30 nm), FL3 (>600 nm long pass filter) and FL4 (661/16 nm) detectors, respectively. The system threshold was set on forward scatter signals and all bacterial analyses were performed at the low flow rate settings (12 µl/min). Data was collected in list mode as pulse height signals (four decades in logarithmic scale each) and 10,000 cells were acquired for further analysis which was performed using CellQuest software (Beckton Dickinson) and/or WinMDI version 2.8 software (http//:facs.Scripps.edu/software.html). The whole hybridization and counting analysis was performed three times for each probe and fecal sample.

Statistical analysis. The analyses performed on the various samples included the variables of methodology (microscopy or flow cytometry), probe type (DAPI, Erec482 probe, or Robe63 probe), individual, and time. Therefore, the coefficient of variation (i.e. the standard variation divided by the mean, termed CV) for the time, for the reproducibility of the counts, and for the different individuals were compared. In addition, regression analysis on the mean counts of fecal samples taken at different time intervals was performed for each individual. Since it appeared that time had no significant effect on the counts, these counts were used as replicates in a three-factor-ANOVA-test to statistically analyze the effect of the other variables.

RESULTS

Probe design and validation. Probes targeting the uncultured *R. obeum*-like bacteria were designed based on comparative analysis of 16S rRNA sequences from the ARB and RDP database and newly deposited sequences from GenBank. Despite the small sequence variation in the hyper-variable V1 region between the different sequences of this uncultured group, a combination of two probes was sufficient to discriminate between this uncultured group and the rest of the sequences in all databases (Table 1). These two probes hybridize at the same target site and were used in a 1:1 mixture during all Robe63 probe hybridizations. Analysis of the 16S rRNA sequence databases showed that the Robe63 probe has only two mismatches with the targets of the *R. obeum* sequence and a sequence of a 16S rDNA clone from a rumen sample (Table 1). All other sequences have three or more mismatches with the probe target site. No match was found with the adhufec171 sequence since the first non-discriminative bases of the Robe63 probe target were not present in this sequence (15). Furthermore, no match was found with any of the 23S rRNA sequences. Thirty-two reference strains (Table 2), including *R. obeum*, did not hybridize with the Robe63 probe, while all of them showed strong hybridization with the Eub338 probe as determined by microscopic and flow cytometric analysis. For the microscopic analysis, hybridization buffer containing 20% formamide was chosen since a few cells (~1% of Eub338 positive cells) of the *Peptostreptococcus micros* culture and about 10-20% of Eub338 positive *R. obeum* cells hybridized with the Robe63 probe when no formamide was added. For flow cytometric analysis Cy5-labeled probes, which give emission in the far red, were used since no adverse autofluorescence was observed in this detector channel (FL4) when fecal samples or reference strains were analyzed. However, autofluorescence of unlabeled fecal samples was observed in all other detectors (FL1 to FL3), which detect green, yellow, and red fluorescent signals, respectively (data not shown). It appeared that the addition of formamide in the hybridization

buffer could not be used during flow cytometric analysis when Cy5-labeled probes were used. No hybridization signal could be detected when formamide was added even when the reference strains were hybridized with the Eub338 probe. However, washing of Robe63 probe-hybridized cells with the low-salt washing buffer was sufficient to render all negative reference strains unlabeled, while maintaining specific hybridization in fecal samples. Only the cells of *R. obeum* showed some weak positive hybridization with the Robe63 probe under these conditions, although the signal was more than ten-fold lower than positively hybridized cells in a fecal sample.

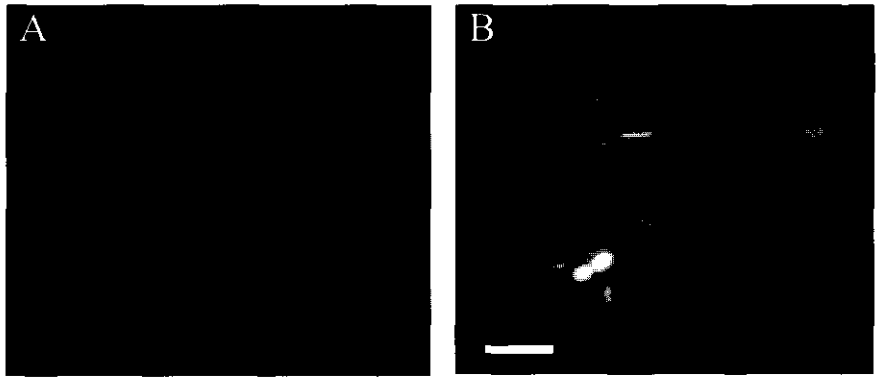

Fig. 2. Photographs of fecal cells in one microscopic field hybridized with (A) the Cy3-labeled Robe63 probe and (B) the FITC-labeled Erec482 probe. The bar scale is 5 μm.

Since no isolate is available to serve as a positive control strain for the Robe63 probe, a double hybridization of fecal samples was performed using the Robe63 and Erec482 probes. This latter probe targets the *Clostridium coccoides* - *Eubacterium rectale* group to which the uncultured group of *R. obeum*-like bacteria belongs. Microscopic analysis revealed that all cells that hybridized with the Robe63 probe also hybridized with the Erec482 probe (Fig. 2). All Robe63-positive cells were morphologically similar in all fecal samples examined (a relatively large duplococcus; Fig. 2A), while the Erec482-positive cells exhibited many different morphotypes (Fig. 2B). Flow cytometric analysis revealed similar results as obtained with microscopic examination (Fig. 3). Although the Erec482 probe was double FITC-labeled, it could be used in combination with the Cy5-labeled Robe63 probe, since the Robe63-positive cells did not show autofluorescence in the FL1 detector, which detects the FITC-labeled cells. The Cy5-labeled Robe63-positive cells were also detected as Erec482-

positive cells and could clearly be discriminated from unlabeled cells (Figs. 3A and 3B). When these double labeled cells were gated, they appeared as a very small cluster in the flow cytometric dot blots of the forward-angle light scatters (FSC) versus side-angle light scatters (SSC), indicating that these cells exhibited limited morphological variability (Fig 3C). This combination of microscopic and flow cytometric analyses indicate that it is very likely that the right approach is taken to detect the uncultured *R. obeum*-like bacteria.

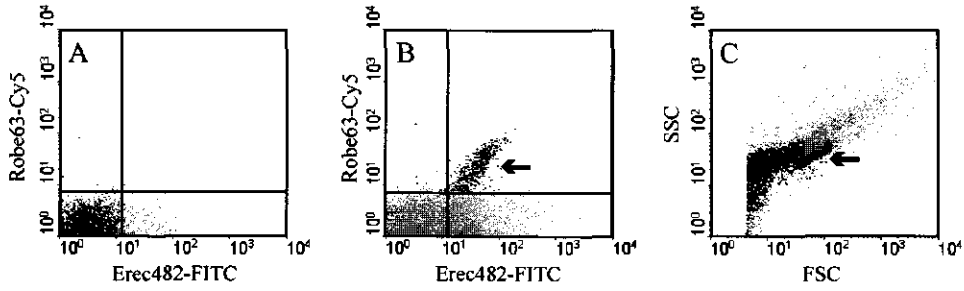

Fig. 3. Flow cytometric analysis of fecal samples showing the dot plots representing the Robe63-Cy5 fluorescence intensity and Erec482-FITC fluorescence intensity of (A) unhybridized bacteria and (B) Robe63 plus Erec482 hybridized bacteria in feces. Cells labeled with both probes (as indicated by the arrow in plot B) were marked with a dark color and appeared as a small cluster in dot plots of the forward-angle light scatters (FSC) versus side-angle light scatters (SSC) of the total community (C), indicating limited morphological variability among these bacteria.

Comparison between microscopic and flow cytometric analysis. Microscopic and flow cytometric counts were determined and compared to determine the total number of bacteria, the number of uncultured *R. obeum* bacteria and the *Clostridium coccoides - Eubacterium rectale* group in feces (Table 3). To visualize the difference between the two approaches the ratio of the mean counts for each probe per sample was determined. The ratios varied between 0.64 and 1.47 and only one relatively high ratio (1.93), which was found for a Robe63 count. The mean percentage of the *Clostridium coccoides – Eubacterium rectale* group varied between 16.9% and 13.5% as determined by microscopy and flow cytometry, respectively. Similarly, the percentage of the uncultured *R. obeum* group was found to be 2.6% and 2.2% of the total community, and 15.6% and 16.7% of the *Clostridium coccoides – Eubacterium rectale* group. This indicated that both groups were predominantly present in fecal samples

Table 3. DAPI and PI total cell counts, Erec482 probe hybridization counts, and Robe63 probe hybridization counts of fecal samples from three individuals (A, B, C) taken over a four weeks period as determined by microscopy (Mic) and flow cytometry (FCM).

		Mean counts ± SD[a]								
Individual	Time (weeks)	**DAPI / PI (10^{11})**			**Erec482 (10^{10})**			**Robe63 (10^{10})**		
		Mic	FCM	Ratio[b]	Mic	FCM	Ratio[b]	Mic	FCM	Ratio[b]
A	0	1.87 ± 0.17	2.40 ± 0.54	0.78	3.95 ± 0.63	2.80 ± 1.16	1.41	1.06 ± 0.17	0.55 ± 0.36	1.93
	2	3.27 ± 0.40	4.98 ± 0.52	0.66	4.32 ± 0.77	5.69 ± 0.73	0.76	1.06 ± 0.30	1.33 ± 0.31	0.79
	4	4.06 ± 0.47	5.20 ± 2.60	0.78	4.90 ± 1.22	5.16 ± 0.75	0.95	1.06 ± 0.25	0.72 ± 0.12	1.47
B	0	3.55 ± 0.41	4.72 ± 0.71	0.75	8.35 ± 1.00	7.16 ± 1.76	1.17	0.56 ± 0.07	0.88 ± 0.30	0.64
	2	3.42 ± 0.37	3.30 ± 0.59	1.04	5.93 ± 0.69	5.15 ± 1.45	1.15	0.88 ± 0.20	1.31 ± 0.37	0.67
	4	3.24 ± 0.33	3.15 ± 0.95	1.03	8.30 ± 1.90	4.98 ± 1.51	1.67	0.71 ± 0.17	0.75 ± 0.12	0.94
C	0	2.27 ± 0.41	2.06 ± 0.64	1.10	3.55 ± 0.58	4.45 ± 1.91	0.80	0.71 ± 0.25	0.57 ± 0.19	1.25
	2	2.43 ± 0.25	2.41 ± 0.97	1.00	2.75 ± 0.46	2.94 ± 0.91	0.94	0.38 ± 0.06	0.31 ± 0.01	1.22
	4	2.23 ± 0.45	2.24 ± 1.50	1.00	2.49 ± 0.62	2.96 ± 1.00	0.93	0.54 ± 0.15	0.41 ± 0.16	1.30

[a]The mean and SD of the counts are represented as cells per gram dry weight of feces.

[b]The Ratio is calculated by dividing the mean microscopic count by the mean flow cytometric count.

and that the uncultured *R. obeum*-like bacteria comprised a significant fraction of the *Clostridium coccoides – Eubacterium rectale* group.

Table 4. Three-factor-ANOVA-test of the counts performed by microscopy and flow cytometry. The degrees of freedom (*df*), mean square (*MS*), the *F*-ratio and the *p*-value of the variables methodology (M; microscopy and flow cytometry), probe (P; DAPI/PI, Erec482 probe, Robe63 probe) and individual (I) are represented. [a] indicates a significant effect of the variable ($p<0.05$).

Source	*df*	MS	*F*-ratio	*p*-value
M	1	2.61×10^{21}	1.03	0.316
P	2	5.05×10^{23}	199.82	<0.001[a]
I	2	1.73×10^{22}	6.85	0.003[a]
M X P	2	3.44×10^{21}	1.36	0.269
M X I	2	1.93×10^{21}	0.76	0.474
P X I	4	9.85×10^{21}	3.90	0.010[a]
M X P X I	4	1.96×10^{21}	0.77	0.549
Error	36	2.53×10^{21}	-	-

Regression analysis was performed on the mean of the total and specific counts separately as determined by microscopy and flow cytometry from the three time samples taken from each individual. None of the eighteen analyses resulted in a significant relationship between time and counts ($P>0.05$). In addition, the CV for time was found to be comparable to the CV for reproducibility of the counts (data not shown). In only half of the counts the CV for time was higher than the CV for reproducibility. These results indicated that the bacterial composition is relatively stable over time. Since no significant differences between the time samples were found within each individual, these counts were used as replicates in a three-factor-ANOVA-test in order to determine the effect of the other variables (Table 4). As may have been expected, a significant difference in the counts was found when DAPI (or PI), the Erec482 probe or the Robe63 probe was used (Table 4). In addition, a significant effect of the individual on the different counts was observed. Furthermore, a significant effect on the interaction between the different probes and individuals was observed, which indicated that the different probe counts varied significantly per individual. These individual differences were most clearly when the CV among individuals was calculated for each probe at each sampling time. In 17 out of the 18 cases, the CV among

individuals was higher than the CV for the assay (data not shown). This suggests that the feces of each individual had a different bacterial composition. Furthermore, it was observed that the different counting approaches did not have a significant influence on the outcome of the counts.

DISCUSSION

This study describes the development, validation and application of the Robe63 probe to detect, examine, and quantify uncultured *R. obeum*-like bacteria in human fecal samples. We used a microscopic and flow cytometric approach in order to characterize the specificity of the probe, the morphology of the cells, and the number of cells in different fecal samples. All reference strains used as negative control in this study did not hybridize with the Robe63 probe and all Robe63 probe-labeled fecal cells also hybridized with the Erec482 probe that is specific for the *Clostridium coccoides – Eubacterium rectale* group (5). This indicates that the right target group was detected. While many different morphotypes of bacteria hybridized with the Erec482 probe, only one morphotype, hybridized with the newly developed Robe63 probe. The Robe63 probe did not hybridize with the type strain *R. obeum*, which has of all known sequences the least (only two) mismatches at the target site. The Robe63 probe was designed and validated using currently available 16S rRNA sequences including several hundreds of 16S rRNA sequences derived from cultured and uncultured GI tract bacteria. Since various *R. obeum*-like sequences have been found frequently in fecal clone libraries from different human individuals (15, 23), we conclude from the present data that the probe can specifically detect a new and numerically important group of intestinal bacteria.

For microscopic analysis and flow cytometric analysis of FISH samples different protocols were required due to the difference in handling procedures. Despite these different approaches our data showed that the counts obtained for the Robe63 and the Erec482 probes with either method were very similar (Table 3). Moreover they were reproducible. This indicates that both approaches will give similar results when quantifying the number of specific bacteria in environmental samples. In general, about 16% of the total bacterial community in the fecal samples belonged the *Clostridium coccoides – Eubacterium rectale* group, which is slightly lower than reported in another study (5). This difference may be due to host-specific factors as observed previously (23). The percentage of the uncultured *R. obeum*-like bacteria in the investigated samples was about 2.5%. This indicates that they

comprise a significant fraction of the predominant bacterial community in feces as observed previously using PCR-based approaches (15, 23). Based on statistical analysis, we could clearly observe a host-specific effect on the counts, while the counts did not vary significantly in time. These results are in line with previous observations based on PCR and TGGE analysis of fecal samples (23).

The results described in this study reveal that a group of uncultured *R. obeum*-like bacteria, whose presence was only suggested repeatedly by 16S rDNA cloning studies of fecal samples, is predominantly present in fecal samples as determined by FISH analysis using a newly developed specific 16S rRNA targeted probe. Although the power of the 16S rRNA probe hybridization has been demonstrated in many independent studies, its definitive value in studying the ecology of uncultured bacteria requires additional systematic research. The application of FISH combined with flow cytometry was demonstrated and offers a possibility to sort the *R. obeum*-like bacteria and other uncultured populations in order to study them in detail. Such studies may ultimately be helpful in refining our knowledge about the ecology of the microorganisms in the human GI tract.

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Chapter 9

Victivallis vadensis gen. nov. sp. nov., a cellobiose-degrading bacterium from human feces

Erwin G. Zoetendal, Caroline M. Plugge, Antoon D. L. Akkermans, and Willem M. de Vos

Abstract

A novel anaerobic cellobiose-degrading bacterium, strain Cello^T was isolated from a human fecal sample by combining enrichments in liquid and soft agar basal media. A noteworthy characteristic was its inability to grow on normal agar plates and in roll tubes. The cells were coccus shaped and non-motile. An extracellular slime layer was surrounding the cells. Growth of strain Cello^T occurred between 20 and 40°C with optimal growth rate at 37°C. The pH range for growth was 5 – 7.5 with optimum at 6.5. In pure culture, strain Cello^T could only grow on a variety of sugars. Glucose was converted to acetate, traces of propionate, H_2, and an unidentified compound. The doubling time on glucose was 0.5 h. In a syntrophic coculture with *Methanospirillum hugatei* strain JF 1 strain Cello^T converted glucose to acetate and H_2. The G+C content of strain Cello^T was 59.2 mol%. 16S rDNA analysis revealed that strain Cello^T was phylogenetically related to a cluster of uncultured bacteria from a variety of ecosystems belonging to the environmental_clone_WCHB1-41_subgroup. The closest relatives of strain Cello^T were two uncultured bacteria from anaerobic digestors, both with 94% 16S rDNA sequence similarity. We propose the name *Victivallis vadensis* for strain Cello^T as a new species of a new genus.

INTRODUCTION

The human gastrointestinal (GI) tract is a very complex ecosystem in which bacteria are in close contact with each other and with the host's cells. Communication between the host and the bacteria, from which the majority is strictly anaerobic, seems to be very important in this ecosystem (2, 4, 5). Despite many intensive cultivation trials in order to isolate bacteria from the human GI tract, it is estimated that at the moment only 10 to 50% of the bacteria in the human GI tract can be obtained in culture (7, 10, 16, 17). Several reasons for this unculturability may include the unknown growth requirements of the bacteria, the selectivity of the very rich media which have mainly been used, the stress imposed by the cultivation procedures and the necessity of strictly anoxic conditions, and the interactions of the bacteria with other microbes and host cells. Many novel bacteria have been detected in feces using several culture-independent approaches based on the variability of the 16S rRNA gene (16-18). This implies that the physiological characteristics and function of the majority of the human GI tract community is unknown.

In this study, we describe the isolation of a novel species belonging to a new genus, strain Cello^T from the GI tract by using an alternative cultivation approach. Exposure of the fecal sample to oxygen was minimized and a combination of liquid and soft agar basal medium having cellobiose as the carbon source was used for isolation and cultivation.

MATERIALS AND METHODS

A fecal sample (~0.5 g) from a healthy Dutch man (25 years) was transferred within one minute after defecation without homogenization into 10 ml sterile anoxic Ringers solution by using a syringe. This fecal suspension was vortexed and 10,000 times diluted in anaerobic ringers. From this dilution 0.3 ml was used to inoculate 30 ml of a bicarbonate-buffered anaerobic medium as described previously by Stams *et al.* (14), supplemented with 10 mM cellobiose and 0.7% (vol/vol) clarified, sterile rumen fluid. This medium was subsequently used to isolate and cultivate strain Cello^T and to prepare plates and roll tubes by supplementation of 2% agar (agar noble: Difco, Detroit, MI, USA) and soft agar (0.75% agar noble). For the plates the concentration of the phosphates was 5 times increased to enhance the buffering capacity. Other media used to cultivate the isolated strain Cello^T include: Wilkins-Chalgren broth (WC broth; Oxoid; 16 g l^{-1}), medium (KA medium) as described by Kamlage and colleagues (6) with minor modifications [no hemin, bacteriological peptone (Oxoid) instead of tryptic peptone from meat], both supplemented with 0.7% clarified, sterile rumen fluid, and Biolog Universal Anaerobic (BUA) agar plates (Stag, St. Katalijne Waver, Belgium). Antibiotics streptomycin (100 mg l^{-1}) and polymyxin B (20 mg l^{-1}) were used to remove a fecal contaminant. Aerobic LB medium (9) was used to check the presence of

the contaminant. All incubations were done at 37°C. Basal media containing several carbon sources were used to determine the utilization of different carbon sources by strain Cello^T. The optimal growth temperature of strain Cello^T was determined in basal medium of pH 7 containing cellobiose ranging from 4°C to 45°C. The pH range was determined in WC broth at 37°C ranging from pH 5 to pH 8 (with 0.5 pH unit intervals, adjusted with NaOH or HCl). HPLC and GC analyses were performed as described previously (14).

Methanospirillum hungatei JF1 (DSM 864) was used as partner organism in syntrophic cultures. For the cultivation of *M. hungatei* a gas phase of 182 kPa H_2/CO_2 (80:20, v/v) was used and after growth the gas phase was exchanged to N_2/CO_2 and the bottles were inoculated with strain Cello (1%, v/v) and glucose (approx. 10 mM final concentration).

Gram staining and light microscopy were performed as described elsewhere (11). For transmission electron microscopy, cells were fixed in 2.5% glutaraldehyde in cacodylate buffer at 0°C for 2 h. After rinsing with cacodylate buffer a post-fixing was performed in a 2:1 mixture of 1% OsO_4 and 5% $K_2Cr_2O_7$ at room temperature for 2 h. After rising with water the cells were post-stained with 1% uranyl acetate overnight, dehydrated in graded ethanol series, and embedded in Epon (Glycid ether (Serva)). Ultra thin coupes were post-stained with lead citrate (3) and analyzed using a Philips CM10 TEM.

DNA isolation, PCR and sequencing of the complete 16S rDNA, and determination of the G+C content was performed as reported recently (12). BLAST was used for homology searches (1) and the ARB and RDP programs were used for phylogenetic analysis (8, 15). PCR and DGGE analysis of the V6 to V8 regions of 16S rDNA of the enrichment cultures was performed as described before (19).

RESULTS AND DISCUSSION

Anaerobic basal medium with cellobiose as sole carbon source was inoculated with a diluted fecal suspension in Ringers. After two days of incubation at 37°C growth was observed. Microscopic analysis revealed that a coccus-shaped bacterium became predominant. In addition, a rod-shaped organism was observed. This rod-shaped bacterium grew very well under oxic conditions in LB medium at 37°C. Repeated transfers in serial dilution of the enrichment to fresh media showed a decrease in number of rod-shaped bacteria. However, it was never outcompeted completely, since the rod-shaped bacteria still could be found in parallel incubations in LB medium. DGGE analysis of 16S rDNA revealed that this rod-shaped bacterium is very likely an *E. coli* strain. Therefore, the enrichment was transferred to medium containing streptomycin and polymyxin B to inhibit growth of the rod-shaped bacterium. After one week of incubation growth of the coccus-shaped bacterium was observed while no growth of the enrichment culture was observed in LB medium. This could also be visualized when the V6 to V8 regions of the 16S rDNA from the cultures were analyzed by DGGE (Fig 1).

Before the antibiotic treatment there is still a tiny band representing the rod-shaped bacterium present, while it is absent after the antibiotic treatment. This indicates that the rod-shaped bacterium was not present anymore in the enrichment. Cultivation of the enrichment in two different very rich media for growing fermentative bacteria (KA medium and WC broth), resulted only in the growth the same coccus-shaped bacterium after one and two days respectively as indicated by microscopic and DGGE analysis (Fig 1).

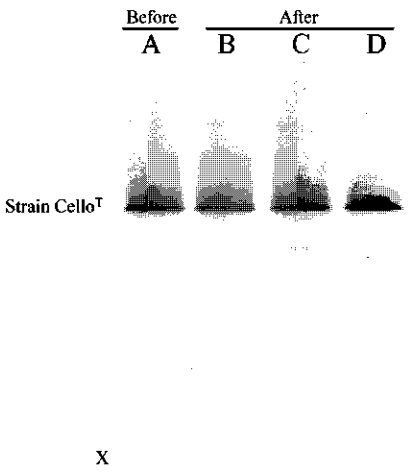

Fig. 1. DGGE analysis of amplified V6 to V8 regions of the 16S rRNA gene of strain Cello^T cultures before (A) and after (B-D) the antibiotic treatment. Lanes B, C, and D represent the cultures grown on minimal medium with cellobiose, KA medium, and WC broth, respectively. The contaminant in culture A before the antibiotic treatment is indicated (X).

Despite several attempts to cultivate this isolate on solid media containing 2% agar (plates and roll tubes) having the same components as the liquid media, no colonies were obtained. In addition, no colonies were formed on BUA agar plates, which are regarded as universal media for anaerobic bacteria. The lack of growth on solid media might be a reason why this fermentative bacterium has never been cultivated so far. In addition, this lack of growth on agar plates can be seen as a confirmation of its purity, since it is very unlikely that a random contaminant cannot grow on general solid media as well. Incubation in soft agar (0.75%) was more successful. Serial dilution of the enrichment in basal medium having cellobiose as carbon source resulted after 10 days in the development of beige, shiny, lens-

shaped colonies. Single colonies were transferred to liquid media followed by incubation in soft agar. This procedure was performed three times and always the same colony, cell shape, and DGGE band position of its 16S rRNA gene were observed. Therefore, it was concluded that the strain was pure and this pure culture was designated as strain Cello^T, and characterized further. Incubation of Cello^T in basal medium without rumen fluid did not affect its growth and therefore rumen fluid was omitted from the following experiments.

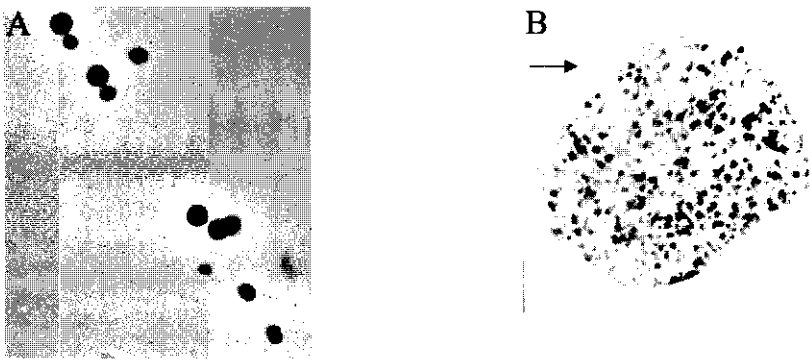

Fig. 2. Phase Contrast microscopic (a) and transmission electron microscopic (b) photographs of strain Cello^T. The arrow in photograph B indicates the extracellular slime layer.

The Gram type of strain Cello^T was determined using Gram staining combined with light microscopy and transmission electron microscopy. The light microscopic analysis revealed that strain Cello^T stained Gram-negative and that the coccus shaped cells were variable in size (Fig 2A). Moreover, many time halos surrounding the cells were observed, which indicated the presence of an extracellular slime layer. Electron microscopy confirmed that the cell wall of strain Cello^T was Gram-negative, although clear visualization of the cell membrane was hampered by the extracellular slime layer, which completely surrounded the coccus-shaped cells (Fig 2B). In addition, the electron microscopy revealed that the cells were varying in diameter from 0.5 μm to 1 μm and the presence of many intracellular electron-dense structures. These structures are most likely intracellular protein precipitates, since they are too large to be considered as ribosomes.

DNA was isolated from strain Cello^T and the complete 16S rRNA gene was amplified. The nucleotide sequence (1456 bp) of the amplified 16S rRNA gene was determined in duplo and revealed that strain Cello^T belongs to the environmental_clone_WCHB1-41_subgroup of

the *Prosthecobacter* group according to the tree-of-life of the RDP (8). It is noteworthy that this subgroup has no cultured reference organism and that it only consists of sequences from different environments retrieved by PCR and cloning approaches. A neighbor-joining tree was constructed using the closest related 16S rDNA sequences of the Cello^T sequence, *Verrucomicrobium spinosum* and *Prosthecobacter* species to represent the *Prosthecobacter* group (Fig 3). This tree demonstrates that strain Cello^T is closest related to clones from anaerobic digestors (clones AA08, vadinHB65, and vadinBE97), rumen (clones RFN4 and RFN44), and a deep-sea sediment (clone BD2-3). The closest relatives of strain Cello^T were clone AA08 and clone vadinHB65 with only 94% sequence similarity which is below the 97% threshold for species discrimination (13). No cultured strain is closely related to strain Cello^T which indicates that we isolated a bacterium belonging to a yet undescribed genus. The G+C content of strain Cello^T was 59.2 mol%.

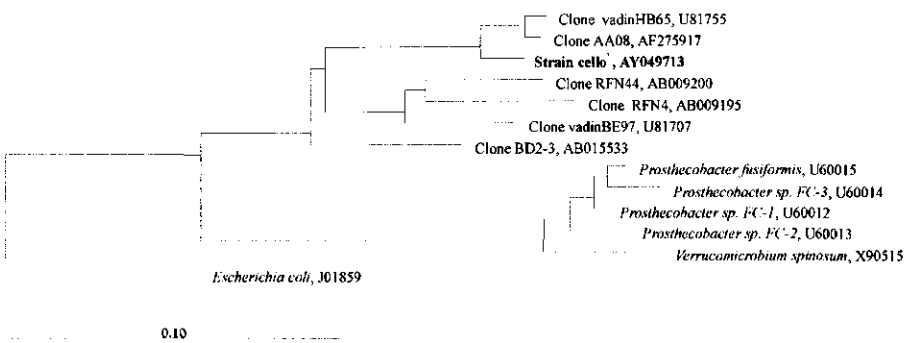

Fig. 3. Neighbor-joining tree showing the phylogenetic relationship of 16S rDNA sequences from strain Cello^T (indicated in bold) and it's closest relatives in the DNA databases. The bar represents 10% sequence divergence. Clones vadinBE97, vadinHB65 and AA08 originate from anaerobic digestor samples, clones RFN4 and RFN44 originate from rumen samples.

Since no cultivable bacterium is closely related to strain Cello^T, a detailed physiological comparison with distantly related bacteria is not possible. To characterize strain Cello^T it was cultured in basal liquid media supplemented with several carbon sources. A variety of sugars and other carbon sources were tested (table 1). It appeared that strain Cello^T grows well on a variety of sugars, while no growth was observed on alcohols, proteins, pyruvate, and fumarate.

Growth of strain Cello^T on cellobiose was found between 20°C and 40°C, with optimum growth rate at 37°C. The growth on WC broth was observed between pH 5 and 7.5 with an optimum at pH 6.5. These optimal temperature and pH conditions are in line with the conditions found in the human colon. Strain Cello^T did not grow under micro-aerophilic conditions. In pure culture strain Cello^T converted glucose (12.8 mM) to acetate (16.3 mM), hydrogen (6 mM) and an unknown reduced compound. The yield on glucose was 26 – 30 gram per mole of glucose. The doubling time was approximately 0.5 hours, when grown on glucose plus 0.02% yeast extract. In syntrophic coculture with *Methanospirillum hungatei strain* JF 1 strain Cello^T converted glucose (12.1 mM) to acetate (20.6 mM) and H_2 (36 mM).

Table 1. Substrate specificity of strain Cello^T. Indicated are + (growth), – (no growth).

Sugars	Growth[a]	Sugars	Growth[a]	Other substrates	Growth[a]
Cellobiose	+	Maltose	+	Alcohols[b]	-
Cellulose	-	Mannitol	+	Casamino acids	-
Galactose	+	Rhamnose	+	Fumarate	-
Gelatine	-	Ribose	+	Oleate	-
Glucose	+	Sucrose	-	Proteins[c]	-
Lactose	+	Xylose	+	Pyruvate	-

[a] Incubations were done at 37°C for at least 14 days.

[b] Methanol, ethanol, 1-propanol, 2-propanol, and 2-butanol were tested.

[c] Casein, peptone, and tryptone were tested.

Despite many cultivation trials in the past decenia, it is estimated that only a minority of the bacteria surrounding us can be obtained in culture. One of the reasons for GI tract bacteria could be a combination of strict anaerobic conditions and specific growth requirements in order to win the competition for substrates in the media used. In this study, we used an alternative approach to enrich and isolate fecal bacteria, which resulted in the isolation of strain Cello^T. Instead of plating the bacteria on rich media, we enriched the bacteria in basal liquid medium containing cellobiose as the carbon source. Only rumen fluid was added as a supplement of unknown growth factors, although it seemed not to be necessary for growth in the latter enrichment procedure.

Strain Cello^T belongs to a new genus and its 16S rDNA sequence clusters in a group

which only comprised of many cloned sequences from different ecosystems. Since most cultivation trials of bacteria from ecosystems including the human GI tract are performed on agar media and since strain Cello^T grows relatively slow in soft-agar media, we speculate that this is the main reason why Cello^T like bacteria have never been isolated before. The limited carbon source specificity of strain Cello^T could also be a reason why such strains have not been obtained in culture before. Therefore, we suggest that alternative cultivation approaches, such as the approach described here, may result in the isolation of novel species. Remarkably, the closest uncultured relatives of strain Cello^T derive from the rumen and anaerobic digestors fed with wastewater containing plant material. Since cellulose is a major component of plant material consisting of cellobiose units, on which strain Cello^T was isolated, cellobiose could be used as carbon source in cultivation trials of similar strains from the rumen and reactors.

Description of *Victivallis* gen. nov. *Victivallis* (Vic.ti.val'. lis. M.L. m. *Victus*, N. L. m. food. *Vallis*, N.L.f. valley). *Victivallis* referring to the Wageningen "Food Valley", which includes Wageningen and surroundings, an area in The Netherlands in which Food Science is a major research topic.

Non motile, Gram-negative cocci. Non-spore forming. Strictly anaerobic. Cells produce extracellular material. Growth on sugars. Type species: *Victivallis vadensis*.

Description of *Victivallis vadensis* sp. nov. *Victivallis vadensis* (va.den'.sis M.L.n. *Vada*, Wageningen, M.L.adj). *vadensis* referring to Wageningen. The complete name *Victivallis vadensis* refers to the Wageningen Food Valley.

Gram-negative non-motile cocci. Single cells diameters vary between 0.5 and 1 μm. In pure culture the cells can grow anaerobically on cellobiose, galactose, glucose, lactose, maltose, mannitol, ribose, rhamnose, and xylose. Growth on 2% agar media is not observed. Cells grow optimal at 37°C and pH 6.5. The DNA G+C base composition is 59.2 mol%. The type strain is Cello^T.

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Chapter 10

Concluding Remarks and Future Perspectives

The results described in this thesis are all based on a 16S rRNA approach which revealed new insights into the ecology of the human GI tract. The major achievements will be highlighted and discussed in the following paragraphs. Figure 1 gives a schematic representation of the major findings and the possibilities for future research.

1.) COMPARISON BETWEEN PCR-DEPENDENT AND FISH APPROACHES

The combination of TGGE analysis, cloning and sequencing of 16S rDNA revealed that the majority of GI tract bacteria have not been obtained in culture (**Chapter 3**). Similar observations were confirmed by a study in which fecal 16S rDNA was randomly cloned and sequenced (25). Although the fecal clone libraries from different studies (25, 32, **Chapter 3**) show variations in composition, they all had in common that the majority of GI tract bacteria belongs to the low G+C Gram positive species, which is in line with studies based on FISH analysis using 16S rRNA targeted probes (7, 27). In addition, bacteria resembling *Fusobacterium prausnitzii* and *Ruminococcus obeum* were found regularly in the fecal TGGE profile of one individual and the clone libraries of different individual (25, **Chapter 3**) and FISH analysis confirms that these bacteria are present in high numbers in fecal samples (7, 26, **Chapter 8**). Notably, no bifidobacteria were detected in these clone libraries, while this group represents a few percentages of the total community based on FISH quantification (7, 16). This may be a result of biased PCR amplification and cloning to the disadvantage of high G+C Gram positive species. On the other hand, bifidobacteria could easily be detected in DGGE profiles of babies and healthy individuals (4) and after amplification using genus-specific primers (23). Surprisingly, *Bacteroides* species were not detected in the TGGE profile of the single individual that was analyzed in detail. *Bacteroides* 16S rDNA showed one mismatch (10 bases from 3' end) with the U968-GC primer, which is used to amplify the predominant bacterial community for DGGE and TGGE analysis. However, it is unlikely that the absence of *Bacteroides* is a result of preferential amplification of the other bacteria. The sequence analysis of some clones which did not correspond to the TGGE profile did also not result in the retrieval of *Bacteroides* sequences, while *Prevotella* sequences having the same mismatch with the primer could be visualized in the TGGE profile (**Chapter 3**). Furthermore, the detection limit for PCR-DGGE analysis of *Bacteroides fragilis* was comparable to that of

some high and low G+C Gram positive GI tract species **(Chapter 4)** and competitive PCR using complete 16S rDNA of *Bacteroides fragilis* and *Clostridium clostridiiforme* as template DNA revealed that both could be visualized after DGGE analysis (33). However, it has to be mentioned that *Bacteroides fragilis* and also clones corresponding to *Prevotella* species gave fussy bands in DGGE and TGGE profiles and therefore they may fall into the background of sharp bands, which hampers their detection. FISH analysis revealed that the number of *Bacteroides* varies considerably (from approximately 0.1% to 20%) between individuals (1, 7). Cultivation of *Bacteroides* and total anaerobic bacteria from feces of the single individual indicated that the total community only harbors a few percent of *Bacteroides* (33). Therefore, it is likely that the absence of bands corresponding to *Bacteroides* species in the TGGE profile and clone library of that individual is caused by the low numbers of *Bacteroides* in the feces of the individual studied.

In our study we combined cloning and TGGE analysis. A major difference between this approach and random clone library analysis is that the first approach will only select for the dominant 16S rDNA sequences from a community. A numerically important group of bacteria may consist of many underrepresented species. If for example these species separately represent less than 1% of the community but about 10% as a group than some of these underrepresented species will be selected by random cloning while they will not be visualized by TGGE or DGGE analysis.

TGGE, DGGE, and randomly cloning of 16S rRNA genes are all PCR-based approaches and therefore the outcome of these approaches cannot be converted to bacterial numbers. FISH can be used to specifically detect and quantify bacteria using 16S rRNA targeted probes, but it has to be realized that FISH is dependent on the available sequences in the DNA databases. In addition, FISH analysis can be hampered by cross-reactions during hybridization, the inaccessibility of the target, and a low number of target molecules.

In conclusion, comparison between the PCR-DGGE, random cloning, and FISH approaches indicated that these approaches showed a high overlap in the detection of the different groups of bacteria in feces. Since all methods on their own have their limitations, we suggest that they should be combined to study the GI tract composition, since they supplement each other with valuable information.

2.) *LACTOBACILLUS*-GROUP SPECIFIC PCR-DGGE

Lactobacillus and related lactic acid bacteria are suggested to have beneficial effects to the host and are regularly used as probiotics in clinical trials. De Roos and Katan reviewed and discussed the studies on the health effects of probiotics between 1988 to 1998 and concluded that the only clear probiotic effect was the shortening of the course of rotavirus infection by *Lactobacillus* GG (22). The authors argue that well-designed placebo-controlled studies with validated outcome variables are needed to determine the other health effects of probiotics. Recently, it was observed that *Lactobacillus* GG was effective in prevention of early atopic disease in children at high risk (14).

The effect of such probiotics on the predominant bacterial community is still questionable and could easily be demonstrated using the PCR-DGGE approach. Two independent studies revealed that the probiotic strains tested did not affect a shift in the human GI tract community (27, 29). Although these bacteria did not affect the dominant community, they might affect the composition of related *Lactobacillus* and other lactic acid bacteria in the GI tract. Since these lactic acid bacteria consist normally less than 1% of the total community, they will not be visualized in DGGE profiles when general 16S rDNA primers are used for PCR. Therefore, a primer specific for the *Lactobacillus* group was developed to detect and monitor this group of bacteria in the GI tract. The primer appeared to be suitable for specifically amplifying 16S rDNA from *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species and the amplicons could be used for DGGE analysis when a nested PCR approach was used (**Chapter 5**).

Another pair of primers to amplify the 16S rDNA from these lactic acid bacteria has been described previously by Walter and colleagues (31). The authors claimed that only a single PCR was sufficient to specifically detect these bacteria and analyze the amplicons by DGGE. However, the validation of the primers was not stringent. Furthermore, a degenerated primer having a C/T wobble base close to the 5' end was used, which may result in an overestimation of the diversity of the *Lactobacillus* group. The bias caused by degenerated primers has been reported for nitrifying bacteria (15).

Using our newly developed primer, several known *Lactobacillus* species were detected in fecal samples and in addition some food-associated *Lactobacillus* and *Leuconostoc* species were found in fecal and other GI tract samples, which is in line with previous observations (31). During a probiotic trial, shifts in the *Lactobacillus* group

communities were found and the probiotic strain could be detected in feces. Recently, this primer was also successfully applied in studying the shift in community structure during the production of Ragusano cheeses (21). Therefore, we think that the newly developed primer set will be very useful in studying the *Lactobacillus* group community in the human GI tract and other ecosystems.

3.) HOST-RELATED IMPACT ON THE BACTERIAL COMMUNITY IN THE HUMAN GI TRACT

Based on comparisons between fecal TGGE profiles, it was observed that these profiles were host-specific and stable in time (**Chapter 3**). Similar observations were made previously by a cultivation approach (13). These findings were confirmed during feeding trials using probiotic strains with humans (27, 29) and pigs (24) based on DGGE approaches. In line with this, it has been established that each individual harbors specific strains of *Helicobacter pylori* or *Bifidobacterium* and *Lactobacillus* species (3, 17).

We also observed that host-genotype related factors may have a major impact on the bacterial community structure in the GI tract as based analysis of fecal samples from adult individuals with varying degrees of genetic relatedness and of colonic biopsy samples (**Chapters 6** and **7**). The important role of the host on the bacterial community has been discussed in several other studies. To our knowledge the first study with feces from mono- and dizygotic twins was performed by van de Merwe and colleagues (19). They observed a significant difference between mono- and dizygotic twins based on difference in cell morphology of selected colonies from plates. It has to be mentioned that the twins in this study include only children of varying ages. Therefore, the presence of a climax bacterial community in these children is still questionable.

A stronger argument for a major role of the host genotype was given by Hackstein and colleagues (8, 9). They performed studies in which the methane production in the intestine of several vertebrates and invertebrates was measured. They found that the presence or absence of methane production in the intestine is a phylogenetic character that obeys 'Dollo's rule', i.e. traits that are lost in the course of evolution do not appear in any of the descendants of the common ancestor that lost these traits. In line with this, only a fraction of humans harbor significant methanogens in the intestine (20). On the other hand, comparisons between mono- and dizygotic twins revealed that shared environmental factors have a major impact on the

methane production (6). However, the twin pairs consisted of children in their puberty and therefore it is likely that they still do not harbor a climax community in their intestine, making the enhancing influence of environmental factors possible.

A major impact of the host genotype on the bacterial community was also observed in mice. Toivanen and colleagues studied the bacterium-derived cellular fatty acids in fecal samples of mouse strains cogenic for the major histocompatability complex (28). In line with the above-mentioned studies they suggested that the bacterial composition in the GI tract is genetically regulated.

The host-related influences on the GI tract community have been observed by a variety of studies, based on different microbiological approaches. These studies argue strongly that host-related factors have a major impact on the bacterial community in the GI tract. However, we can so far only speculate what these factors are. The GI tract is a very complex ecosystem in which interactions between the bacteria, the immune system, the epithelium, host secreted compounds, and food take place. A major effect of the host may be related to receptors in the epithelium, the action of the immune system, the secretion of specific compounds or a combination of these. Recent studies indicate that communication between bacteria and the host are important for a stable GI tract community (2, 10, 11). The first molecular details of the cross talk between *Bacteroides thetaiotaomicron* and its murine host have been elucidated. Therefore, future studies on the GI tract ecosystem should include those trying to unravel the molecular details of the interactions between host and bacterial cells.

4.) ALTERNATIVE APPROACHES TO STUDY THE ROLE OF UNCULTURED GI TRACT BACTERIA

Using 16S rRNA approaches many novel species have been detected in the human GI tract (25, 32, **Chapter 3**). Despite, our knowledge about the majority of the GI tract bacteria remains still limited. To understand the ecology of the GI tract the physiological activity of these uncultured bacteria should be determined. In **Chapter 9** we describe a strain isolated from feces which belongs to a novel genus. This strain was isolated using an alternative cultivation method with liquid basal medium and cellobiose as major carbon source. The majority of isolates from the GI tract have been obtained by using (selective) solid media (reviewed by 5). Total anaerobic counts are mostly done after incubation of feces in very rich

media for a few days at 37 °C. A striking characteristic of our novel isolate is its inability to grow on conventional plates solidified with agar. However, after 10 days of incubation it appears as colonies in soft agar. This characteristic may be a reason why bacteria belonging to this genus have never been found before. Therefore, one way to obtain novel isolates from the human GI tract may include the enrichment in liquid basal media using several carbon sources. Another possibility for retrieving new isolates might be a longer incubation of the plates at 37°C and look for colonies derived from slower-growing bacteria.

Despite many cultivation trials, it may of course still be possible that important members of the GI tract community will not be isolated, because we simply do not know their growth requirements or they have obligate syntrophic interactions. In **Chapter 8** we compared a microscopic and a flow cytometric quantification of *Ruminococcus obeum*-like bacteria. Since both methods gave similar results, the flow cytometric approach may give new opportunities to study uncultured bacteria. Hybridized cells could specifically be sorted and used for further analysis.

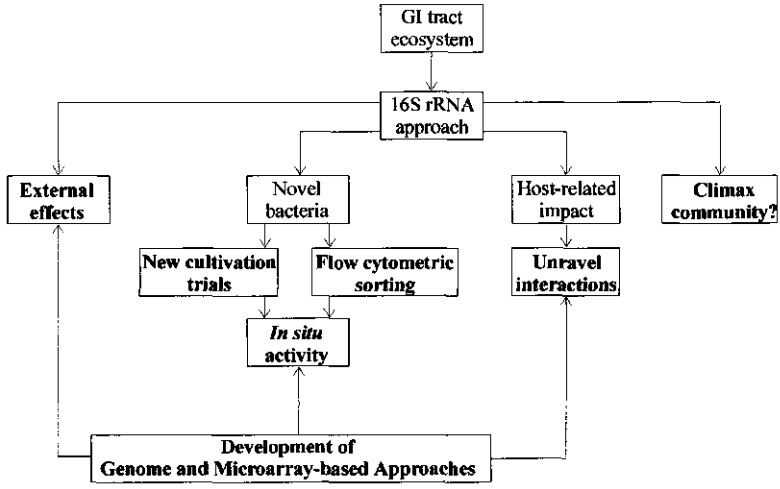

Fig. 1. Schematic overview of the findings obtained during this study and the possibilities for future research (bold) in the GI tract ecology.

FUTURE PERSPECTIVES

The current study demonstrated that molecular approaches based on the 16S rRNA sequence

variability gave novel insights into the bacterial diversity in the GI tract. These approaches could be instrumental for studying the development of a host-specific bacterial community until a climax host-specific community is reached (Figure 1). In addition, these approaches could be used to study effects of external factors such as prebiotics, probiotics, antibiotics, food additives, and GI tract disorders.

Several studies indicated already the power of combining flow cytometry and 16S rDNA analysis. Wallner and colleagues (30) showed that specific groups of bacteria could be sorted from several environmental samples and afterwards the sorted fractions could be used for further molecular analysis. Such flow cytometric approaches will be very instrumental to analyze uncultured bacteria from the human GI tract.

An explosive increase in the availability of various genome sequences in DNA databases and the development of microarrays and related technological developments occurred during the past years. These developments are promising for studying the role of the bacterial community, the host, and external effects on the GI tract ecosystem (Figure 1). It should be possible to correlate changes in bacterial gene expression and in host gene expression using the sequence information of genomes and microarray technology and relate the observations to the physical state of the GI tract or the role of food, probiotics, and other external effects. Hooper and colleagues used a microarray approach to study the transcriptional responses of the host cells on colonization of *Bacteroides thetaiotaomicron* (11). In addition, they suggested to use the collective genomes of the microbes in the GI tract, a so-called microbiome, for future studies (12). Such studies may help in unraveling the role of the bacteria in the human GI tract and expend our knowledge about the ecology of the human GI tract.

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Chapter 11

Summary

The human gastrointestinal (GI) tract is a complex ecosystem in which host and microbial cells live in close contact with each other. The microbial community in the human GI tract has an important nutritional and protective function (reviewed by 8). After birth, the germ-free GI tract and is being colonized by the first invading microbes and this community is successively changing in time, ultimately resulting in a stable climax community. Although the details of obtaining and sustaining a complex microbial community in the GI tract are unknown, recent studies indicate that interactions on molecular level between microbes and host cells are important in this ecosystem (1-3). One of the major problems that hampers studying the bacterial composition in the GI tract is the inability to obtain the predominant bacteria in culture. Estimates of culturability vary between 10% and 50% (4, 6, 7, 9). One way to overcome these culturability problems is the use of culture-independent approaches to detect, monitor, and quantify bacteria in the human GI tract. Approaches using the sequence variability of the 16S ribosomal (r)RNA gene have become widely applied, since the 16S rRNA gene is an ideal phylogenetic marker consisting of variable and conserved regions. In the middle of the year 2001 there were up to 20,000 nucleotide sequences of different 16S rRNA genes available in several databases, which is far more than for any other gene (5).

In the present study, the bacterial communities in the human GI tract were investigated using a culture-independent approach based on the sequence variability of bacterial 16S rRNA genes. Our main questions to be answered were related to the composition of the bacterial community in the GI tract, its stability in time, and the effect of the host on the GI tract community (**Chapter 1**).

Based on estimates of culturability we expected a majority of uncultured bacteria to be detected in the human GI tract. Therefore, our first approach was based on direct DNA isolation from feces followed by PCR of 16S rDNA. Temperature and denaturing gradient gel electrophoresis (TGGE and DGGE, respectively) combined with cloning and sequence analysis of amplified 16S rDNA was performed to analyze and monitor the bacterial diversity in human GI tract samples. The application of such fingerprinting techniques, their benefits, and their drawbacks to describe bacterial communities in ecosystems like the human GI tract have extensively been discussed in **Chapter 2**.

Chapter 3 describes the application of TGGE and sequence analysis of 16S rRNA and rDNA amplicons to characterize and monitor the predominant bacterial community in human fecal samples. Before starting this culture-independent analysis of varying GI tract

samples from humans, we optimized and validated carefully the nucleic acid isolation from feces, the subsequent reverse transcriptase (RT-) and regular PCR amplification, and the generation of TGGE profiles from the bacterial communities. TGGE analysis of the V6 toV8 regions of fecal 16S rRNA and rDNA revealed that the bacterial communities were host-specific and stable in time. Only slight differences in band intensities were observed when DNA and rRNA derived profiles were compared, indicating that the predominant bacterial 16S rRNA genes are also predominantly expressed in the colon. Since TGGE profiles revealed to be host-specific, the bands of a fecal profile from one individual were identified by a cloning and sequencing approach. Somewhat more than half of the clones corresponded to one of the predominant amplicons in the TGGE profile and most of them appeared to originate from novel Gram-positive species which is in line with cloning-based observations (7, 9).

An improved procedure to isolate DNA from samples containing a small number of cells, such as biopsy samples from the human colon is described in **Chapter 4**. Compared to feces which may contain up to 10^{12} cells per gram the number of cells in biopsy samples is very small (approximately 10^6 cells per biopsy) and therefore the DNA recovery must be optimal and the bias introduced by the cell lysis procedure has to be minimized. The recovery of DNA during the precipitation could be improved by adding glycogen as co-precipitant and a detection limit of 10^5 cells was reached for the PCR-DGGE approach. Although the detection limit varied between the different species, this bias was relatively small compared to another DNA isolation protocol in which cells were lyzed by boiling in a Triton-X100 solution. The improved DNA isolation procedure revealed to be accurate for generating reproducible DGGE profiles from biopsy samples.

Chapter 5 describes the specific detection of *Lactobacillus* species and related lactic acid bacteria in the GI tract by using a specific primer. Lactic acid bacteria may have a beneficial effect on the human GI tract but their number hardly exceeds 1% of the total community and therefore lactic acid bacteria will not be detected in DGGE profiles reflecting the predominant bacterial community. A specific 16S rDNA primer to target lactobacilli was developed, validated, and used for DGGE analysis. This primer appeared to be specific for the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Weissella*. While also *Eubacterium biforme*-like sequences were detected, these could be excluded when a second primer was used, which did not match the 16S rDNA of *Eubacterium biforme*. The utility of the *Lactobacillus* group-specific primer was demonstrated by cloning and DGGE analysis of 16S

rDNA from feces of adults and children, although a nested PCR approach was necessary to obtain sufficient PCR product for DGGE analysis, since very stringent PCR conditions were required. In addition, the primer was successfully used to monitor the fate of a probiotic *Lactobacillus* strain in a clinical trial.

Based on DGGE analysis of 16S rDNA from fecal samples originating from different individuals it revealed that the human GI tract communities were stable in time and host-specific. The study described in **Chapter 6** was performed to estimate the impact of the host and of environmental factors on the GI tract community of human adults. DGGE profiles of fecal samples from individuals with varying degrees of genetic relatedness were performed and similarity indices of the profiles were calculated for comparisons. A significant positive relationship was found between the genetic relatedness of the hosts compared and the similarity indices of the fecal profiles. In addition, no significant difference was found between unrelated individuals and marital partners, which are sharing similar environmental conditions. These data suggest that host genotype-related factors have a major impact on the bacterial composition in the GI tract of human adults.

Although fecal samples may reflect the community structure at the end of the GI tract, they do not necessarily reflect the bacterial community at other parts in the intestine. Therefore, the bacterial composition of feces was compared to that of biopsy samples taken from the ascending, transverse, and descending colon (**Chapter 7**). For the DGGE profiles reflecting the predominant bacterial composition, remarkably high similarity indices were found when biopsy samples from the different locations within single individuals were compared, indicating that the mucosa-associated bacterial community is equally distributed along the colon, while significant differences were observed between feces and the biopsy samples. Together with the observation that these communities were host-specific, we concluded that host-related factors have a high impact on the bacterial composition. Although we observed some differences between healthy and individuals and those with a diagnosed GI tract disorder, the groups of volunteers were too small to draw a definite conclusion. Similar results were obtained when analyzing the *Lactobacillus* group-specific profiles, although it was noteworthy that a *Lactobacillus gasseri*-like bacterium was observed as the predominant lactic acid bacterium of the mucosa-associated community in most individuals.

Most of the above-described analyses are based on the isolation of DNA from feces and the amplification of 16S rDNA. Unfortunately, these data cannot be converted to cell numbers. A group of uncultured bacteria which appeared dominantly present in the TGGE

profiles of several individuals and clone libraries of two individuals (7, **Chapter 3**) are related to *Ruminococcus obeum*. **Chapter 8** describes the development, validation, and application of probe Robe63, a 16S rRNA targeted probe which could be used for fluorescent *in situ* hybridization (FISH) to quantify this uncultured *R. obeum*-like bacteria. Microscopic and flow cytometric counts revealed that this group of bacteria makes up approximately 2.5% of the total community. The predominance of this group of bacteria confirms the PCR-based observations.

Chapter 9 gives a description of a fecal isolate, strain cello^T belonging to a novel genus. Strain cello^T was obtained using a cultivation approach in which anaerobic basal liquid medium with cellobiose as main carbon source was used instead of the canonical plating on extremely rich media. The 16S rDNA sequence of strain cello^T clusters with sequences of cloned 16S rDNA from several environments, while no cultured relative was closely related. The difficulty to grow strain cello^T on agar media might be an explanation that this and related bacteria have never been detected before. We propose the name *Victivallis vadensis* to identify strain cello^T. *Victivallis vadensis* refers to the Wageningen "Food Valley".
In **Chapter 10** the main achievements of the current study is discussed.

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Chapter 12

Samenvatting

Dit hoofdstuk geeft de achtergrondinformatie en samenvatting van dit proefschrift weer.

DE DARMFLORA

Het menselijke maagdarmkanaal kan beschouwd worden als een complex ecosysteem waarbij de darmcellen en micro-organismen heel dicht bij elkaar leven. Deze micro-organismen spelen een belangrijke rol bij het omzetten van voedselcomponenten en de bescherming van de darm tegen de invasie van pathogene bacteriën. Bij de geboorte is er nog geen enkele bacterie aanwezig in de darm. De eerste bacteriën die via het maagdarmkanaal binnenkomen vestigen en vermeerderen zich in de darm. In de loop der tijd zullen er steeds meer verschillende bacteriën zich gaan vestigen, terwijl andere bacteriën verdwijnen. Dit zal uiteindelijk resulteren in een complexe, maar stabiele leefgemeenschap van verschillende bacteriën in de darm. Deze leefgemeenschap staat ook wel bekend als de "darmflora". Er wordt geschat dat het aantal bacteriecellen 10 maal meer is dan het aantal menselijke cellen, zodat het aantal bacteriën in de darm gigantisch moet zijn. Hoewel de exacte details voor de ontwikkeling en het behoud van een stabiele darmflora onbekend zijn, wordt er vermoed dat de bacteriën en darmcellen met elkaar communiceren door middel van het uitwisselen van signaalstoffen. Een van de nadelen die het bestuderen van de samenstelling van de darmflora bemoeilijkt, is het kweken van deze bacteriën in het laboratorium. Men weet niet precies wat iedere bacterie nodig heeft om te kunnen groeien. Men schat dat hierdoor maar 10 tot 50% van alle darmbacteriën in het laboratorium te kweken is. Dit betekent dat de kweekmethoden ontoerijkend zijn, wanneer men de samenstelling van de darmflora wilt onderzoeken. Om de gehele samenstelling van de darmflora te karakteriseren zijn dus kweek-onafhankelijke methodes nodig. Een van de mogelijkheden om dit te doen is door een specifieke karaktereigenschap van de bacteriën aan te tonen, en zodoende de verschillende bacteriën te identificeren.

DE BACTERIECEL

Voordat het kweek-onafhankelijk aantonen van de bacteriën wordt uitgelegd, is het nodig om wat basiskennis van de bacteriecel te verwerven. Om een idee te krijgen hoe bacteriën eruit zien, is een microscopische foto van bacteriën en een foto van bacteriën op een voedingsbodem (of plaat) in figuur 1 weergegeven.

Iedere cel, en dus ook een bacteriecel heeft DNA waarop de erfelijke eigenschappen beschreven staan in de vorm van genen. Dit DNA is een lange dubbelstrengs keten waarbij de afzonderlijke ketens opgebouwd zijn uit vier verschillende bouwstenen. Deze bouwstenen zijn de nucleotiden adenine (A), cytosine (C), guanine (G) en thymine (T). Doordat er waterstofbruggen tussen de nucleotiden gevormd worden, wordt de dubbelstrengsketen gestabiliseerd. In een dubbelstrengsketen ligt een A tegenover een T (A-T paar) en een G tegenover een C (G-C paar). Genen bestaan dus uit specifieke volgordes van deze nucleotiden. Om de genen om te zetten naar een eigenschap van de bacterie worden de genen eerst overgeschreven naar een zogenoemd boodschapper RNA (messenger RNA of mRNA). Dit mRNA wordt vervolgens door de ribosomen oftewel "eiwitfabriekjes" omgezet in een eiwit, wat een belangrijke rol speelt bij een bepaalde eigenschap van de bacterie.

Fig. 1. A. Voorbeeld van bacteriën die groeien op een agar voedingsbodem en daar zogenoemde kolonies vormen. **B.** Een microscopische foto van bacteriën.

RIBOSOMEN ALS BASIS VOOR DE DETECTIE EN IDENTIFICATIE VAN BACTERIËN

De ribosomen spelen een cruciale rol in het leven van de bacterie en zijn dus in alle bacteriën aanwezig. Een onderdeel van een ribosoom is een molecuul dat het 16S ribosomaal RNA of kortweg 16S rRNA wordt genoemd. Net als genen bestaat dit 16S rRNA ook uit een specifieke volgorde van de vier nucleotiden, waarbij geen thymine maar uracil (U) als nucleotide wordt gebruikt. In tegenstelling tot het DNA is het 16S rRNA enkelstrengs, maar stabilisatie vindt plaats doordat er nucleotideparen gevormd worden tussen specifieke regionen van het 16S rRNA. Door het bepalen van de nucleotide volgorde of sequentie van 16S rRNA is gebleken dat dit 16S rRNA van alle bacteriën regionen heeft die erg veel op

elkaar lijken (conservatieve regionen), waardoor het 16S rRNA eenvoudig te herkennen is. Daarnaast bevat het16S rRNA 9 variabele regionen (V-regionen) die bacterie-specifiek zijn en daarom gebruikt kunnen worden om de bacteriën van elkaar te onderscheiden. Aan de hand van een database is waarin wel 20.000 16S rRNA sequenties van verschillende bekende bacteriën staan, kun je dus al redelijk betrouwbaar zeggen of de bacterie die je detecteert een bekende is of niet. Op deze manier kunnen alle bacteriën van de darmflora geïdentificeerd worden zonder ze te kweken. De 16S rRNA benadering kan men vergelijken met het nemen van een vingerafdruk. Ieder mens heeft vingers die qua vorm op elkaar lijken en daarom te herkennen zijn als onderdeel van de mens, terwijl je alle mensen uit elkaar kunt houden aan de hand van de verschillen in de vingerafdrukken. Doordat er al vele vingerafdrukken in verschillende databases staan, kun je dus zien of het om een bekende gaat of niet.

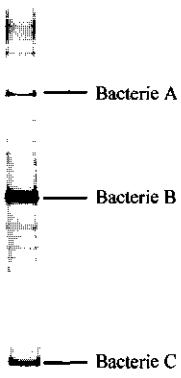

Fig. 2. Voorbeeld van een DGGE streepjescode van de darmflora. Ieder streepje geeft een specifiek 16S rRNA molecuul weer. Door de nucleotide sequentie van de afzonderlijke 16S rRNA streepjes te analyseren, kunnen de bacteriën waarvan ze afkomstig zijn geïdentificeerd worden, zoals in dit geval bacteriën A, B en C.

KARAKTERISATIE VAN DE DARMFLORA MET BEHULP VAN HET 16S rRNA

Dit proefschrift gaat over het gebruik van 16S rRNA informatie voor het karakteriseren van de menselijke darmflora. De vragen die we hiermee proberen op te lossen zijn met name gericht op de samenstelling van de darmflora van verschillende mensen, of deze samenstelling verandert in de tijd, en wat de effecten van de mens zelf zijn op deze darmflora.

Op basis van literatuurgegevens over de kweekbaarheid van de bacteriën in de darm zijn we ervan uitgegaan dat er veel onbekende bacteriën aanwezig zijn in de darmflora. Er is daarom

een analytische methode gekozen waarin in een oogopslag de verschillen en overeenkomsten tussen de darmflora van verschillende mensen zichtbaar gemaakt kan worden. Het principe van deze methode is dat eerst de verschillende 16S rRNA moleculen of het gen daarvan (het 16S rDNA) uit de bacterie geïsoleerd wordt, waarna het kunstmatig wordt vermeerderd (wordt in dit proefschrift aangeduid met PCR) en vervolgens van elkaar gescheiden wordt (aangeduid met TGGE en DGGE). Door gebruik te maken van de DGGE en TGGE technieken wordt een streepjescode gemaakt van de darmflora waarbij ieder streepje afkomstig is van het 16S rRNA of 16S rDNA van een bepaalde base sequentie en dus een specifieke bacterie voorstelt. Figuur 2 geeft een voorbeeld van DGGE streepjescodes van de darmflora van een persoon.

IEDER MENS HEEFT EEN UNIEKE DARMFLORA

Het gebruik van TGGE en DGGE technieken om de menselijke darmflora te onderzoeken is eerst geoptimaliseerd en vervolgens gebruikt om monsters uit de darm (ontlasting of feces) van verschillende mensen te onderzoeken. Uit dit onderzoek is gebleken dat deze samenstelling per individu nauwelijks verandert in de tijd, en daarnaast dat ieder volwassen mens zijn eigen unieke streepjescode heeft voor de samenstelling van de darmflora.

Omdat iedere mens een unieke en redelijk stabiele bacteriële samenstelling heeft in de darm, is vervolgens gekeken of dit gerelateerd kan worden aan de genetische achtergrond van de mens of zijn directe leefomgeving. Daarom werd feces verzameld van mensen behorende tot verschillende families, inclusief een- en tweeeiige tweelingen. Eeneiige tweelingen zijn genetisch gezien identiek terwijl tweeeiige tweelingen dat niet zijn. Bij het verzamelen van de feces werd ervoor gezorgd dat alle mensen die genetisch verwant waren met elkaar niet in hetzelfde huis leefden. Echtparen waren dus wel qua omgeving maar niet qua genetische achtergrond verwant aan elkaar. Uit de DGGE analyses is gebleken dat er geen significant effect waar te nemen is van de omgeving waarin de mensen leven, maar dat er wel een positief verband bestaat tussen een toenemende genetische verwantschap van de mensen en de overeenkomst in de samenstelling van de darmflora. Het lijkt er dus op dat de genetische achtergrond van de mens een belangrijke factor is bij de samenstelling van de darmflora. Wat precies deze factoren zijn zal uit vervolgonderzoek moeten blijken.

DE SAMENSTELLING VAN AANGEHECHTE BACTERIËN

De bacteriële samenstelling in feces geeft weer hoe de darmflora eruit ziet aan het eind van de dikke darm, maar zegt in principe niets over de samenstelling in de verschillende delen van de dikke darm. Om dit te onderzoeken werden monsters van de darmwand van de dikke darm en feces met elkaar vergeleken. De monsters van de darmwand zijn genomen via endoscopie en bevatten dus de bacteriën die aan de darmwand gehecht zijn. Uit DGGE analyse is gebleken dat bacteriële gemeenschap die aan de darmwand gehecht is overal hetzelfde is in de dikke darm, maar verschilt per individu. Dit komt overeen met wat er voor fecale monsters gevonden is en bevestigt dat gastheer-specifieke factoren belangrijk zijn voor de samenstelling van de darmflora. Ondanks dat er verschillende leefcondities voor de bacteriën zijn aan het begin en het eind van de dikke darm, bleek dit geen invloed te hebben op de samenstelling van de bacteriën die zich aan de darmwand hechten. Als echter de bacteriële samenstelling van de darmwand vergeleken wordt met die van feces dan blijkt dat er significante verschillen zijn.

DETECTIE VAN ONBEKENDE BACTERIËN

De DGGE streepjescode van de feces van een persoon is ontrafeld door de afzonderlijke streepjes te identificeren. Uit deze analyse bleek dat er veel 16S rDNA van onbekend origine aanwezig is. Dit is dus afkomstig van tot nu toe onbekende of nieuwe bacteriesoorten. Twee typen van bacteriën bleken veelvuldig voor te komen in de darmflora, namelijk bacteriën die lijken op *Fusobacterium prausnitzii* en *Ruminococcus obeum*. Deze bacteriën zijn ook in andere laboratoria aangetoond in feces van mensen en blijken dus veelvuldig voor te komen bij verschillende mensen. Helaas kun je uit de streepjescode niet berekenen van hoeveel bacteriën zo'n streepje afkomstig is. Daarom is een andere techniek gebruikt waarbij deze bacteriën specifiek geteld konden worden. Er werd een probe (kunstmatig kort stukje DNA) gemaakt welke alleen maar bindt aan het 16S rRNA van de *Ruminococcus obeum*-achtige bacteriën. Door een fluorescerend label aan deze probe te hangen zullen deze *Ruminococcus obeum*-achtige bacteriën een fluorescerend signaal geven als ze onder een fluorescente microscoop bekeken worden of met een flow cytometer (complexe machine waarmee o.a. fluorescerende bacteriën worden geteld) geanalyseerd worden. Op deze manieren van tellen bleek dat zo'n 2.5% van de totale darmflora bestaat uit bacteriën die op *Ruminococcus obeum*

lijken. Op een vergelijkbare manier is door andere onderzoekers aangetoond de totale darmflora voor 10 tot 15% bestaat uit *Fusobacterium prausnitzii*-achtige bacteriën. Deze specifieke tellingen geven aan dat de gebruikte DGGE methoden een goede weergave zijn van de menselijke darmflora.

ISOLATIE VAN EEN NIEUWE BACTERIE

Vanaf het eind van de 19de eeuw zijn er veel bacteriën uit de menselijke darm geïsoleerd en beschreven. De meest gebruikte methode om zoveel mogelijk bacteriën met verschillende eigenschappen te isoleren is door vaste agar voedingsbodems te gebruiken met zoveel mogelijk ingrediënten (zoals in figuur 1 weergegeven is). Hierop zullen de afzonderlijke bacteriën gaan groeien, wat betekent dat ze zich vermenigvuldigen. Uiteindelijk resulteert dit in de vorming van een zichtbare kolonie op de voedingsbodem. Ruwe schattingen geven weer dat je op deze manier maar 10 tot 50% van de totale darmflora kunt kweken. In dit proefschrift is een onderzoek beschreven waarin een alternatieve methode is geprobeerd waarbij geen voedingsbodem, maar een vloeibaar medium is gebruikt om de bacteriën te kweken. Daarnaast is het medium specifieker gemaakt door alleen maar cellobiose (een suiker) als hoofdbestanddeel te gebruiken. Dit heeft uiteindelijk geresulteerd in de isolatie van een nog niet eerder gekweekte soort. Verwante soorten zijn alleen maar met kweek onafhankelijke technieken aangetoond in verschillende ecosystemen (zoals de maag van koeien en bioreactoren), maar niet in humane feces. Dit nieuwe isolaat werd *Victivallis vadensis* genoemd, wat "Wageningen Food Valley" betekent. De isolatie van deze nieuwe bacterie geeft aan dat er mogelijkheden bestaan om nieuwe soorten te isoleren, wanneer alternatieve kweekmethoden bedacht en toegepast worden.

DETECTIE VAN MELKZUURBACTERIËN IN DE DARM

Melkzuurbacteriën worden veel gebruikt bij o.a. de productie van levensmiddelen, zoals bijvoorbeeld kaas. Verder zie je in de supermarkten ook steeds meer producten waar levende melkzuurbacteriën inzitten (zoals Yakult en Mona Vifit). Van deze zogenoemde probiotische producten wordt gezegd dat ze goed zijn voor de darmflora en voor de gezondheid. Uit experimenten in verschillende laboratoria is gebleken dat de betreffende melkzuurbacterie weliswaar in feces aangetoond kan worden, maar dat het nauwelijks een effect heeft op

samenstelling van de darmflora. Hierbij moet opgemerkt worden dat melkzuurbacteriën bij de mens in lage aantallen in de darmflora aanwezig zijn en daardoor niet te herkennen zijn met een DGGE analyse waarmee alle bacteriën aangetoond worden. Een probiotische melkzuurbacterie kan dus wel een effect hebben op de al bestaande melkzuurbacteriën in de darmflora. Om de samenstelling van deze melkzuurbacterie-populatie en het effect van een probiotische melkzuurbacterie te onderzoeken, is eerst het 16S rRNA van melkzuurbacteriën in de darmflora geïsoleerd en specifiek vermenigvuldigd. Vervolgens werd dit met behulp van DGGE geanalyseerd zodat de streepjescodes van de samenstelling van de melkzuurbacteriën in de darmflora bekend werd. Deze methode bleek zeer geschikt te zijn voor het analyseren van feces van volwassenen en baby's en ook voor het analyseren van darmwand monsters. Op deze manier werden zowel bekende als onbekende melkzuurbacteriën gevonden in de verschillende darmmonsters. Een bacterie met de naam *Lactobacillus gasseri* bleek een veelvoorkomende melkzuurbacterie te zijn in de monsters van de darmwand. Tijdens consumptie van de probiotische producten kon de probiotische bacterie aangetoond worden in de feces en dus een verschuiving in de darmpopulatie van melkzuurbacteriën veroorzaken, maar zodra de consumptie van het probiotische product gestopt werd verdween deze bacterie ook weer snel uit de darm. Een permanente vestiging had in dit geval dus niet plaatsgevonden. Met deze resultaten is aangetoond dat het effect van probiotische producten op de bestaande melkzuurbacterie populatie in de darm onderzocht kan worden met behulp van DGGE analyses.

.....TOT SLOT

In bovenstaande samenvatting staat beschreven wat er uit mijn onderzoek naar voren is gekomen. De toepassing van nieuwe kweek onafhankelijke technieken heeft ons nieuw inzicht gegeven in de samenstelling van de darmflora van verschillende mensen en de belangrijke invloed van de mens zelf op deze samenstelling. Vervolgonderzoek naar de rol van de darmflora zal gericht moeten worden op het ontrafelen van de functie van met name de aangetoonde onbekende bacteriën in de darm.

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Nawoord

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Erwin

Curriculum Vitae

Erwin Gerard Zoetendal werd in Drachten geboren op 22 december 1971. Na zonder bijzondere noemenswaardigheden de kleuterschool doorlopen te hebben, heeft hij zijn lagere school opleiding eerst in Drachten en later in Joure gevolgd en met succes afgerond. Daaropvolgend werd in 1990 het Atheneum diploma behaald aan het Nassau College te Heerenveen. Daarna werd het wel eens tijd om de horizon voorbij de grenzen van Friesland te gaan verkennen, en daarom is Erwin in datzelfde jaar Biologie aan de Rijksuniversiteit Groningen in Haren gaan studeren. Geboeid door de vakken Microbiologie en Ecologie, heeft hij besloten zijn afstudeervakken in de Microbiële Ecologie te kiezen. Als Microbieel Ecoloog studeerde hij af in 1996. Op 1 Mei 1996, twee maanden voor zijn afstuderen, begon Erwin als AIO bij het laboratorium voor Microbiologie aan de Wageningen Universiteit met zijn promotie onderzoek, wat gericht was op het ontrafelen van de samenstelling van bacteriën in de menselijke darm. In 1998 werd hij als "adoptie AIO" aangenomen bij het Wageningen Centre for Food Sciences (WCFS). Eind 2000 was nagenoeg al het praktisch werk afgerond en werd een aanvang genomen met schrijven, wat heeft geresulteerd in dit proefschrift. Via het WCFS zette hij vanaf oktober 2001 zijn onderzoek voort aan de Universiteit van Kuopio in Finland.

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