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Identification of Dominant Bacteria in Feces and Colonic Mucosa from Healthy Spanish Adults by Culturing and by 16S rDNA Sequence Analysis

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The aim of this work was to examine by culturing the changes in the total and indicator populations of the feces of two individuals over 1 year and to identify the dominant microbial components of a single sample of feces from each donor. Populations and dominant bacteria from a sample of colonic mucosa from a further individual were also assessed. The culture results were then compared to those obtained with the same samples by 16S rDNA cloning and sequencing. High interindividual variation in representative microbial populations of the gastrointestinal tract (GIT) was revealed by both the culture and the culture-independent techniques. Species belonging to *Clostridium* clusters (XIVa, IV, and XVIII) predominated in both the fecal and the mucosal samples (except in the mucose cultured isolates), members of *Clostridium coccoides* cluster XIVa being the most numerous microorganisms. Species of γ-proteobacteria (*Escherichia coli* and *Shigella* spp.), bifidobacteria, and actinobacteria appeared in lower numbers than those of clostridia. From the mucosal cultured sample, only facultative anaerobes and bifidobacteria were recovered, suggesting destruction of the anaerobe population during processing. In accordance with this, the microbial diversity revealed by 16S rDNA sequence analysis was greater than that revealed by culturing. Despite large interindividual differences, distinct human communities may have group-associated GIT microbiota characteristics, such as the low number of *Bacteroides* seen in the subjects in this study.

KEY WORDS: human GIT; fecal microbiology; mucosal microbiology; 16S rDNA sequences; culture-independent microbiology.

In recent years, interest has grown in the complex microbial ecosystem of the human gastrointestinal tract (GIT). The microbial composition of this system and the beneficial or detrimental activities of each of its component are important to human health. The human GIT is colonized from birth by a complex and diverse collection of microbial species. The number and types of bacteria are different in each part of the GIT, ranging from very low numbers and low diversity in the stomach to a high density and diversity in the rectum (1, 2). The constituents of this microbiota are known to influence several biochemical, physiological, and immunological characteristics of their hosts, thus contributing to the overall health status (3–5). Besides type and number, the position of the microorganisms may also determine their influence. Thus, mucosa-associated bacteria are thought to have a stronger interaction with the host than luminal bacteria (6).

The pioneering culture-based work of Moore and Holdeman (7), Savage (8), Finegold *et al.* (9), and Noack-Loebel *et al.* (10) has been complemented in the last decade by studies involving a number of molecular techniques (11–17). The main conclusion of both approaches is that the prominent GIT populations are composed of strict anaerobic bacteria. Further, each individual is thought to carry a personal microbial community that is

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rather stable over time and is only transiently modified by external disturbances.

However, the literature contains discrepancies with respect to the genera and species that dominate the different areas of the GIT. These may arise through the use of different methodologies but could also be due to host-specific differences (age, sex, physiological state, genotype, etc.) (18–21) or diet-related phenomena (10, 22). At the same time, although individual differences persist, similarities are seen in the bacterial constituents of people from the same community (7, 9, 23). In some instances, correlations have been found between GIT bacterial composition and an increased risk of suffering certain diseases (5, 19). However, neither the microbial types involved in these effects nor the exact nature of their beneficial or detrimental effects is well established (5, 20, 24).

Given the complexity of the GIT microbial ecosystem and its role in human nutrition and health, some authors believe that the intestinal microbiota of many human communities should still be examined (2, 25). This paper reports the evolution in the dominant and indicator microbial populations of the feces of two healthy Spanish individuals during a 1-year period. The microbiology of the mucosa of a third individual is also reported. The predominant microbial components in the mucosal and in one single fecal sample from each donor were characterized by culture and culture-independent methods.

MATERIALS AND METHODS

Sampling and Processing of Samples

Selection of donors and sampling were carried out as recommended by the Regional Ethics Committee (Principality of Asturias, Spain). Volunteers considered themselves healthy and followed no special dietary routines. None had recently received antibiotic treatment.

Monthly fecal samples were collected in sterile containers from two individuals. These were transported to the laboratory in anaerobic jars containing Anaerocult A (Merck, VWR International, Darmstadt, Germany) as a reducing agent. Six samples from individual A (male, 46 years of age) and 10 samples from individual B (male, 60 years of age) were analyzed.

A third individual, subject C (male, 45 years of age), who underwent a routine diagnostic colonoscopy due to a family history of polyps, provided the mucosal sample. Prior to the examination, a liquid diet was provided to this subject for 2 days to minimize stool formation, and an electrolyte solution (Bohm) was administered 1 day prior to the test. This procedure renders a water enema unnecessary. The material used in the exploration included an Olympus cf-vl colonoscope (Olympus Corporation, New Hyde Park, NY, USA) and a pair of large (8-Fr) biopsy forceps with a central bayonet. Biopsy samples (∼0.5 mg) from two distinct parts of the descending colon were pooled and stored in reduced saline solution (0.9% NaCl, 0.1% peptone, 0.1% Tween 80, and 0.02% cysteine) until use.

The fecal and mucosal samples were processed within 2 hr of collection or placement in an anaerobic chamber (Mac500; Down Whitley Scientific, West Yorkshire, UK) containing an anoxic atmosphere (10% H_2, 10% CO_2, 80% N_2). Homogenized fecal samples were serially diluted in a reducing medium containing brain heart infusion (BHI) broth (Merck) supplemented with 0.5% glucose, 0.5% yeast extract (Merck), 0.25% cysteine (Merck), 10 μg L^{-1} vitamin K_1 (Merck), and 0.02 g L^{-1} hemin (Sigma Chemical Co., St. Louis, MO). A small part of the fecal homogenates from samples A5 (sample 5 from individual A) and B10 (sample 10 from individual B) was frozen at −80°C and stored for DNA isolation. Biopsy samples were thoroughly washed in saline solution and homogenized in BHI reducing medium. Dilutions were processed as per the fecal samples. The remaining homogenate was frozen at −80°C and stored for DNA isolation.

Microbial Analyses

Direct microbial counts. Total cell counts were determined by direct examination of dilutions using an Olympus phase contrast microscope (Olympus Optical Co., Hamburg, Germany) and a Pretroff-Hausser counting chamber.

Total Bacterial Counts. Total bacterial counts were determined on BHI agar plates supplemented as indicated above and incubated anaerobically at 37°C for up to 5 days. Colonies representing all morphotypes from fecal samples A5 and B10 (from the highest dilutions cultured on BHI) and from the mucosal sample of individual C were isolated by subculturing and then classified.

Clostridia. Clostridia were counted on reinforced clostridium agar (RCA) (Merck) with 20 μg ml^{-1} of polymixin B (Sigma) after incubation under anaerobic conditions at 37°C for 48 hr.

Bacteroides. The bacteroides group was enumerated on esculine bile agar (EBA; Merck) with 100 μg ml^{-1} kanamycin (Sigma) and 7.5 μg ml^{-1} vancomycin (Sigma). Incubations were performed anaerobically at 37°C for 48 hr.

Lactobacilli and Bifidobacteria. Bifidobacteria and lactobacilli were enumerated on Man, Rogosa, and Sharpe (MRS) agar (Merck) containing 0.25% cysteine. All incubations were performed anaerobically at 37°C for 72 hr.

Enterobacteria and Coliforms. Violet red bile lactose (VRBL) agar (Merck) was used to enumerate enterobacteria and coliforms (aerobic incubation for 24–48 hr at 37°C).

Enterococci. Enterococci were scored after 24–48 hr of aerobic incubation at 44°C in Slanetz and Bartley (S-B) agar (Merck), a medium containing 10 g L^{-1} triphenyltetrazolium chloride (TTC).

Staphylococci. Dilutions were plated on Baird/Parker (B-P) agar (Merck) with 50 ml L^{-1} of tellurite egg yolk solution (Merck) and aerobically incubated for 24 hr at 37°C. Black colonies with or without egg yolk clearing were recorded.

Yeasts and Molds. Dilutions of the samples were plated on yeast extract/ chloramphenicol/glucose (YCG) agar (Merck) and incubated aerobically for 3–5 days at 25°C.

Classification of Isolates by Sequencing of Partially Amplified rDNA

Representative colonies from the BHI agar plates used for total counts were dispersed in TE buffer (10 mol L^{-1} Tris–HCl, 1 mol L^{-1} EDTA, pH 8.0), washed, and subjected to a temperature of

98°C for 10 min. After centrifugation at 14,000 rpm for 10 min, cell extracts were stored at −20°C until use. PCR primers Y1 (5′-TGG CTC AGG ACG AAC GCT GGC GGC-3′) (positions 20–43 on 16S rDNA; *Escherichia coli* numbering) and Y2 (5′-CCT ACT GCT GCC TCC CGT AGG AGT-3′) (positions 361–338) (26), based on the prokaryotic conserved regions embracing the V1 and V2 regions of the 16S rDNA gene, were used to amplify a 348-bp segment of DNA. Cell extracts were used as a source of the template. Amplification was performed in a PCR Sprint thermocycler (Hybaid; Thermoelectron Co., Waltman, MA, USA). The amplification conditions were 95°C for 5 min, 30 cycles at 95°C for 45 sec, 58°C for 1 min, and 72°C for 45 sec, and a final extension step at 72°C for 10 min. Amplicons were purified using Microcon PCR filters (Millipore, Bedford, MA) to remove unincorporated primers and nucleotides, then sequenced by cycle extension in an ABI 370 DNA sequencer (Applied Biosystems, Foster City, CA, USA) using Y2 as a primer. Sequences were finally compared to those in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) and those of the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) using the BLAST program (27). Sequences with a percentage identity of 97% or higher were considered to represent the same species (28).

DNA Isolation, PCR, and Cloning of 16S rDNA Amplicons

Fecal and biopsy homogenates were pelleted and diluted 1:10 in PBS (100 mol L^{-1} phosphate buffer, 0.9% NaCl, pH 7.4). Glass beads 106 μm in diameter (Sigma) were then added. Microbial cells were detached from particles and the mucosa using a Minibead Beater (Biospec Products Inc., Bartlesville, OK, USA) at low speed (5000 rpm) for 3 min. Pelleted cells were washed with PBS, suspended in 1 ml of lysis buffer (6.7% sucrose, 50 mol L^{-1} Tris–HCl, 10 mol L^{-1} EDTA, pH 8.0), and incubated at 37°C for 1 hr with lysozyme (20 mg ml^{-1}; USB), mutanolysine (1500 U ml^{-1}), and RNase (100 μg ml^{-1}). Cell lysis was accomplished by adding 20 μl of SDS (20%, w/v, in water). Proteinase K (20 mg/ml) was added to the solution and incubated at 60°C for 1 hr. DNA was finally purified by phenol and phenol/chloroform extractions and precipitated by ethanol.

DNA from both biopsy and fecal samples was used as a template to amplify the V1 and V2 regions of the 16S rDNA with primers Y1 and Y2. To avoid preferential amplification (13, 29), the number of cycles was reduced to 10. DNA was the purified and cloned in a TA-vector (pCR 2.1; Invitrogen, Carlsbad, CA, USA) following the supplier's recommendations. Plasmid DNA from positive clones was isolated and purified using a commercial kit (GenElute Plasmid Mini-prep kit; Sigma), then sequenced. The sequences were compared as above.

Nucleotide Sequence Accession Numbers

The 16S rDNA sequences obtained in this work were deposited in the EMBL data library under accession numbers AY669225 to AY669316.

RESULTS

Microbial Analyses

In some fecal samples, the total number of cultivatable microorganisms surpassed 1×10^{10} cfu g^{-1} (Figure 1). Counts of the different microbial groups showed the major populations in the feces of both subjects to be strict anaerobes of the clostridium, bifidobacterium, and bacteroides groups (Figure 1). The relative numbers of some populations remained constant throughout the experimental period, but their absolute numbers did not. Direct counting by microscopic observation gave results approximately 1 logarithmic unit greater than those obtained by culturing (mean, $1.23 \times 10^{11} \pm 0.58$ cfu g^{-1} of feces), which may indicate that an important proportion of the microorganisms in feces is in a noncultivable state.

The most striking distinction between the microbiology of the feces of individual A and that of individual B was that, in the former, the anaerobic populations were 1 logarithmic unit larger than those of facultative anaerobes. In contrast, individual B showed a consistent high population of coliforms which sometimes surpassed the counts for strict anaerobes.

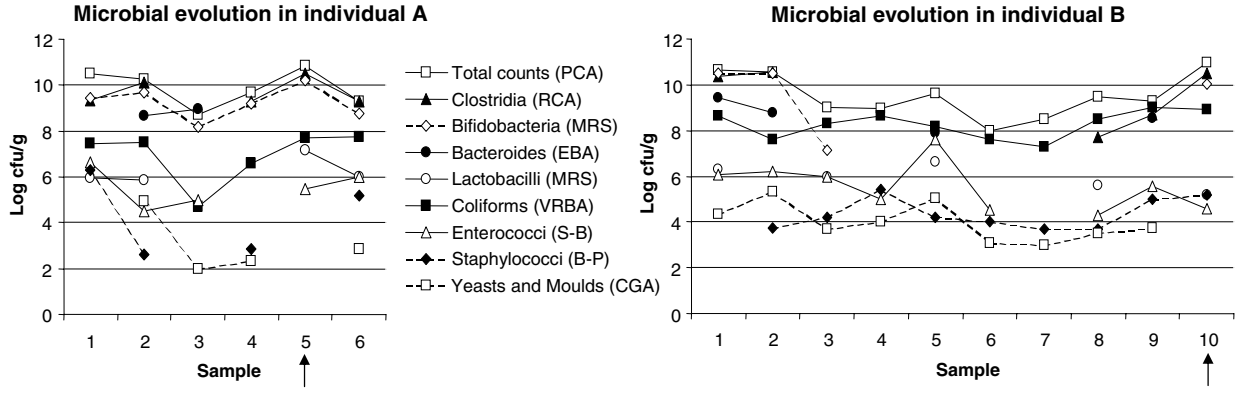

Fig 1. Composition and evolution of several microbial populations in the faeces of individuals A and B. Some populations could not be counted in several samples and, for this reason, lines appear discontinued. The media used for counting the different populations is indicated in parenthesis. Arrows indicate the two fecal samples in which the predominant microorganisms were analyzed by culturing and 16S rDNA sequence analysis.

Total microbial counts from the mucosa sample were slightly over 1×10^7 cfu g^{-1}. Coliforms were as numerous as clostridia (around 1×10^6 cfu g^{-1}). Bifidobacteria reached some 5.0×10^5 cfu g^{-1}. Neither bacteroides nor lactobacilli were recovered from this sample (their numbers must thus have been $<10^5$ cfu g^{-1}). The lactobacilli were counted by microscopic examination of the colonies, because bifidobacteria grew in the MRS agar plates under all conditions assayed. Enterococci and staphylococci appeared at around 7.5×10^3 and 3×10^3 cfu g^{-1}, respectively. The necessary preparation of the mucosa dilutions was extremely difficult and lengthy, therefore some of the most anoxic anaerobic bacteria may have entered a nonrecoverable state.

Identity of Predominant Cultured Bacteria

The identity of the dominant cultivable bacteria was examined in two specific samples of feces from each donor and in the mucosal sample of subject C. Thirty-two colonies from the cultured samples were chosen as representatives of all the morphologies encountered in the highest dilutions on BHI. Nine came from sample A5, 12 from sample B10, and 11 from the mucosal sample of individual C. The isolates were classified by amplification and sequencing of a DNA segment of their 16S rDNA gene with primers Y1 and Y2 and comparison with those in databases. Table 1 shows the results of these comparisons. The phylogenetic group to which the isolates were assigned and the highest percentages of identity to sequences from cultivated and noncultivated bacteria are also indicated.

Isolates from the feces of individual A and B differed, and both were different from those obtained from the mucosal sample. Most of the isolates from individual A (seven isolates) belonged to *Clostridium coccoides* cluster XIVa, as defined by Collins *et al.* (30). Three strains showed ~96% identity to cultivated strains; another four showed <93% identity. For all these sequences, ~98% identity with sequences from uncultured bacteria in the databases was seen (Table 1). Two strains related to the *Bifidobacterium longum/infantis* species were found in isolates from subject A; these showed 99% identity to the type strains of this species. Isolates from individual B were more diverse and included components from *Clostridium* cluster XIVa (five isolates), γ-proteobacteria (five isolates), and one isolate each from the actinomycetes and bacteroidetes groups. Except for three isolates distantly related to the *Eubacterium rectale* species (88% identity), all showed >98% identity to sequences of cultivated species.

All biotypes recovered from the mucosa were facultative anaerobes belonging to either the *Bifidobacterium, Lactobacillus, Streptococcus* (BLS) (six isolates) or the γ-proteobacterium (five isolates) groups. Several members of the Enterobacteriaceae and *Streptococcus* spp. strains showed ≥98% identity to sequences of cultivated microorganisms.

Cloned 16S rDNA Sequences

Three partial 16S rRNA gene libraries were constructed after amplification from total DNA from samples A5, B10, and C. Sixty clones were analyzed from the mucosal and from two selected fecal samples. The diversity of phylotypes and species recorded by this molecular method was higher than that obtained from cultures of all three (Table 1). Twenty-three different phylotypes were encountered (9 from the mucosal clones and 15 from the feces).

Sixteen clones came from fecal sample A5. Most of the sequences were associated with *Clostridium* clusters: 11 with *Clostridium* cluster XIVa and 2 with *Clostridium leptum* cluster IV. The dominant phylotypes in this sample were related to *Ruminococcus obeum* (six sequences), followed by *Eubacterium rectale* relatives (three sequences). Other well-known microbial inhabitants of the feces were also encountered, such as *Faecalibacterium prausnitzii* and *Eubacterium halii.* Finally, two *B. longum* sequences, plus another related to the actinobacterium *Collinsella aerofaciens*, were also seen.

Twenty-three sequences of the 24 clones analyzed from sample B10 were associated with *Clostridium* clusters. The identity between the sequences of these clones and those of cultivated bacteria varied between 88 and 100%. Sixteen sequences were related to members of cluster XIVa, four to cluster IV, and two to *Clostridium ramosum* of cluster XVIII. One sequence showed maximum identity to *Catenibacterium mitsoukai*. As already described for the cultivatable species from sample B10, *E. rectale*-related sequences (five) were dominant among the clones.

Ten different phylotypes were found in 20 clones analyzed from the mucosal sample (C). Identities to sequences from cultivated microorganisms ranged between 94 and 100%. Twelve phylotypes were again related to *Clostridium* cluster XIVa, and two more to *Clostridium* cluster IV, together accounting for 70% of the sequences. Of these, eight sequences were related to those of *Clostridium nexile.* However, since identities were <97%, they might belong to a new, noncultured species. Two β-proteobacterium and two γ-proteobacterium clones were also found, as well as one clone belonging to the bacteroides group. Only γ-proteobacteria and members of the BLS group were recovered from cultures (Table 1).

TABLE 1. PERCENTAGES OF SEQUENCE IDENTITY BETWEEN ISOLATES AND CLONES FROM THE DIFFERENT FECAL AND MUCOSAL SAMPLES AND SEQUENCES IN DATABASES.

Isolate/ Clone	Maximun similarity to a cultivated microorganism	%	Maximum similarity to a sequence from databases	%	Phylogenetic group
Strains isolated from feces (sample A5)					
SA101 (3)*	**L34421** *Eubacterium ventriosum* **L76604** *Ruminococcus torques*	92	**AB080865** Uncultured human intestinal bacterium clone JW1A1	97	*Clostridium* cluster XIVa†
SA105 (2)	**AY169422** *Clostridium clostridioforme*	96	**AB064730** Uncultured firmicute clone NB2A8	99	*Clostridium* cluster XIVa
SA106 (1)	**AJ508452** *Clostridium bolteae*	92	**AY328370** Uncultured bacterium inhufecA-44	98	*Clostridium* cluster XIVa
SA107 (2)	**AY166537** *Bifidobacterium longum*	98	**AF253392** Uncultured bacterium (human infant) N108D	99	Actinobacteria
SA1011 (1)	**L14676** *Roseburia cecicola*	96	**AB064887** Human intestinal firmicute CO13	98	*Clostridium* cluster XIVa
Clones from fecal sample A5, CA101 and CA1010					
CA101 (1)	**X85022** *Faecalibacterium prausnitzii*	96	**AF052411** Uncultured bacterium A10	99	*Clostridium* cluster IV
CA102 (2)	**AY166537** *Bifidobacterium longum*	99	—	—	Actinobacteria
CA105 (2)	**AY169419** *Ruminococcus obeum*	96	**AF052417** Uncultured bacterium A20	99	*Clostridium* cluster XIVa
CA106 (3)	**AY169428** *Eubacterium rectale*	98	**AB064884** *Eubacterium* sp. CB4	99	*Clostridium* cluster XIVa
CA1010 (1)	**AY169429** *Faecalibacterium prausnitzii*	95	**AJ408989** Uncultured bacterium clone HuCB5	99	*Clostridium* cluster IV
CA1014 (1)	**AY169411** *Ruminococcus obeum*	94	**AB064754** Uncultured *Ruminococcus* clone N044	98	*Clostridium* cluster XIVa
CA1015 (1)	**AB011816** *Collinsella aerofaciens*	97	**AJ131149** *Coriobacterium* sp. CCUG 33917	99	Actinobacteria
CA1018 (2)	**L34621** *Eubacterium halii*	98	**AJ409000** Uncultured bacterium clone HuCB26	99	*Clostridium* cluster XIVa
CA1020 (1)	**X85101** *Ruminococcus obeum*	95	**AB064895** *Ruminococcus* sp. CO7	97	*Clostridium* cluster XIVa
CA1022 (1)	**X85101** *Ruminococcus obeum*	95	**AB064752** Uncultured *Ruminococcus* sp. clone NO54	97	*Clostridium* cluster XIVa
CA1023 (1)	**AB064895** *Ruminococcus* sp. CO7 (*R. obeum*)	97	**AB064753** Uncultured *Ruminococcus* sp. clone NB2F4	97	*Clostridium* cluster XIVa
Strains isolated from feces (sample B10)					
SB28 (5)	**AE016770** *Escherichia coli*	99	—	—	γProteobacteria
SB121 (3)	**AY169428** *Eubacterium rectale*	88	**AF499841** Uncultured bacterium clone LCLC2	99	*Clostridium* cluster XIVa
SB1213 (2)	**AY169428** *Eubacterium rectale*	98	**AB064884** *Eubacterium* sp. CB4	99	*Clostridium* cluster XIVa
SB1214 (1)	**AB011816** *Collinsella aerofaciens*	98	**AJ131150** *Coriobacterium* sp. strain CCUG 33918	99	Actinobacteria
SB1216 (1)	**AB050110** *Bacteroides uniformis*	98		—	Bacteroides
Clones from fecal sample B10, CB128 (2)					
CB121 (2)	**X73441** *Clostridium spiriforme*	89	**AB099797** Uncultured clostridium clone OLDB-C1	99	*Clostridium* cluster XVIII
CB122 (1)	**L76604** *Ruminococcus torques*	94	**AF371597** Uncultured bacterium clone p-1561-b5	98	*Clostridium* cluster XIVa
CB124 (1)	**AY169428** *Eubacterium rectale*	90	**AB080865** Uncultured human intestinal bacterium clone JW1A1	99	*Clostridium* cluster XIVa
CB125 (1)	**AY341237** *Ruminococcus gnavus*	94	**AF371597** Uncultured bacterium clone p-1561-b5	98	*Clostridium* cluster XIVa
CB126 (1)	**X85099** *Ruminococcus bromii*	100	—	—	*Clostridium* cluster IV
CB127 (3)	**L34619** *Dorea formicigenerans*	96	**AF371595** Uncultured bacterium clone p-596-a5	99	*Clostridium* cluster XIVa
CB128 (2)	**AY169427** *Faecalibacterium prausnitzii* clone 1-79	99	**AF371715** Uncultured bacterium clone p-2559-9F5	100	*Clostridium* cluster IV
CB129 (1)	**AB030225** *Catenibacterium mitsuokai*	97	**AF445227** Swine fecal bacterium FPC54	98	Clostridia
CB1210 (1)	**AY169411** *Ruminococcus obeum*	99	—	—	*Clostridium* cluster XIVa
CB1211 (1)	**AY169411** *Ruminococcus obeum*	96	**AB064753** Uncultured *Ruminococcus* sp. clone NB2F4	98	*Clostridium* cluster XIVa
CB1212 (4)	**AY169428** *Eubacterium rectale*	98	**BBA270475** Butyrate-producing bacterium A1-86	99	*Clostridium* cluster XIVa
CB1215 (1)	**L34619** *Dorea formicigenerans*	94	**AB064740** Uncultured firmicute clone NO62	99	*Clostridium* cluster XIVa

TABLE 1. CONTINUED

Isolate/ Clone	Maximun similarity to a cultivated microorganism	%	Maximum similarity to a sequence from databases	%	Phylogenetic group
CB1216 (1)	**AY169415** *Clostridium nexile* **L76604** *Ruminococcus torques*	95	**AF371596** Uncultured bacterium clone p-2431-55G5	99	*Clostridium* cluster XIVa
CB1218 (2)	**AY169415** *Clostridium nexile*	99	—	—	*Clostridium* cluster XIVa
CB1221 (1)	**L34625** *Eubacterium siraeum*	99	—	—	*Clostridium* cluster IV
CB1226 (1)	**X96964** *Shigella sonnei* **AE016991** *Shigella flexneri*	97	—	—	γProteobacteria
Strains isolated from mucosa (sample C)					
SC91 (3)[1]	**M58839** *Streptococcus salivarius*	99	—	—	BLS‡
SC93 (3)	**AE016770** *Escherichia coli*	100	—	—	γProteobacteria
SC95 (2)	**X96963** *Shigella flexneri* **AY186041** *Escherichia coli*	100	—	—	γProteobacteria
SC97 (1)	**AY188352** *Streptococcus salivarius* **AF393762** *Streptococcus mitis* **AY188354** *Streptococcus thermophilus*	98	**AF253343** Uncultured bacterium (human infant) D29A	99	BLS
SC98 (2)	**AF003933** *Streptococcus parasanguinis*	98	**AB028427** Unidentified oral bacterium RP55-19	99	BLS
Clones from mucosal sample C, CC918 (2)					
CC911 (1)	**M58839** *Streptococcus salivarius*	100	—	—	BLS
CC912 (3)	**X73443** *Clostridium nexile*	96	**AB099735** Uncultured bacterium clone OLDB-F3	98	*Clostridium* cluster XIVa[2]
CC913 (1)	**AY341237** *Ruminococcus gnavus*	100	—	—	*Clostridium* cluster XIVa
CC915 (1)	**AJ420107** *Megamonas hypermegale*	96	**AB064837** Uncultured intestinal Gram-positive clone NB4B4	100	Bacteroides
CC916 (1)	**AE016767** *Escherichia coli*	99	—	—	γProteobacteria
CC917 (1)	**AE016991** *Shigella flexneri*	99	—	—	γProteobacteria
CC918 (2)	**AJ413954** *Faecalibacterium prausnitzii*	94	**AF371724** Uncultured bacterium clone p-2840-6C5	98	*Clostridium* cluster IV
CC919 (2)	**L37785** *Sutterella wadsworthensis*	95	**AB064866** Uncultured ß-proteobacterium clone NB2C10	100	βProteobacteria
CC922 (1)	**L34619** *Dorea formicigenerans*	96	**AJ408996** Uncultured bacterium clone HuCB21	99	*Clostridium* cluster XIVa
CC9110 (3)	**AY169415** *Clostridium nexile*	96	**AJ270484** Butyrate-producing bacterium A2-231	99	*Clostridium* cluster XIVa
CC9115 (1)	**Y18184** *Clostridium indolis*	95	**AB064773** Uncultured firmicute clone NS2F9	99	*Clostridium* cluster XIVa
CC9118 (2)	**AY169415** *Clostridium nexile* **L76604** *Ruminococcus torques*	95	**AF371596** Uncultured bacterium clone p-2431-55G5	99	*Clostridium* cluster XIVa
CC9117 (1)	**AY169428** *Eubacterium rectale*	98	**AB064884** *Eubacterium* sp. CB4	99	*Clostridium* cluster XIVa

*In parentheses, number of identical clones.
†Roman numerals indicate phylogenetic clusters of *Clostridium* as defined by Collins *et al.* (30).
‡BSL, group of *Bifidobacterium, Lactococcus*, and *Streptococcus.*

DISCUSSION

Some of the results obtained agree well with those found in the literature. For instance, it is well established that anaerobic populations belonging to the clostridium, bifidobacterium, and bacteroides groups seem to dominate the colonic positions of the GIT, including the mucosa and the feces (1, 2, 31). It has also been stated that the numbers of facultative anaerobic bacteria found are usually far below those of strict anaerobes. However, high interindividual variations have been reported (15, 32; Delgado and Mayo, unpublished). Since the biopsy samples were very small and their homogenization process took longer than that of the feces, they were more exposed to oxygen during sampling and this may have reduced the number of viable anaerobes they harbored.

The main microorganisms in the fecal samples in this study belonged to anaerobes of the low-G+C Gram-positive branch. In the mucosal sample, the amount of oxygen diffused from the colonic mucosa may favor the presence of facultative anaerobic microorganisms (33). However, their presence in the clones suggests that the anaerobe populations were probably destroyed during processing. Whatever the case, bacteria belonging to the Clostridia class formed the majority in most samples. Although this includes several genera (*Clostridium, Coprococcus, Eubacterium, Lachnospira*, and *Ruminococcus*), all these organisms are phylogenetically similar (30). The members of *Clostridium* cluster XIVa are reported to be major components of the human fecal flora (14, 17, 22, 32, 34). In these reports,

however, species composition varied widely between individuals.

The bacteroides populations found were somewhat lower than those reported for other human communities. In fact, they have been reported to be the predominant populations in studies using both culture-dependent and culture-independent methods (7, 9, 13, 15, 16, 22, 32, 34), although high interindividual differences in numbers and species have been noted. In the present study, it was first thought that the differences in these numbers could be attributed to the excessive selectivity of the EBA medium (Delgado and Mayo, unpublished). However, this could be discarded, as two phylotypes related to the bacteroides group were recovered, one from the cultures and another from the clones. Somehow, our human community seems to be characterized by a small bacteroides-like population (Delgado and Mayo, unpublished). Since high bacteroides populations are thought to be a risk factor for a number of diseases (19, 32), the fecal microbiota of our community may be of a rather healthy type. The genetic background of the Spanish volunteers and the widely recognized healthy Mediterranean diet (35) may account for differences with respect to other human groups.

Bifidobacterium spp. are easily recovered by culture techniques, but their sequences are not commonly detected in 16S rDNA libraries (13, 16, 29, 34, 36). However, sequences of bifidobacteria appeared in two of the three healthy men examined by Hayashi *et al.* (22), which strengthens the idea that there is high interindividual/intercommunity variation.

The use of a reduced number of cycles in PCR ensures an unbiased picture of the GIT microbiota (13). Phylogenetic analyses based on partial 16S rDNA sequences have been proven to give similar results to those for complete sequences (15, 16, 29). The diversity found in this study by the 16S rDNA analysis was higher than that revealed by culture. This has been reported by other authors (13, 16, 22, 29, 36). The bacterial diversity found in this work (as a function of the number of sequences analyzed: 92) is some 40–50% (as estimated by comparing the number of present sequences to those reported in other studies [16, 29]).

According to some authors, the majority of the fecal flora is cultivatable (7, 9). Others, however, report plate counts of total anaerobes to be 5- to 10-fold lower than total cell counts (16, 37, 38). In our work, despite the number of isolates being small, it should be emphasized that they were chosen as representative of all morphotypes on BHI. Differences between the number of cultivatable and that of noncultivatable bacteria similar to those seen in this work have been reported elsewhere (11, 15). However, these differences could be due either to the presence of bacterial groups (or species) that are not cultivatable by current culture techniques (sampling, equipment, media, etc.) or to the presence of a proportion of cultivatable bacteria being in a nonrecoverable state. Comparison of the results obtained with the culture and the molecular techniques in the present work suggests that both possibilities may account for these differences. This is further supported by the fact that the phylogenetic relationships of isolates and clones from both feces and mucosa branch at different positions (data not shown), in agreement with other published results (39). Consequently, culture-independent techniques seem to be indispensable for obtaining an unbiased picture of the microbial constituents of the GIT.

Given the high interindividual variation, a large number of isolates and clones from many individuals belonging to different human groups will need to be analyzed if the human GIT microbiota is to be defined with precision. To unravel the components of the dominant populations and their relationships with the human host is of crucial importance in order to decipher their actual effects on healthy and disease states.

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REFERENCES

1. Conway PL: Microbial ecology of the human large intestine. *In* Human Colonic Bacteria: Role in Nutrition, Physiology, and Pathology. GR Gibson, GT Macfarlane (eds). Boca Raton, FL, CRC Press, 1995, pp 1–24
2. Tannock GW: Analysis of the intestinal microflora: a renaissance. Antonie van Leeuwenhoek 76:265–278, 1999
3. Gill HS: Stimulation of the immune system. Int Dairy J 8:535–544, 1998
4. Salminen S, Bouley C, Boutron-Ruault MC, Cummings JH, Franck A, Gibson GR, Isolauri E, Moreau MC, Roberfroid M, Rowland I: Functional food science and gastrointestinal physiology and function. Br J Nutr 80:S147–S171, 1998
5. Parodi PW: The role of intestinal bacteria in the causation and prevention of cancer: modulation by diet and probiotics. Aust J Dairy Technol 54:103–121, 1999
6. Hooper LV, Gordon JI: Comensal host-bacteria relationships in the gut. Science 292:115–1118, 2001
7. Moore WEC, Holdeman LV: Special problems associated with their isolation and identification of intestinal bacteria in fecal flora studies. Am J Clin Nutr 27:1450–1455, 1974
8. Savage DC: Microbial ecology of the gastrointestinal tract. Annu Rev Microbiol 31:107–133, 1977
9. Finegold SM, Attebery HR, Sutter VL: Effect of diet on human fecal flora: comparison of Japanese and American diets. Am J Clin Nutr 27:1456–1469, 1974

10. Noack-Loebel C, Küster E, Rush V, Zimmermann K: Influence of different dietary regimen upon the composition of the human fecal flora. Prog Food Nutr Sci 7:127–131, 1983
11. McCartney AL, Wang W, Tannock GW: Molecular analysis of the composition of the bifidobacterial and lactobacilli microflora of humans. Appl Environ Microbiol 62:4608–4613, 1996
12. Wang RF, Cao WW, Cerniglia CE: PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl Environ Microbiol 62:1242–1247, 1996
13. Wilson KH, Blitchington RB: Human colonic biota studied by ribosomal DNA sequence analysis. Appl Environ Microbiol 62:2273–2278, 1996
14. Franks AH, Harmens HJM, Raangs GC, Jansen GJ, Schut F, Welling GW: Variation of bacterial populations in human feces measured by fluorescent *in situ* hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. Appl Environ Microbiol 64:3336–3345, 1998
15. Zoetendal EG, Akkermans ADL, 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 64:3854–3863, 1998
16. Suau A, Bonnet R Sutren M, Godon JJ, Gibson GR, Collins MD, Doré J: Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol 65:4799–4807, 1999
17. Sghir A, Gramet G, Suau A, Rochet V, Pochart P, Doré J: Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Appl Environ Microbiol 66:2263–2266, 2000
18. Holdeman LV, Cato EP, Moore WEC: Human fecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. Appl Environ Microbiol 32:359–375, 1976
19. Moore WE, Moore LH: Intestinal flora of populations that have a high risk of colon cancer. Appl Environ Microbiol 61:3202–3207, 1995
20. Hopkins MJ, Sharp R, Macfarlane GT: Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. Gut 48:198–205, 2001
21. Zoetendal EG, Akkermans ADL, Akkermans van-Vliet WM, de Vos WM: The host genotype affects the bacterial community in the human gastrointestinal tract. Microb Ecol Health Dis 13:129–134, 2001
22. Hayashi H, Sakamoto M, Benno Y: Fecal microbial diversity in a strict vegetarian as determined by molecular analysis and cultivation. Microbiol Immunol 46:819–831, 2002
23. Koornhof HJ, Richardson NJ, Wall MD, Moore WE: Fecal bacteria in South African rural blacks and other population groups. Isr J Med Sci 15:335–340, 1976
24. Mackie RI, Sgir A, Gaskins HR: Developmental microbial ecology of the neonatal gastrointestinal tract. Am J Clin Nutr 69:S1035–S1045, 1999
25. Salminen S, Ouwehand AC, Isolauri E: Clinical applications of probiotic bacteria. Int Dairy J 8:563–572, 1998
26. Young JPW, Downer HL, Eardly BD: Phylogeny of the prototrophic *Rhizobium* strain BTAil by polymerase chain reaction-based sequencing of a 16S rRNA segment. J Bacteriol 173:2271–2277, 1991
27. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: Gapped BLAST and PSI-BLAST: a new generation of protein databases search programs. Nucleic Acids Res 25:3389–3402, 1997
28. Stackebrandt E, Goebel BM: Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44:846–849, 1994
29. Wang X, Heazlewood SP, Krause DO, Florin THJ: Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. J Appl Microbiol 95:508–520, 2003
30. Collins MD, Lawson PA, Willems A, Córdoba JJ, Fernández-Garaizábal J, García P, Cai J, Hippe H, Farrow JA: The phylogeny of the genus *Clostridium:* proposal of five new genera and eleven new species combinations. Int J Syst Bacteriol 44:812–826, 1994
31. Mitsuoka T: The human gastrointestinal tract. *In* The Lactic Acid Bacteria, Vol. 1. The Lactic Acid Bacteria in Health and Disease. BJB Wood (ed). London, Elsevier Applied Science, 1992, pp 69–114
32. Hayashi H, Sakamoto M, Benno Y: Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. Microbiol Immunol 46:535–548, 2002
33. Hill MJ: The normal gut bacterial flora. *In* Role of the Gut Bacteria in Human Toxicology and Pharmacology. MJ Hill (ed). London, Taylor Francis, 1995
34. Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, Oyaizu H, Tanaka R: Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Envion Microbiol 68:5445–5451, 2002
35. Trichopoulou A, Lagiou P: Healthy traditional Mediterranean diet: an expression of culture, history, and lifestyle. Nutr Rev 11:383–389, 1997
36. Hold GL, Pryde SE, Russell VJ, Furrie E, Flint HJ: Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. FEMS Microbiol Ecol 39:33–39, 2002
37. Langendijk PS, Schut F, Jansen GJ, Raangs GC, Kamphuis GR, Wilkinson MHF, 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 61:3069–3075, 1995
38. Harmsen HJ, Gibson GR, Elfferich P, Raangs GC, Wideboer-Veloo AC, Argaiz A, Roberfroid MB, Welling GW: Comparison of viable cell counts and fluorescence in situ hybridization using specific rRNA-based probes for the quantification of human fecal bacteria. FEMS Microbiol Lett 183:125–129, 2000
39. Zoetendal EG, von Wright A, Vilpponen-Salmela T, Ben-Amour K, Akkermans ADL, de Vos WM: Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl Environ Microbiol 68:3401–4407, 2002