The mucosa-associated bacteria from the sigmoid colon of nine healthy 60 years old individuals, identified by bacterial 16S rDNA

Pettersson, Bertil; Ahrné, Siv; Wang, Mei; Jeppsson, Bengt; Uhlén, Mathias; Molin, Göran

Published in:
Preprint without journal information

2003

The mucosa-associated bacteria from the sigmoid colon of nine healthy 60 years old individuals, identified by bacterial 16S rDNA.

Running title: 16S rDNA from mucosa of sigmoid colon.

Bertil Pettersson[1], Siv Ahrné[2], Mei Wang[2], Bengt Jeppsson[3], Mathias Uhlén[1] and Göran Molin[2]*.

Department of Biotechnology, Royal Institute of Technology, Stockholm[1], Food Hygiene, Dept. Food Technology, Engineering and Nutrition, Lund University, Lund[2], and Departments of Surgery, Lund University, Malmö University Hospital, Malmö[3], Sweden.

* Correspondence to: Göran Molin, Mailing address: Food Technology, Chemical Center, P.O. Box 124, SE-221 00 Lund, Sweden. *Telephone:* +46 46 2228327. *Fax:* +46 46 2224532. *E-mail:* goran.molin@appliednutrition.lth.se

Comment

This work was run experimentally during 1998-2000 and the manuscript in its present form was finished 2002. The method of direct gene identification of bacteria in environmental samples by cloning of 16S rRNA genes was relatively new at that time, and the method as such, and the way the results were presented without a phylogenic analysis were called in question and given as the major reason for rejection by three different journals. Then, the manuscript was put aside, but in perspective of the fact that the method now is generally accepted and the work still has certain unique merits (relatively numerous and long sequences; samples in form of colonic biopsies from healthy 60 years old humans) it has been made public in LUP.
/ Göran Molin, Lund, May 2010

ABSTRACT

The bacterial flora of the gastro intestinal (GI) tract may be involved in chronic inflammation and colon cancer and affected by antibiotics, cytotoxic drugs and radiotherapy, trauma and intensive care therapy. It is important to map the mucosa-associated flora in healthy individuals to clarify the pathogenic risk under stressed conditions. The aim was to achieve an overview of the mucosa-associated bacterial flora in the sigmoid colon by direct 16S rDNA identification by sampling nine 60-years old volunteers, without clinical symptoms or medication. The bacterial flora was estimated by sequence analysis of cloned 16S rDNA as enriched by PCR from biopsies. 26% of the clones had ≥99% similarity to known species (36% had ≥98% similarity). The largest number of identified clones was related to *Escherichia coli*, *Bacteroides vulgatus* and *Ruminicoccus torques*. Most frequently distributed between the volunteers were *Bacteroides uniformis* and *Bacteroides vulgatus* (7 individuals). *Bacteroides caccae*, *Bacteroides distasonis*, *Bacteroides putredinis*, *Bacteroides thetaiotaomicron* and *Ruminicoccus torques* were found in 5 persons. Opportunistic pathogens found in more than one individual were *Bacteroides fragilis, Escherichia coli* and *Bilophila wadsworthia*. *Acinetobacter baumannii, Brachyspira aalborgi, Cardiobacterium hominis, Clostridium perfringens, Klebsiella pneumoniae* and *Veillonella parvula* were found in single individuals. A majority of the individuals had a heterogeneous flora but in one person, 91% of the clones were related to *E. coli*. The GI-flora differs between healthy individuals in respect to both composition and diversity, and it can include several opportunistic pathogens.

INTRODUCTION

The bacterial flora of the gastrointestinal (GI) tract plays an important role in human physiology, and the anatomic sites of highest bacterial concentration are also the sites most frequently affected by inflammation in patients with inflammatory bowel disease (IBD) (25) and intestinal cancer. The clinical importance of the bacterial GI-flora in IBD and ways to achieve therapeutic benefits by altering the flora is under discussion (2, 4, 5). A baseline for such discussions is the so called normal bacterial flora.

By the use of phenotypicl identification methods and anaerobic culture technique, the GI-flora has been reported to be dominated by genera such as *Bacteroides*, *Eubacterium*, *Clostridium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, *Bifidobacterium* and *Fusobacterium* (8, 22). However, culture based methods provide an incomplete assessment of the bacterial GI-flora and phenotypic features are not always reliable or sufficiently descriptive in bacterial systematics. Sixty to eighty percent of the GI-bacteria have been claimed to be uncultivable (18). A way to circumvent the need for cultivation is the use of amplification by PCR and subsequent cloning of 16S rRNA genes, followed by identification (13).

Studies of the bacterial GI-flora in humans have predominantly been performed on faeces (28, 32). Even more interesting than the faecal flora, and also less studied, is the flora associated to the intestinal mucosa. Those are the bacteria in closer contact with the body which are more likely to interact with human physiology. Therefore, the aim of the present study was to use sequence identification of cloned 16S rRNA genes, as enriched by PCR from biopsy material, to achieve an overview of the

bacterial flora of the mucosa in the sigmoid colon. Nine 60-years old volunteers without clinical symptoms participated in the study. The risk of colon cancer is increasing at this age.

MATERIAL AND METHODS

Volunteers

Samples were collected within a pilot study of 60-years old volunteers where the value of screening for malignancies by sigmoidoscopy was evaluated. The volunteers had been selected at random from the Swedish Registry, and they were without symptoms or medication. The study was approved by the Human Ethic's Committee at Lund University.

Sampling

Five to ten biopsies per person from the sigmoid colon were taken in the evening at sigmoidoscopy, 30 cm to 50 cm above the anal verge, and directly after a small enema (Bisacodyl, 10 mg; Toilax® Orion Pharma, Sollentuna, Sweden). The biopsies from 9 individuals with a normal sigmoidoscopy (6 women and 3 men), were directly transmitted to TE-buffer (TE; 10 mM Tris, 1 mM EDTA, at pH 8.0) (19). The different biopsies from the same individual were pooled into the same tube. The samples were frozen in liquid nitrogen, and stored until processing in –80^{o}C.

Isolation and purification of DNA

Biopsies (5-10 pieces) in 2 ml of TE-buffer were thawed on ice and ultrasonicated for 5 min in a beaker with TE-buffer submerged into an ultrasonication-bath (Millipore®, Sweden), followed by 2 min vortexing. The samples were centrifuged for 2 min at 1150 times gravity and the supernatants were collected into two Eppendorf tubes. The bacteria were pelleted at 14000 times gravity for 5 min, washed twice with TE-buffer and re-suspended in 0.25 ml TE-buffer. The cells were disintegrated by shaking together with 6-8 glass beads (2 mm in diameter) for 45 min at 4°C in an Eppendorf Mixer (Model 5432, Eppendorf, Hamburg, Germany). After centrifugation at 14000 times gravity for 5 min, clear supernatants were collected and the DNAs to be used as template in the subsequent amplification by PCR were isolated by using Dynabeads DNA DIRECT™ system I (DYNAL®, Oslo, Norway) as recommended by manufacturer and eluted in 20μl TE-buffer. One millilitre of TE-buffer was treated in parallel to each of the extraction procedures serving as a negative control in each of the sample preparations.

Amplification of 16S rDNA with universal primers

Amplification of the 16S rDNA was carried out with primers designed to anneal to conserved regions of bacterial 16S rRNA genes. The forward primer ENV1 (5' AGA GTT TGA TII TGG CTC AG 3') corresponded to positions 8 to 27 of *Escherichia coli* 16S rRNA, and the reverse primer ENV2 (5' CGG ITA CCT TGT TAC GAC TT 3') corresponded to the complement of positions 1511 to 1492 (3). The primers were designed with guidance from aligned sequences as available in the Ribosomal Database Project. The PCR reactions contained 0.2 μM of each primer, 5 μl of template DNA, 5 μl of 10XPCR reaction buffer (100 mM Tris-HCl, 500 mM KCl, at pH 8.3), 2.5 mM $MgCl_2$, 200 μM each deoxyribonucleotide

triphosphate and 2.5 U of Taq DNA polymerase (Roch Diagnostics GmbH, Mannheim, Germany) in a total volume of 50 μl. The reaction mixtures were overlaid with mineral oil and incubated in a "DNA thermal cycler" (Model 480, Perkin Elmer, Norwalk, USA) as follows: denaturation at 96°C for 15s, annealing at 48°C for 30s and extension at 72°C for 90s with an additional extension time of 10 min on the final cycle for a total of 30 cycles.

Cloning of 16S rDNA

The PCR products were checked individually by electrophoresis on 1.2% (w/v) agarose gels in TAE buffer. PCR products from 3 to 4 individual PCR:s were then pooled and electrophoresed on 1.2% (wt/vol.) agarose gels in TAE-buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, at pH 8.3) (19) and viewed by ethidium bromide staining. Bands with the proper size (1.5 kb) were excised and purified with a GENECLEAN® Kit (Bio 101, California, USA). The purified products were ligated into pGEM®-T Easy Vector (Promega, Madison, USA). The ligation products were transformed into *E. coli* JM109 high efficiency competent cells according to the manufacturer's protocol (Promega, Madison WI, USA). Colonies which contained plasmid with an insert were blue/white screened on LB-agar with ampicillin (100 μg/ml; Sigma, St. Louis Mo, USA), X-gal (80 μg/ml) and IPTG (0.5 mM; Bio-Rad, Hercules CA, USA). White colonies were randomly picked and stored in freezing medium at –80°C. They were then amplified by PCR using vector primers RIT28 and RIT29 (14).

Sequencing of cloned DNA and identification

The 5'– and 3'-ends of the constructs were sequenced using universal sequencing primers (the universal sequencing primer and the reverse sequencing primer; Amersham Pharmacia Biotech, Uppsala, Sweden) flanking the cloning sites. These partial sequences were searched against GenBank (National Center for Biotechnology Information, Rockville Pike, Bethesda, Md.) using the Advanced BLAST similarity search option (1) accessible from the homepage at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Multiple alignment for part of groups with uncertain identity was performed with the CLUSTAL X program (31). The partial sequences were mostly around 500 base pairs (range 400-1450). All nucleotide sequences were determined using the MegaBACE 96 capillary system.

Statistical evaluations

Significant differences between individuals was evaluated by Independent-samples T-test. This and the calculation of standard deviation were done with the programme package SPSS (SPSS Sweden AB, Sundbyberg).

RESULTS

Sequence identification was performed for 1421 clones of 16S rRNA genes enriched from the sigmoid colonic mucosa of 9 volunteers. Thirty-six percent of the sequenced clones were closely related to a known species as judged from a 16S rDNA sequence similarity of ≥98%, 26% showed ≥99% similarity (Table 1). The largest number of identified clones was related to *E. coli* (8% of the clones), *Bacteroides vulgatus* (6%) and *Ruminicoccus torques* (4%). Most frequently distributed amongst the volunteers were *Bacteroides uniformis* and *B. vulgatus* that

were found in seven individuals and *Bacteroides caccae*, *Bacteroides distasonis*, *Bacteroides putredinis*, *Bacteroides thetaiotaomicron* and *Ruminicoccus torques* that were found in five persons (Table 1). Sixty-four percent of the clones were not related to any known species. About 20 % of these clones were related to *Bacteroides*, 11% to *Clostridium*, 6% to *Verrucomicrobium* (distantly) and 4% to *Ruminococcus*. *Bacteroides* were found in all individuals while *Clostridium* and *Ruminococcus* were found in 8 individuals, and *Verrucomicrobium* in 5. About 7% of the clones showed only a low similarity to known genera (<90%) and about 14% of the clones could only be affiliated to a division or phylum (data not shown).

The opportunistic pathogens *Bacteroides fragilis* and *E. coli* were found in 3 persons, *Bilophila wadsworthia* in 2 persons and *Acinetobacter baumannii*, *Brachyspira aalborgi*, *Cardiobacterium hominis*, *Clostridium perfringens*, *Klebsiella pneumoniae* and *Veillonella parvula* in single subjects (Table 1).

The bacterial flora varied between individuals, e.g. volunteer A shared only the species *Bacteroides uniformis* with F (Table 1) and subjects A and H showed a high proportion of facultatively anaerobic species which deviated them from the other volunteers which harboured mainly strict anaerobes (Table 1). The diversity, calculated on the basis of identified phylotypes, differed between the volunteers (Table 2). Volunteer H had a significantly less diverse flora than the others and subject B harboured a significantly higher number of phylotypes than the others, even if a relatively low proportion of the phylotypes in B could be identified (Table 2).

DISCUSSION

Direct PCR-based analysis of 16S rDNA should, theoretically out of statistical reasons, reflect the composition of the dominant bacterial groups in the sample, i.e. the type of 16S rDNA that dominates should also be the one most frequently found in the clones (7, 27). However, the PCR amplification can be biased (10, 29), and this can be influenced by the primer pair that are being used (29). The primer pair for universal 16S rDNA amplification in the present study has succeeded to amplify a large spectrum of different groups of bacteria with huge phylogenetic differences between them. The accuracy of the method is also strengthened by the fact that the recorded composition of different bacterial groups differ much between the samples. For example, most of the clones of person H were related to *E. coli*. This might indicate that the PCR-amplification favoured *E. coli*, but in other samples, *E. coli* was either absent or present with only few clones. The same comparison, leading to similar results, can be made for many of the other taxa (Table 1). Hence, we believe that the frequency of clones, related to a certain taxa, to some extent reflects the distribution of this taxa in the original sample, i.e. all the cloned 16S rDNA are representing a relatively dominant part of the 16S rDNA present in the mucosa. Thus, when we find an overwhelming domination of a certain 16S rDNA sequence amongst clones, as with *E. coli* in person H, it means that the organism dominates the bacterial flora. However, the frequency might be somewhat biased for some taxa with abnormally high or low number of operons.

In the present study, partial sequences above 400 bases were evaluated. This is 100 bases fewer than that applied by Keswanit & Witeman (15) but in agreement with others (28). The relationships of the clones to described taxa in the database are given in Table 1. It has been suggested that a similarity in 16S rDNA of >97% indicates closely related species (30). However, Keswanit & Whitman (15) showed that there was only 50% chance for two 16S rDNA to belong to the same species if the similarity was >99.8. Thus, the specification of Table 1 has a certain uncertainty and the taxa, defined here, is not necessarily true species but can be called phylotypes (17) or molecular species (28). Only clones with ≥97% similarity were regarded with reasonably certainty to be related to a specific genus. For example, clones related to *Verrucomicrobium* showed a similarity <90% to this genus and can only be said to belong to the order *Verrucomicrobiales*.

Holdeman *et al* (11) followed volunteers during a five month period and comparing the results with older data, they concluded "that the variations in faecal flora reported previously, but based on the study of only one specimen from each person, more certainly reflect real differences (and not daily variation) in the types of bacteria maintained by individual people". Thus, the present variation between individual are presumably reflecting a long term difference in composition of the bacterial GI-flora.

Some of the subjects in the present study showed a mucosa flora that corresponded fairly well with the faeces flora reported by Suau *et al*. (28), i.e. individuals with many clones of *Bacteroides* spp. and *Clostridium*-groups as persons E and F while others as persons A and H differed greatly (many facultatively aerobic taxa). The number of different species inhabiting the GI-tract has been estimated to around 400-500 (33). However, only around 13 to 30 different species were identified per faecal sample by Moore and Holdeman (22), while Suau *et al*. (28) defined 82 molecular species (≥98% similarity) from 284 sequenced clones from faeces. In the present study, the number of taxa defined with ≥98% similarity to a known species varied considerably between the subjects (Table 2). It should be noted that an inclusion of unnamed phylotypes will increase the numbers in most of the subjects (Table 2). Using traditional pure culture technique and phenotypic traits, Holdeman *et al* (11) showed that *B. vulgatus, B. thetaiotaomicron, B. distasonis* made up a substantial part of the faecal flora. These species were also frequently found in the present study together with *B. uniformis*. Using both species specific 16S rDNA primers on isolates and direct PCR with extracted 16S rDNA, the same *Bacteroides* spp. together with *Bacteroides ovatus* were found to predominate in normal faecal samples (21).

Clones of the opportunistic pathogens *E. coli*, *B. fragilis* and *B. wadsworthia* were identified in several volunteers (Table 1). The prevalence of *E. coli* in some individuals is hardly surprising. It is perhaps more unexpected that *E. coli* was not found in five of the volunteers. *B. fragilis* is a well known opportunistic pathogen frequently involved in secondary infections after surgery, and the species has drawn attention for the occurrence of enterotoxicogenic strains (6, 24). *B. wadsworthia* is associated with intra-abdominal infections but has also been recovered from a wide variety of other infections (9). The spirochete *B. aalborgi* can cause intestinal spirochetosis (20), but is difficult to detect by traditional culture techniques, and might therefore, escape being sampled. Actually, only two isolates of this species

have been reported (12, 16). *B. aalborgi* and *B. aalborgi*-like organisms were found in person D (Table 1). The genetic variation between the brachyspiral clones has been phylogenetically evaluated (23).

Microbial agents appear to be involved in the pathogenesis of IBD and intestinal bacteria seem to be an important factor in their development and chronicity (2, 5, 26). In these conditions there is a complex interaction of bacteria, mucosa and immune system and this interaction is far from clarified (5). The knowledge on gastrointestinal micro-ecology will enter a new phase with the advent of comparative analysis of cloned 16S rDNA sequences for identification. However, before any implications of association between different diseased states and bacterial components of the mucosal flora can be concluded, the normal one has to be characterized. On the other hand, the present study shows that the bacterial GI-flora can vary within wide limits between individuals, both in respect of composition and diversity. The fact that a high proportion of the bacterial taxa associated to the mucosa have not yet been described also indicates that there could be unknown bacterial elements of relevance for the onset or maintenance of disease states.

ACKNOWLEDGEMENTS

Supported by the Swedish Foundation for Strategic Research. We thank Ausi Boholm and Helena Rönning for excellent technical assistance.

REFERENCES

1. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997; 25: 3389-402.
2. Ardizzone S, Bollani S, Manzionna G, Porro, GB. Inflammatory bowel disease approaching the 3rd millennium: pathogenesis and therapeutic implications. Eur J Gastroenterol Hepatol 1999; 11: 17-32.
3. Brosius J, Palmer JL, Kenneedy JP, Noller HF. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc Natl Acad Sci USA 1978; 75: 4801-5.
4. Campieri M, Gionchetti P. Probiotics in inflammatory bowel disease: New insight to pathogenesis or a possible therapeutic alternative. Gastroenterology 1999; 116: 1246-60.
5. Campieri M, Gionchetti P. Bacteria as the cause of ulcerative colitis. Gut 2001; 48: 132-5.
6. Claros MC, Claros ZC, Tang YJ, Cohen SH, Silva J, Goldstein EJ, Rodloff AC. Occurrence of *Bacteroides fragilis* enterotoxin gene-carrying strains in Germany and United States. J Clin Microbiol 2000; 38: 1996-1997.
7. Diviacco S, Norio P, Zentilin L, Menzo, S, Clementi M, Biamonti G, Riva S, Falaschi A, Giacca M. A novel procedure for quantitative polymerase chain reaction by coamplification of competitive templates. Gene 1992; 122: 313-20.

8. Finegold SM, Jousimies-Somer H. Recently described clinically important anaerobic bacteria: medical aspects. Clinical Infectious Disease 1997; 25 (Suppl 2): S88-S93.
9. Finegold SM, Sutter VL, Mathisen GE. Normal Indigenous flora, In: Hentges DJ, ed. Human intestinal microflora in health and disease. New York: Academic Press, 1983: 3-31.
10. Hansen MC, Tolker-Nielsen T, Givskov M, Molin, S. Biased 16S rDNA PCR amplification caused by interference from DNA flanking the template region. FEMS Microbiol Ecol 1998; 26: 141-9.
11. Holdeman LV, Good IJ, Moore WEC. Human fecal flora: Variation in bacterial composition within individuals and a possible effect of emotional stress. Appl Environm Microbiol 1976, 3**1**: 359-75.
12. Hovind-Hougen K, Birch-Andersen A, Henrik-Nielsen R, Orholm M, Pedersen JU, Teglbjaerg PS, Thaysen EH. Intestinal spirochetosis: morphological characterization and cultivation of the spirochete *Brachyspira aalborgi* gen. nov., sp. nov. J Clin Microbiol 1982; 16: 1127-1136.
13. Hugenholtz P, Goebel BM, Pace NR. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol 1998; 180: 4765-74.
14. Hultman T, Bergh S, Moks T, Uhlén M. Bidirectional solid phase sequencing of in vitro-amplified plasmid DNA. BioTechniques 1991; 10: 84-93.
15. Keswanit J, Whitman WB. Relationship of 16S rRNA sequence similarity to DNA hybridization in prokaryotes. Int J System Evol Microbiol 2001; 51: 667-78.
16. Kraatz W, Pettersson B, Thunberg U, Engstrand L, Fellström C. *Brachyspira aalborgi* infection diagnosed by culture and 16S ribosomal DNA sequencing using human colonic biopsy specimens. J Clin Microbiol 2000; 38: 3555-60.
17. Kroes I, Lepp PW, Relman DA. Bacterial diversity within the human subgingival crevice. PNAS 1999; 95: 14547-52.
18. Langendijk PS, Schut F, Jansen GJ, Raangs GC, Kamphuis MH, Welling GW. Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl Environ Microbiol 1995; 61: 3069-3075.
19. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: a laboratory manual. Cold Spring Harbour: Cold Spring Harbour Laboratory, 1982.
20. Mikosza ASJ, La T, Brooke J, Ward PB, Hiene RG, Guccion JG, de Boer BW, Hampson DJ. PCR amplification from fixed tissue indicates frequent involvement of *Brachyspira aalborgi* in human intestinal spirochetosis. J Clin Microbiol 1999; 37: 2093-8.
21. Miyamoto Y, Watanabe K, Tanaka R, Itoh K. Distribution analysis of six predominant *Bacteroides* species in normal human feces using 16S rDNA-targeted species-specific primers. Microb Ecol Health Dis 2002, 14: 133-6.
22. Moore WE, Holdeman, LV. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl Microbiol 1974; 27: 961-79.
23. Pettersson B, Wang M, Fellström C, Uhlén M, Molin G, Jeppsson B, Ahrné S. Phylogenetic evidence for novel and genetically different intestinal spirochetes resembling *Brachyspira aalborgi* in the mucosa of the human

colon as revealed by 16S rDNA analysis. System Appl Microbiol 2000; 23: 355-63.
24. Prindiville TP, Sheikh RA, Cohen SH, Tang YJ, Cantrell MC, Silva Jr. J. *Bacteroides fragilis* enterotoxin gene sequences in patients with inflammatory bowel disease. Emerging Infectious Disease 2000; 6: 171-174.
25. Sartor RB. Enteric microflora in IBD: pathogens or commensal? Infl Bowel Dis 1997; 3: 230-35.
26. Schutltz M, Sartor RB. Probiotics and inflammatory bowel diseases. Am J Gastroenterol 2000; 95 (Suppl): S19-21.
27. Siebert PD, Larrick JW. Competitive PCR. Nature (London) 1992; 359: 557-8.
28. Suau A, Bonnet R, Sutren M, Godon J-J, Gibson GR, Collins MD, Dore J. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol 1999; 65: 4799-807.
29. Suzuki MT, Giovannoni SJ. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 1996; 62: 625-30.
30. Stackebrandt E, Goebel B. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequnce analysis in the present species definition in bacteriology. Int J Bacteriol 1994; 44: 846-849.
31. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin, F, Higgins, DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997; 25: 4876-82.
32. Wilson KH, Blictchington RB. Human colonic biota studied by ribosomal DNA sequence analysis. Appl Environ Microbiol 1996; 62: 2273-78.
33. Zoetendal EG, Akkermans AD, de Vos WM. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl Environ Microbiol 1998; 64: 3854-3859.

Table 1. Clones related to known species, from mucosa samples of the sigmoid colon of 9 healthy adults by means of PCR-amplification of rDNA, cloning and sequencing. 36% of the studied clones were related to known species.

Identification close to species; Phylotype	*Positive volunteers (A through I), followed by number of positive clones*		*Numbers of clones* [a)]
	≥99% similarity	<99, ≥98% similarity	[n=1421]
Acinetobacter baumannii	A,4	-	4
Actinomyces naeslundii	A,1	-	1
Bacteroides acidofaciens	D,1	-	1
Bacteroides caccae	C,1; D,1; E,2; G,1	A,1; C,1; D,1; E,4	12
Bacteroides distasonis	B,1; E,1; G,1; I,1	B,2; E,5; F,3; G,4; I,1	19
Bacteroides eggerthii	I,1	-	1
Bacteroides fragilis	B,3; E,8; F,2	E,4	17
Bacteroides merdae	B,3; D,1	B,1; G,1	6
Bacteroides putredinis	B,2; C,1; D,1; E,3; F,2	F,1	10
Bacteroides splanchnicus	E,3	C,1	4
Bacteroides stercoris	C,1	C,3	4
Bacteroides thetaiotaomicron	C,2; E,1; G,3; I,1	C,2; E,4; F,1	14
Bacteroides uniformis	A,1; B,3; C,1; D,1	B,1; D,1; E,4; F,1; I,1	14
Bacteroides vulgatus	C,15; D,5; E,2; F,11; G,7; H,3; I,2	C,2; D,3; E,3; F,14; G,8; H,2; I,4	81
Bilophila wadsworthia	B,2; E,1	-	3
Brachyspira aalborgi	D,11	D,7	18
Butyrvibrio crossotus	G,1	G,1	2
Cardiobacterium hominis	A,1	-	1
Clostridium celerecrescens	-	E,1	1
Clostridium lituseburense	-	G,1	1
Clostridium perfringens	B,1	-	1
Clostridium ramosum	E,1	-	1
Clostridium spiroforme	F,1	B,1	2
Clostridium sordelli	B,1	-	1
Clostridium symbiosum	D,1	-	1
Desulfomonas pigra	I,1	-	1
Desulfovibrio fairfieldensis	-	I,1	1
Dolosigranulum pigrum	-	I,1	1
Eubacterium biforme	-	E,1	1
Eubacterium contortum	-	E,1; G,1	2
Eubacterium formicigenerans	I,1	-	1
Eubacterium limosum	B,3	B,2	5
Eubacterium ramulus	-	B,1	1
Eubacterium rectale	C,1	F,2	3
Eubacterium ventriosum	F,1	-	1
Escherichia coli [b)]	B,13; F,15; H,80	F,1; H,3	112

Fusobacterium prausnizii	C,1; D,1; E,2; G;1	B,1; C,1; E,3	10
Granulicatella elegans	B,1	A,1	2
Haemophilus paraphrophilus	G,1	-	1
Holdemania filiformis	B,1; E,1	I,1	3
Klebsiella pneumoniae	B,4	-	4
Lactobacillus gasseri/acidophilus	B,1	-	1
Lactobacillus pentosus	A,2	A,1	3
Lactobacillus plantarum	A,1	-	1
Lactobacillus reuteri	A,2	-	2
Lactobacillus ruminis	B,2	-	2
Lactobacillus salivarius	B,2	-	2
Lactococcus lactis subsp. *cremoris*	A,1	-	1
Moraxella osloensis	A,3	A,1; B,2	6
Peptostreptococcus magnus	-	H,1	1
Peptostreptococcus vaginalis	-	H,1	1
Phascolarctobacterium faecium	-	B,2; G,1	3
Propionibacterium acnes	A,11	A,6; E,1	18
Pseudomonas putida	-	I,1	1
Pseudomonas veronii	H,1	-	1
Ruminococcus bromii	-	C,1	1
Ruminococcus gnavus	I,1	-	1
Ruminococcus lactaris	C,1; D,1; F,1; I,1	-	4
Ruminococcus obeum	B,1	B,1	2
Ruminococcus torques	B,3; C,5; F,32; I,15	D,1; F,5; I,2	63
Streptococcus mitis	A,8; B,1	A,1; B,1	11
Streptococcus pneumoniae	B,1	-	1
Streptococcus salivarius	A,3; B,4	A,1; B,2	10
Staphylococcus auricularis	A,1	-	1
Staphylococcus epidermidis/caprae	A,4; D,1	-	5
Staphylococcus pasteurii/warneri	A,2	-	2
Sutterella wadsworthensis	-	B,1	1
Termitobacter aceticus	B,1	-	1
Veillonella parvula	A,1	A,3	4

[a)] Total number of identified clones per person: A, 62 clones; B, 72; C, 40 clones; D, 38 clones; E, 56 clones; F, 92 clones; G, 32 clones; H, 91 clones; I, 35 clones.

Table 2. The percentage of identified clones and some measurements reflecting the bacteriological diversity between individuals.

Volunteers	*Identified number of clones to total number of clones (%)*	*Number of identified phylotypes*	*Number of uniquely, identified phylotypes* [a)]	*Estimated total number of phylotypes* [b)]	*Diversity (H)* [c)]
Person A (n=133) [d)]	47	18	10	38	2.35
Person B (n=301)	24	28*	12*	117*	2.75
Person C (n=97)	41	12	2	29	1.92
Person D (n=102)	37	12	3	32	1.75
Person E (n=222)	25	16	3	64	2.47
Person F (n=193)	48	13	2	27	1.85
Person G (n=190)	17	11	3	65	1.81
Person H (n=99)	92¤	5	3	5	0.39¤
Person I (n=84)	43	14	7	33	1.91
Mean value [e)]	42 (7.3)	14 (2.1)	5 (1)	46 (11)	1.90 (0.2)
Median	41	13	3	33	1.90

[a)] Phylotypes found in only one individual.

[b)] Estimated total number of phylotypes = Number of identified phylotypes / the ratio of identified number of clones to total number of clones (%).

[c)] The Shannon-Weiner index: $H = -\sum p_i \ln p_i$, where p_i is the frequency of the *i*:th phylotype. Calculated on basis of identified phylotypes (unidentified phylotypes were excluded).

[d)] n = number of clones.

[e)] Standard deviation is given within parenthesis.

* The value is significant different from the others, number of identified phylotypes (p=0.007), number of uniquely identified phylotypes (p=0.035), and estimated total number of phylotypes (p=0.006).

¤ The value is significant different from the others (p=0.003).