ELSEVIER

FEMS Microbiology Ecology 54 (2005) 219–231

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes

Mei Wang [a], Siv Ahrné [a,*], Bengt Jeppsson [b], Göran Molin [a]

[a] *Laboratory of Food Hygiene, Department of Food Technology, Engineering and Nutrition, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden*
[b] *Department of Surgery, Malmö University Hospital, S-205 02 Malmö, Sweden*

Received 19 July 2004; received in revised form 20 January 2005; accepted 30 March 2005

First published online 26 April 2005

Abstract

Bacterial diversity of the mucosal biopsies from human jejunum, distal ileum, ascending colon and rectum were compared by analysis of PCR-amplified 16S rDNA clone libraries. A total of 347 clones from the mucosal biopsies were partially sequenced and assigned to six phylogenetic phyla of the domain *Bacteria*: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia*, and *Actinobacteria*. The jejunum sample had least microbial diversity compared to the other samples and a trend towards highest diversity in ascending colon was observed. The clone libraries of distal ileum, ascending colon and rectum were not significantly different from each other ($P > 0.0043$), but they differed significantly from the jejunum library ($P = 0.001$). The population of sequences retrieved from jejunal biopsies was dominated by sequences closely related to *Streptococcus* (67%), while the population of sequences derived from distal ileum, ascending colon and rectum were dominated by sequences affiliated with *Bacteroidetes* (27–49%), and *Clostridium* clusters XIVa (20–34%) and IV (7–13%). The results indicate that the microbial community in jejunum is different from those in distal ileum, ascending colon and rectum, and that the major phylogenetic groups are similar from distal ileum to rectum.

Keywords: Bacterial diversity; 16S rDNA cloning and sequencing; Human mucosal biopsy

1. Introduction

The microbiota of the human gastrointestinal tract is a complex microbial ecosystem and the bacterial composition in this ecosystem differs along the length of the intestinal tract. Bacterial concentrations in the stomach and the upper two-thirds of the small intestine are 10^2–10^4 bacteria ml^{-1} of gastric or intestinal content [1]. The numbers of microorganisms are controlled in these areas by the acidic pH, short transit times of content, secretion of bile and pancreatic juice. The distal part of the small intestine, the ileum, has a concentration of 10^7–10^8 bacteria ml^{-1} and usually contains bacteria similar to those found in the colon [1,2]. The human colon harbours tremendous numbers of bacteria (10^{10}–10^{11} bacteria g^{-1} intestinal contents) and obligate anaerobes outnumber facultative anaerobes by a factor of 100–1000 [1,2]. The composition and activity of intestinal microbiota play important roles in human health because of its contributions in nutrition, development of immune system and colonization resistance [3–7]. Some species of gut bacteria have been used as probiotics to provide health beneficial effects in humans [8–10]. Intestinal microbes have also been implicated in association with diseases, such as inflammatory bowel disease [11,12], colon cancer [13,14], and multiple organ failure [15]. Our knowledge of intestinal bacterial diversity is critical to understand

* Corresponding author. Tel.: +46 46 222 8327; fax: +46 46 222 9517.
E-mail address: siv.ahrne@livsteki.lth.se (S. Ahrné).

0168-6496/$22.00
doi:10.1016/j.femsec.2005.03.012

these phenomena and to evaluate the effects of dietary treatments aimed at modulating the intestinal bacterial flora.

Current knowledge of bacterial diversity is largely based on the use of classical culture-dependent techniques. It is clear that culture techniques have drawbacks. There is strong evidence that culture-based methods detect only a small fraction of bacteria present in the intestine since some bacteria are not cultivable [16]. In addition, identification and characterization of isolates by classical methods is time-consuming and sometimes lacks accuracy [17]. In recent years, molecular tools based on 16S rRNA, such as PCR cloning [18], denaturing and temperature gradient gel electrophoresis (DGGE and TGGE, respectively) [19,20] and fluorescence in situ hybridization (FISH) [16] have been successfully applied in microbial ecology and made it possible to study the composition and diversity of intestinal flora without need for cultivation. Sequencing of random PCR-amplified 16S rDNA clones has been applied to analyse intestinal microbiota and provides valuable information on bacterial diversity in the human intestinal tract [18,21,22]. However, most studies are based on the analysis of faecal samples, and few authors have attempted to compare the bacterial diversity in different parts of the human intestinal tract within the same individual [23].

To our knowledge, the present work is the first attempt, to use molecular analysis of 16S rRNA genes to examine the microbiota in the mucosa of human jejunum, and to compare the bacterial diversity in mucosal biopsies of jejunum, distal ileum, ascending colon and rectum within one healthy individual.

2. Materials and methods

2.1. Subject and sample collection

The subject of this study was a 54-year-old female with previous good health, no earlier surgery, no medication and with no physical complaints from the gastrointestinal tract. She had a normal diet and took no probiotics and antibiotics for 4 weeks prior to sampling. She underwent a routine oesophago-gastro-duodenoscopy, which proved normal.

The bowel evacuation was performed on the day before the colonoscopy by drinking 45 ml sodium phosphate (Phosphoral®, Ferring, Sweden) twice. Biopsies were taken orally from the upper part of jejunum, just distal to the ligament of Treitz, using a Watson intestinal biopsy capsule (Ferraris Development and Engineering Co, Ltd, Edmonton, London, UK). Biopsies from distal ileum, ascending colon and rectum were taken during a colonoscopy with a biopsy forceps (PCF 160 AL colonoscope and FB24 41 biopsy forceps, Olympus, GmbH, Germany). The time lapse between biopsy taking and retrieval of the specimen is between 10 and 20 s for both methods. Both the capsule and biopsy forceps were sterilised before use. The biopsy channel was flushed with sterile saline after taking each sample. Two biopsies were collected at each location and directly placed in 1.5 ml TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The samples were immediately frozen in liquid nitrogen and stored at −80 °C. The study was approved by the Human Ethics Committee of Lund University.

2.2. DNA extraction

The total DNA from biopsies was isolated and purified by QIAamp DNA Mini Kit (tissue protocol; Qiagen, Hilden, Germany) in combination with glass bead beating. Briefly, biopsies (2 pieces) frozen in TE buffer were thawed on ice, centrifuged and resuspended in 60 μl of phosphate-buffered saline (PBS, pH 7.3, OXOID, Basingstoke, UK). The samples were treated with buffer ALT, proteinase K, RNase A and buffer AL according the manufacturer's instructions. Fifteen glass beads (2 mm in diameter) were added to tube containing the sample, and the tube was shaken for 45 min at 4 °C in an Eppendorf Mixer 5432 (Eppendorf, Hamburg, Germany) to further disintegrate the cells. DNA was precipitated with ethanol, purified on a QIAamp spin column and eluted in 50 μl of AE buffer (10 mM Tris–HCl, 0.5 mM EDTA, pH 9.0). Sixty microlitres of PBS were treated in the same way in each of the extraction procedures to serve as negative control.

2.3. PCR amplification and cloning

Amplification of the 16S rRNA genes was carried out with the universal primers ENV1 (5′-AGA GTT TGA TII TGG CTC AG-3′ *Escherichia coli* numbering 8–27) and ENV2 (5′-CGG ITA CCT TGT TAC GAC TT-3′, *E. coli* numbering 1511–1492) [24]. The PCR reaction mixture contained 0.2 μM of each primer, 1–3 μl of template DNA, 5 μl of 10× PCR reaction buffer (100 mM Tris–HCl, 500 mM KCl, pH 8.3), 200 μM of each deoxyribonucleotide triphosphate, 2.5 mM $MgCl_2$ and 2.5 U of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany) in a final volume of 50 μl. PCR was performed in a DNA Thermal Cycler (Perkin–Elmer, Norwalk, USA) with the following profile: 1 cycle at 94 °C for 3 min, followed by 25 cycles of 96 °C for 15 s, 50 °C for 30 s, and 72 °C for 90 s, with an additional extension at 72 °C for 10 min. PCR products were checked on 1% (wt./vol.) agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) after ethidium bromide staining.

Six reactions were made from each sample, and the PCR products from the same sample were pooled to

minimize PCR bias [25]. The pooled PCR products were run on 1% (wt./vol.) agarose in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3). Band was excised from the gel and the DNA was purified with a GENECLEAN II Kit (Bio 101, Carlsbad, USA). The purified PCR products were ligated into pGEM-T vector system II and then transformed into *E. coli* JM109 high efficiency competent cells according to the manufacturer's instructions (Promega, Madison, USA). Colonies were blue/white screened on Luria–Bertani (LB) agar supplemented with Ampicillin (100 μg ml^{-1}; Sigma, St. Louis, USA), X-gal (100 μg ml^{-1}) and IPTG (0.5 mM; Promega, Madison, USA). White colonies were randomly picked from each sample and stored in glycerol buffer at −80 °C.

2.4. Sequencing and phylogenetic affiliation of clones

The plasmid DNA was isolated from selected clones by Nucleospin MWG-Plasmid Prep 96 (MWG-Biotech, Ebersberg, Germany). The insert DNA was sequenced by MWG-Biotech with the automated ABI 3700 sequencer (Applied Biosystems, Darmstadt, Germany). The sequencing reactions were carried out with primers S-*-Univ-0519-a-A-18 and S-*-Univ-0915-a-A-16 [26], and ABI PRISM BigDye Terminators v3.0 Cycle Sequencing Kit (Applied Biosystems).

The sequences were examined for possible chimeric artifacts using the programs CHIMERA_CHECK [27] and Bellerophon [28]. The retrieval of the same sequence from two independent libraries was considered as evidence of a nonchimeric sequence [21]. The 16S rDNA sequences (mostly around 850 base pairs) were assigned to major phylogenetic groups based on the BLAST [29] search against GenBank. The sequences from both mucosal biopsies and public databases with known phylogenetic affiliation were aligned with the CLUSTAL X program [30], and the alignment was checked and corrected manually using Bioedit Sequence Alignment Editor version 5.0.9 [31]. Phylogenetic analysis was performed with PHYLIP package (version 3.5c; distributed by J. Felsenstein, University of Washington, Seattle). The programs are available online at http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-fr.html. Distance and similarity matrices were calculated with the DNADIST program according to the Jukes-Cantor model. Phylogenetic trees were constructed using the neighbor-joining algorithm [32]. Bootstrap analysis (100 replications) was used to estimate the confidence of tree topologies and the CONSENSE program was used to generate consense trees. The TREEVIEW program was used to draw trees [33].

The clones from mucosal biopsies were initially partially sequenced and the near-full-length sequencing was performed on clones that showed <97% 16S rDNA sequence similarity to GenBank entries. The near-full-length 16S rDNA sequences and the sequences used in phylogenetic analyses have been deposited in the GenBank database with Accession Nos. AY684365–AY684431 and AY862393–AY862394. The reference strains and cloned sequences used in phylogenetic analyses were also from the GenBank.

2.5. Statistical analysis

Rarefaction analysis [34], Shannon index, the reciprocal of Simpson's index [35] and Chao-1 estimator [36] were chosen to characterize the microbial diversity of mucosal samples. Rarefaction compares observed species richness between sites, treatments, or habitats that have been unequally sampled [37]. The rarefaction curves were produced using the software program, Analytic Rarefaction 1.3, which is available online at http://www.uga.edu/~strata/software/index.html. The Shannon index (H') is a general diversity index that is positively correlated with species richness and evenness, and is more sensitive to change in abundance of rare species [35,37]. The Simpson's index (D) is a dominance measure and shows the probability that two clones chosen at random will be from the same species [37]. The index is weighted towards the abundance of the commonest species. The use of $1/D$ instead of the original formulation of Simpson index ensures that the value of the index ($1/D$) increases with increasing diversity [35]. Chao-1 can be used to estimate the total richness of the microbial community from a sample. Chao-1 is particularly useful since a valid expression for the variance exists which can be used to calculate confidence intervals (CIs) [36].

The phylotype compositions of libraries were compared using the Sorensen index, $C_s = 2j/(a + b)$, where j is the number of phylotypes found in both samples A and B, a is the number of phylotypes in sample A, and b is the number of phylotypes in sample B [35]. The significance of difference in the composition between the clone libraries was examined in pairs by using the LIBSHUFF program as described by Singleton et al. [38]. Cramér-von Mises statistic was used to calculate differences between homologous and heterologous coverage curves, and Monte Carlo resampling approach was applied to infer statistic significance. Since the LIBSHUFF does not correct experimentwise error for multiple comparisons of libraries, the Bonferroni correction was used to calculate critical p-value. The two libraries were considered to be significantly different from each other if the lower of the two P-values generated by LIBSHUFF is below or equal to the critical p-value (the critical p-value for four clone libraries is 0.0043). The coverage of clone libraries was calculated with the formula $[1 - (n/N)]$ as described by Good [39], where n is the number of phylotypes represented by one clone and N is the total number of clones. The distance matrices for LIBSHUFF

analysis were generated using the method described in section 2.4.

3. Results

The bacterial diversity of the jejunum, distal ileum, ascending colon and rectum libraries was examined by calculating diversity measures, comparing phylotype compositions between the libraries, and analysing the phylogenetic distribution of 16S rDNA clones in each library.

3.1. Diversity measures

In order to calculate the diversity measures, the 16S rDNA clones were partially sequenced and clones with ⩾98% sequence similarity were grouped into the same phylotype, as defined by Suau et al. [21]. A total of 347 clones from the mucosal biopsies were analysed, 88 from jejunum, 85 from distal ileum, 86 from ascending colon and 88 from rectum. In total, 76 phylotypes were identified, with 22, 33, 37 and 32 detected from the jejunum, the distal ileum, the ascending colon and the rectum biopsies, respectively (Table 1).

Rarefaction curves were obtained by plotting the number of phylotypes observed against the number of clones sequenced (Fig. 1). The decrease in the rate of phylotype detection shown on the curves indicated that the major part of the diversity in the libraries had been detected. Using the formula of Good [39], the coverage of jejunum, distal ileum, ascending colon and rectum libraries were 86%, 80%, 76% and 80%, respectively. The rarefaction curves also suggested that the sequence population was the least diverse in the jejunum and the most diverse in the ascending colon. This conclusion was

Table 1
Diversity indices for 16S rDNA libraries obtained from mucosal biopsies of jejunum, distal ileum, ascending colon and rectum

Community	No. of sequences	No. of phylotypes	Diversity measure		
			H'[a]	$1/D$[b]	Chao-1[c]
Jejunum	88	22	1.897	2.945	40 (26, 93)
Distal ileum	85	33	3.104	18.402	57 (40, 112)
Ascending colon	86	37	3.331	29.959	110 (57, 303)
Rectum	88	32	3.081	19.834	85 (46, 234)

[a] Shannon index.
[b] Reciprocal of Simpson index.
[c] The values in parentheses are the 95% confidence intervals.

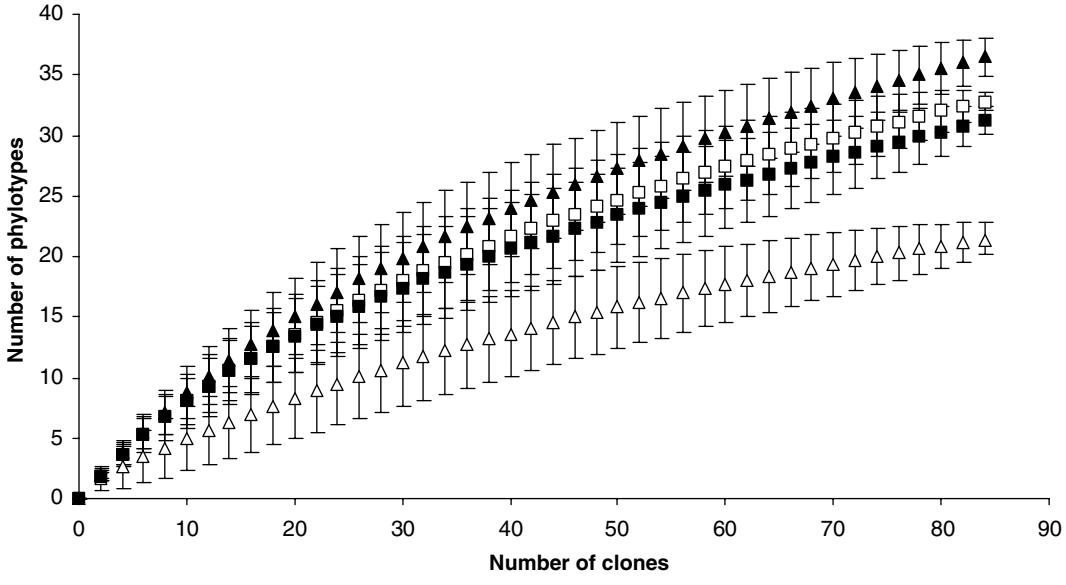

Fig. 1. Rarefaction curves generated for 16S rRNA genes in clone libraries from mucosa biopsies collected at human jejunum (△), distal ileum (□), ascending colon (▲) and rectum (■). Error bars indicated 95% confidence intervals. Clones were grouped into phylotypes at a level of sequence similarity ⩾98%. The numbers of phylotypes were significantly different ($P < 0.05$) between the library of jejunum and libraries of distal ileum, ascending colon and rectum in the range 50 < (number of analysed clones) < 84.

further supported by calculating Shannon and the reciprocal of Simpson's indices (Table 1). The Chao-1 estimator suggested a trend of increasing species richness from jejunum to distal ileum, to ascending colon, and a slight decrease in species richness was observed from ascending colon to rectum (Table 1). Because the 95% confidence intervals (CIs) for the Chao-1 estimator in each intestinal region overlap, the null hypothesis that there is no difference between the richness in the four intestinal regions cannot be rejected at significant level of 0.05.

3.2. Comparison of the microbial compositions between the clone libraries

Bacterial compositions in the clone libraries were compared by calculating the similarity indices and using the LIBSHUFF analysis. The similarities of the phylotype populations in these clone libraries ranged from 0 to 0.6078 (Table 2). The jejunum library shared only a few phylotypes with the libraries of distal ileum and ascending colon, and had no phylotypes in common with the rectum library (the similarity index for the jejunum and the distal ileum libraries was 0.0727, and for jejunum and ascending colon libraries was 0.0339). The library of distal ileum shared similar numbers of phylotypes with ascending colon and with rectum libraries. The highest similarity index (0.6078) was found between the libraries of ascending colon and rectum, although the similarity indices between distal ileum and ascending colon libraries, and between distal ileum and rectum libraries were relatively high (0.5714 and 0.5846, respectively). Paired comparisons of homologous and heterologous coverage curves between the jejunum libraries and other libraries using LIBSHUFF analysis resulted in low P values ($P = 0.001$; Table 3), indicating that the jejunum library was significantly different from the libraries of distal ileum, ascending colon and rectum. Comparisons of distal ileum, ascending colon and rectum libraries suggested that they are not significantly different ($P > 0.0043$, Table 3).

Table 2
Pairwise comparisons of phylotype compositions of 16S rDNA libraries from mucosal biopsies collected at different locations of human intestinal tract

Sampling locations	Similarity index[a] for the libraries from samples collected at the following locations		
	Distal ileum	Ascending colon	Rectum
Jejunum	0.0727	0.0339	0
Distal ileum		0.5714	0.5846
Ascending colon			0.6078

[a] Sorenson similarity index determined as follow: $C_s = 2j/(a + b)$, where a and b are the numbers of phylotypes in sample A and B, respectively, and j is the number of phylotypes found in both samples A and B.

Table 3
P value obtained by comparisons of 16S rRNA gene sequence libraries from mucosal biopsies collected at different locations of human intestinal tract

Sampling locations	P values[a] for the clone libraries from samples collected at the following locations		
	Distal ileum	Ascending colon	Rectum
Jejunum	0.001, 0.001[b]	0.001, 0.001	0.001, 0.001
Distal ileum		0.567, 0.286	0.442, 0.827
Ascending colon			0.329, 0.165

[a] Calculated by using LIBSHUFF program as described by Singleton et al. [38].

[b] Values are presented as X compared to Y, Y compared to X, where X is the library indicated in the stub and Y is the library indicated in the column head. Paired comparisons between the clone libraries indicate that the jejunum library is significantly different from the libraries of distal ileum, ascending colon and rectum ($P = 0.001$). Comparisons of distal ileum, ascending colon and rectum libraries suggest that they are not significantly different ($P > 0.0043$).

3.3. Phylogenetic affiliation of 16S rDNA sequences

All 16S rDNA sequences generated from the mucosal samples were subjected to BLAST search against GenBank. Of the 347 clones in total, 9.5% of the clones (18 phylotypes) showed less than 97% sequence similarity to their nearest database entries and may belong to hitherto unknown phylotypes [40]. Based on the BLAST results, all sequences were assigned to six phylogenetic phyla of the domain *Bacteria*: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia*, and *Actinobacteria* (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html). The majority of the recovered sequences (88%) belonged to the *Firmicutes* and *Bacteroidetes* phyla. The relative abundances of different phylogenetic groups presented in each clone library are shown in Fig. 2. Phylogenetic analysis of sequences showed that the jejunum library was dominated by sequences closely related to the *Streptococcus* genus, whereas the *Bacteroidetes*, and *Clostridium* clusters XIVa and IV of Collins et al. [41] were the predominant bacterial groups identified from the libraries of distal ileum, ascending colon and rectum. A total of 49%, 27% and 43% of the sequences retrieved from the distal ileum, ascending colon and rectum libraries, respectively, were assigned to the *Bacteroidetes* phylum. Statistical analysis using Fisher's exact test revealed that sequences affiliated with this phylum were less abundant in the ascending colon library than in the distal ileum and rectum libraries ($p = 0.003$ and $p = 0.027$, respectively), and no significant difference in relative abundance of this phylum was found between distal ileum and rectum libraries ($p = 0.448$).

3.3.1. Firmicutes

Fifty-seven percent of the sequences were affiliated to the *Firmicutes*. The majority of the sequences in this

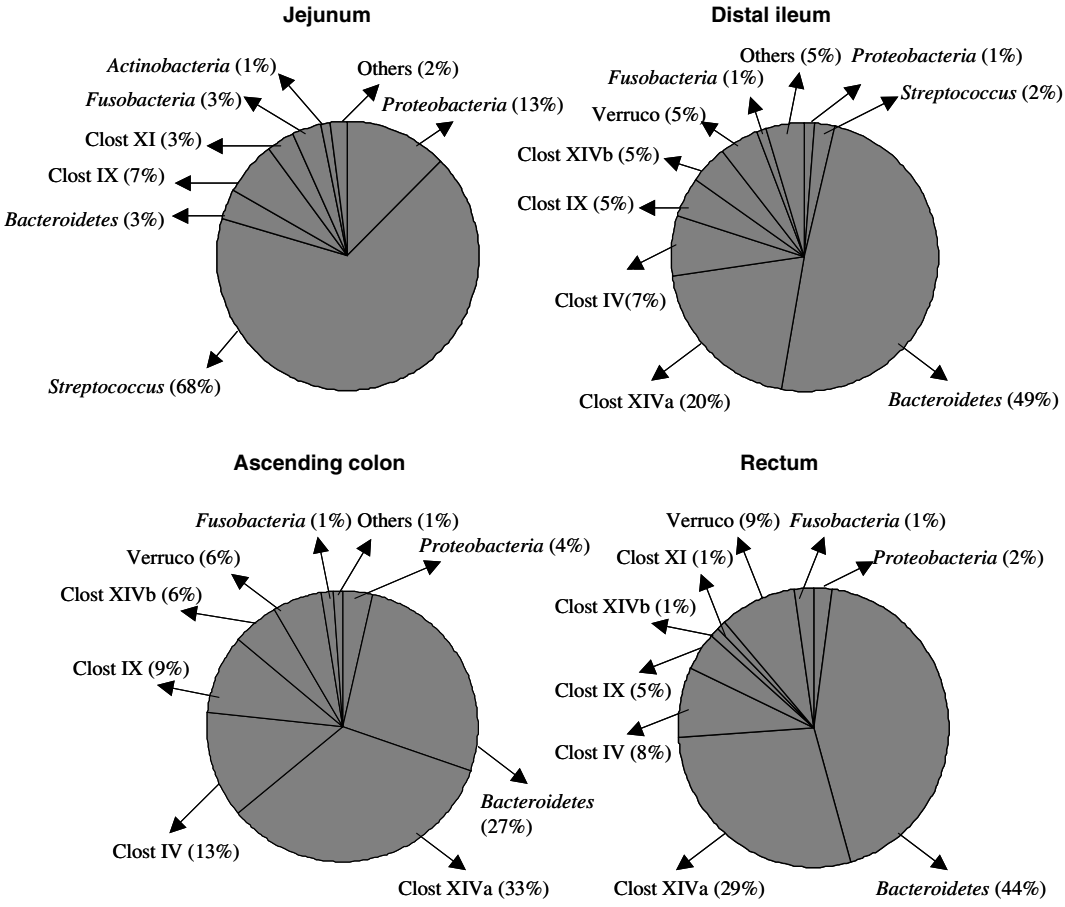

Fig. 2. Phylogenetic distribution of 16S rDNA sequences generated from mucosa biopsies of human jejunum, distal ileum, ascending colon and rectum. Verruco, *Verrucomicrobia*; Clost, *Clostridium* cluster [41]. *Streptococcus*, *Clostridium* clusters and others belong to *Firmcutes*.

phylum were closely related to genus of *Streptococcus* and *Clostridium* clusters of Collins et al. [41].

In the jejunum library, 59 sequences were related to *Streptococcus* species (⩾98% sequence similarity), including *S. mitis* (51 clones), *S. salivarius* (3 clones), *S. oralis* (2 clones), *S. parasanguis* (2 clones) and *S. anginosus* (1 clone). Two sequences derived from distal ileum were closest to *S. mitis* (99.1% sequence similarity) and *S. oralis* (98.2% sequence similarity), respectively. No *Streptococcus* clones were found in the libraries of ascending colon and rectum.

The majority of the sequences in the *Firmicutes* phylum belonged to the *Clostridium* clusters [41]. Seventy-one clones fell into *Clostridium* cluster XIVa (Fig. 3). Clones of this cluster were found in the libraries of distal ileum, ascending colon and rectum, but not in the jejunum library (Fig. 2). Sixty-three clones showed ⩾97% sequence similarity to previously cultured bacteria or reported bacterial clones from human intestine and faeces, and from the intestine of pig [19,21–23,42,43]. Twenty-four clones were assigned to *Clostridium* cluster IV (Fig. 4). Clones in this cluster were found from all sampling sites, except jejunum (Fig. 2). Three and 14 clones, respectively, were identified as *Clostridium orbiscindens* and *Faecalibacterium prausnitzii* (>99% sequence similarity). The remaining sequences in cluster IV showed <97% sequence similarity to GenBank database entries.

Many sequences were also affiliated to *Clostridium* clusters IX, XI and XIVb (Fig. 4). Twenty-two clones were grouped into cluster IX, with 14 clones being identified as *Dialister invisus* (>99% sequence similarity) and 6 clones closely related to *Veillonella* spp. (>98% sequence similarity). Four clones were assigned to cluster XI. All clones in this cluster showed > 98% sequence similarity to GenBank database entries. In cluster XIVb, 8 clones were closest to uncultured bacterium clone p-4247-4Wa3 (>97% sequence similarity) [43] and 2 clones had <94% sequence similarity to GenBank database entries.

Within the *Firmicutes*, seven clones could not be assigned to the *Streptococcus* or *Clostridium* clusters mentioned above. Three of these were identified as *Eubacterium dolichum*, *Gemella haemolysans* and *Abiotrophia para-adiacens* (>99% sequence similarities). The remaining clones were distantly related either to *Peptococcus niger* (90–91% sequence similarity) or to pig intestine clone p-5389-2wb5 (91–92% sequence similarity) [43].

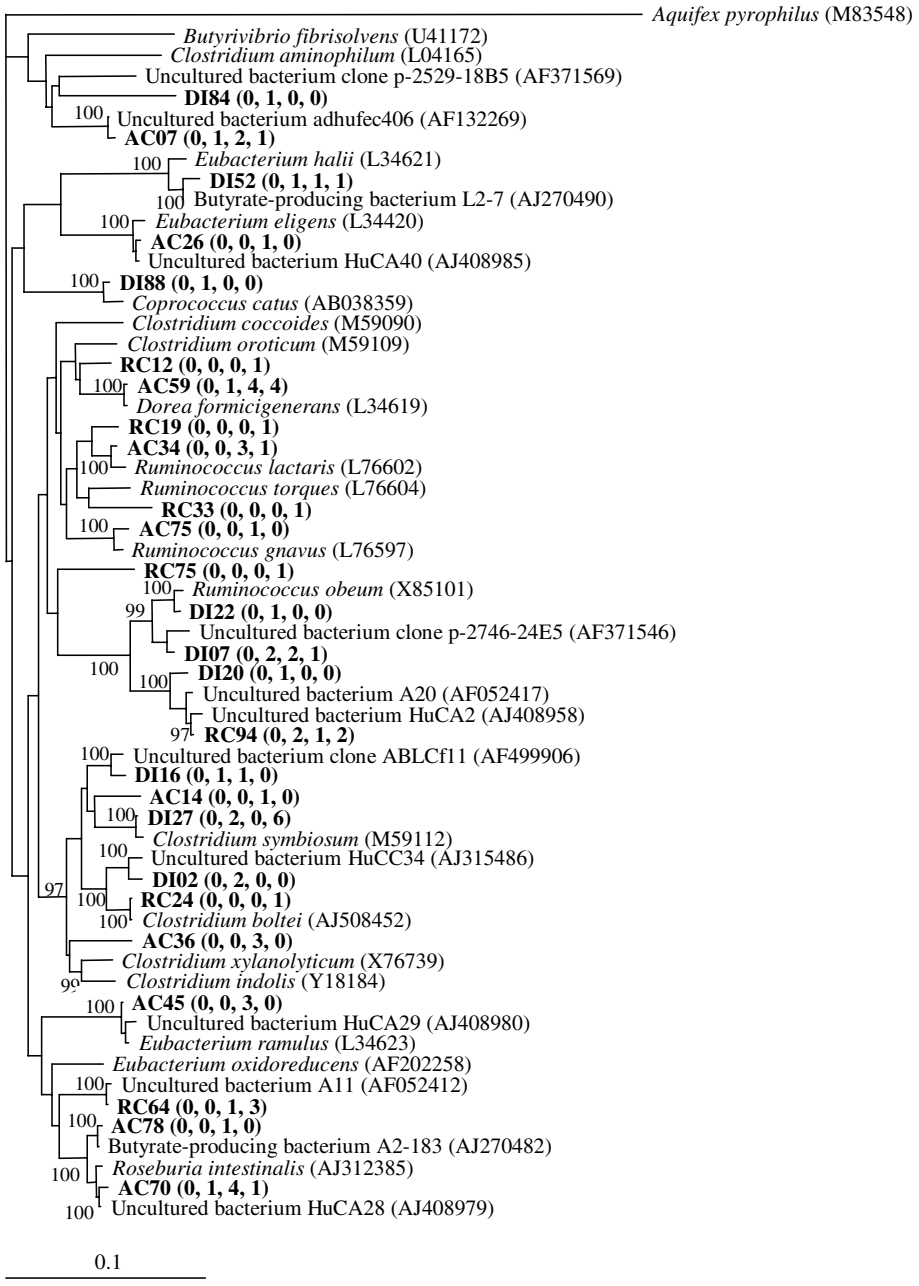

Fig. 3. Phylogenetic tree showing the positions of 16S rDNA phylotypes recovered from mucosa biopsies of human jejunum, distal ileum, ascending colon and rectum for *Clostridium* cluster XIVa of Collins et al. [41]. The tree was constructed using neighbor-joining method based on the partial 16S rDNA sequences (*E. coli* positions 30–875). The bootstrap values (expressed as percentages of 100 replications) are shown at branch points: values of 95% or higher were considered significant. The scale bar represents genetic distance (10 substitutions per 100 nucleotides). *Aquifex pyrophilus* is used as an outgroup. Sequences obtained from this study are in bold type and are prefixed on the basis of origin (JJ = jejunum community; DI = distal ileum; AC = ascending colon; and RC = rectum). The numbers in parentheses indicate the number of times the phylotype was found in each of the libraries, e.g., **AC59** (**0**, **1**, **4**, **4**) was not found in jejunum community and was present 1, 4 and 4 times in the distal ileum, ascending colon and rectum communities, respectively.

3.3.2. Bacteroidetes

One hundred and six clones (31% of the total clone population) were placed into *Bacteroidetes* (Fig. 5). Ninety-five clones from the libraries of distal ileum, ascending colon and rectum were clustered into the *Bacteroides fragilis* subgroup (RDP registration no 2.15.1.2.8). Clones within this subgroup had ⩾98% sequence similarity to GenBank database entries. Twenty-one clones were identified as *Bacteroides uniformis* (⩾99 sequence similarity) and 28 were closest to *Bacteroides thetaiotaomicron* (⩾98% sequence similarity). Three phylotypes (27 clones) were closely related to uncultured bacterium clones of human colonic 2samples such as HuCB3, HuCC30 and HuCA21 [22].

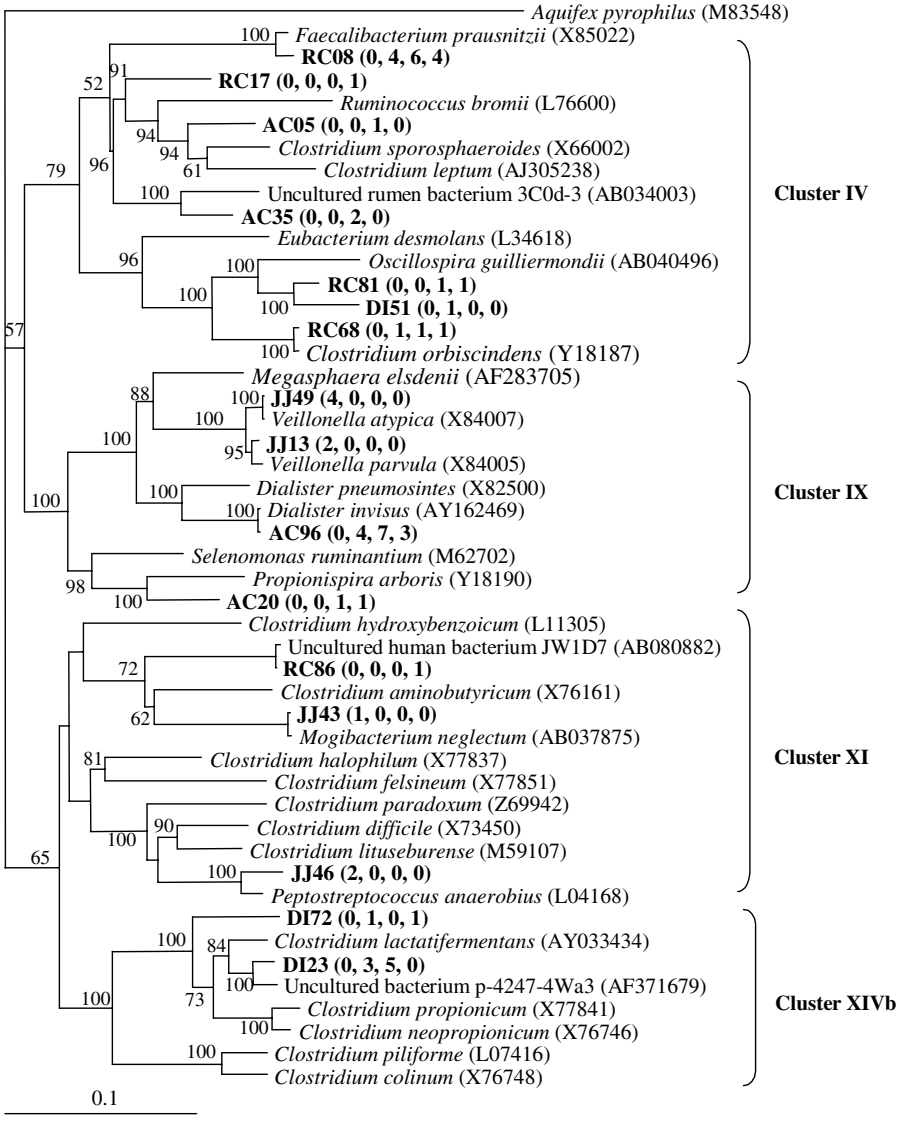

Fig. 4. Phylogenetic tree showing the positions of 16S rDNA phylotypes recovered from mucosa biopsies of human jejunum, distal ileum, ascending colon and rectum for *Clostridium* cluster IV, IX, XI and XIVb of Collins et al. [41]. The tree was constructed using neighbor-joining method based on the partial 16S rDNA sequences (*E. coli* positions 30–875). The bootstrap values (expressed as percentages of 100 replications) higher than 50% are shown at branch points. See legend of Fig. 3 for explanation.

Nineteen clones had ⩾99% sequence similarity to the uncultured bacterium adhufec367 reported by Suau et al. [21].

Of the remaining clones, one recovered from rectum had a sequence closest to *Bacteroides distasonis* (97% sequence similarity). Seven sequences were grouped together with *Rikenella microfusus* with two similar to uncultured bacterium adhufec52.25 (>99% sequence similarity) [44] and five being closest (>95% similarity) to uncultured bacterium clone HuCB 23 [22]. All three clones recovered from jejunum were closely related to the *Prevotella* genus (>97% sequence similarity).

3.3.3. Proteobacteria

Seventeen sequences (5% of the total clone population) were placed into *Proteobacteria* (Fig. 6). Within β-*proteobacteria*, three sequences were identified as *Neisseria subflava* (>99% sequence similarity) and two sequences were closely related to *Sutterella wadsworthensis* (96% and 99% sequence similarity, respectively). Twelve clones were identified in γ-*proteobacteria* group and all the clones in this group were closely related to previously cultured bacteria (>98.5% sequence similarity) including *Haemophilus parainfluenzae*, *E. coli*, *Acinetobacter johnsonii*, *Acinetobacter haemolyticus*, *Acinetobacter lwoffii* and *Pseudomonas putida*.

3.3.4. Other phyla

The remaining phyla comprised 7% of the total number of sequences recovered from mucosal biopsies. Seven sequences were assigned to *Fusobacteria* phylum

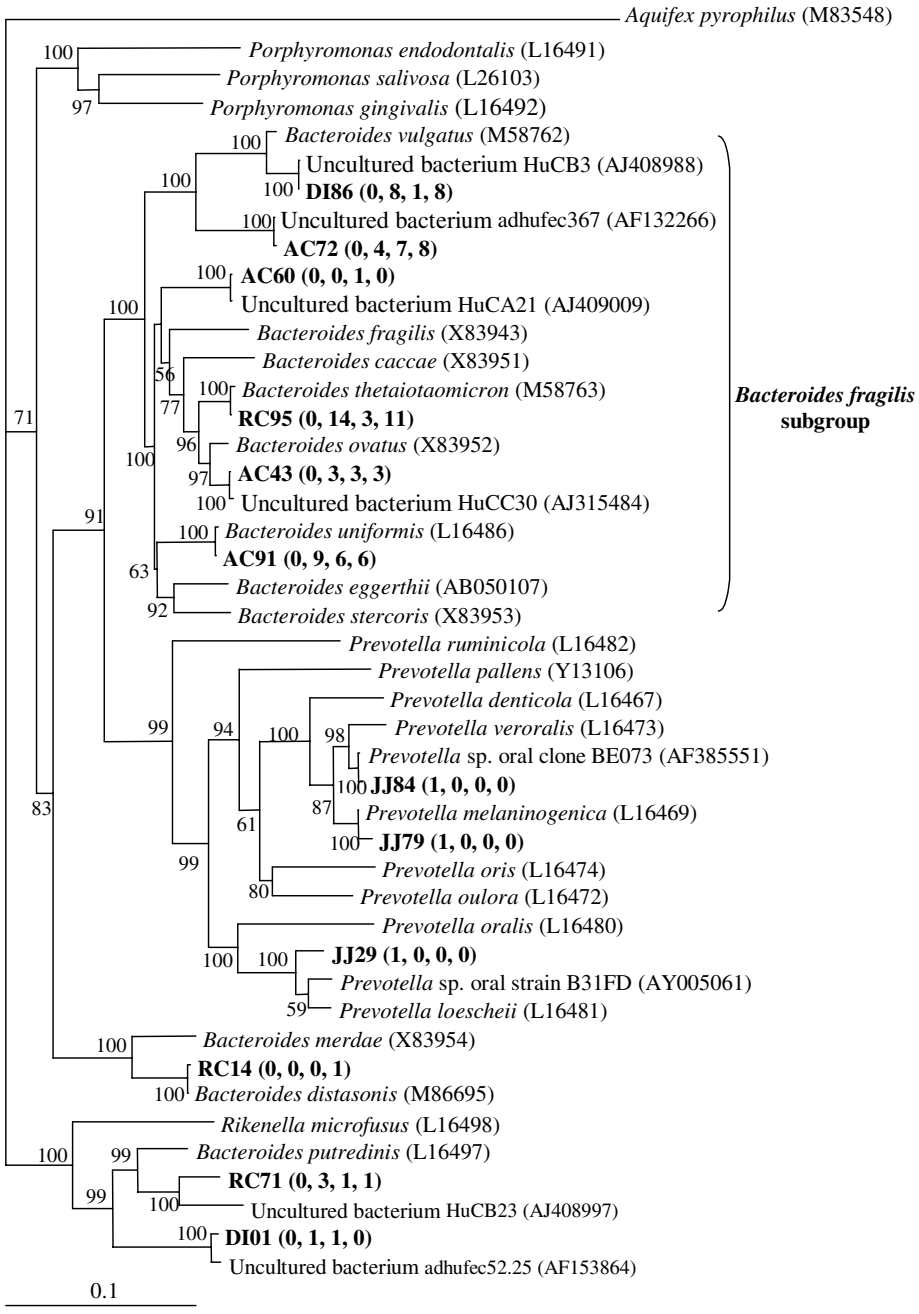

Fig. 5. Phylogenetic tree showing the positions of 16S rDNA phylotypes recovered from mucosa biopsies of human jejunum, distal ileum, ascending colon and rectum for *Bacteroidetes*. The tree was constructed using neighbor-joining method based on the partial 16S rDNA sequences (*E. coli* positions 30–875). The bootstrap values (expressed as percentages of 100 replications) higher than 50% are shown at branch points. See legend of Fig. 3 for explanation.

and 17 clones were placed into *Verrucomicrobia* phylum (Fig. 6). Clones in *Verrucomicrobia* phylum were detected from distal ileum, ascending colon and rectum, but not from jejunum (Fig. 2). Only one clone was clustered into *Actinobacteria* (Fig. 6) and this clone was identified as *Micrococcus mucilaginosus* (99% sequence similarity).

4. Discussion

In the present study, 16S rDNA sequence analysis was used to compare the bacterial diversity in the different parts of the human intestinal tract. While other 16S rRNA-based techniques, such as DGGE and FISH, have proved to be valuable tools in characterizing

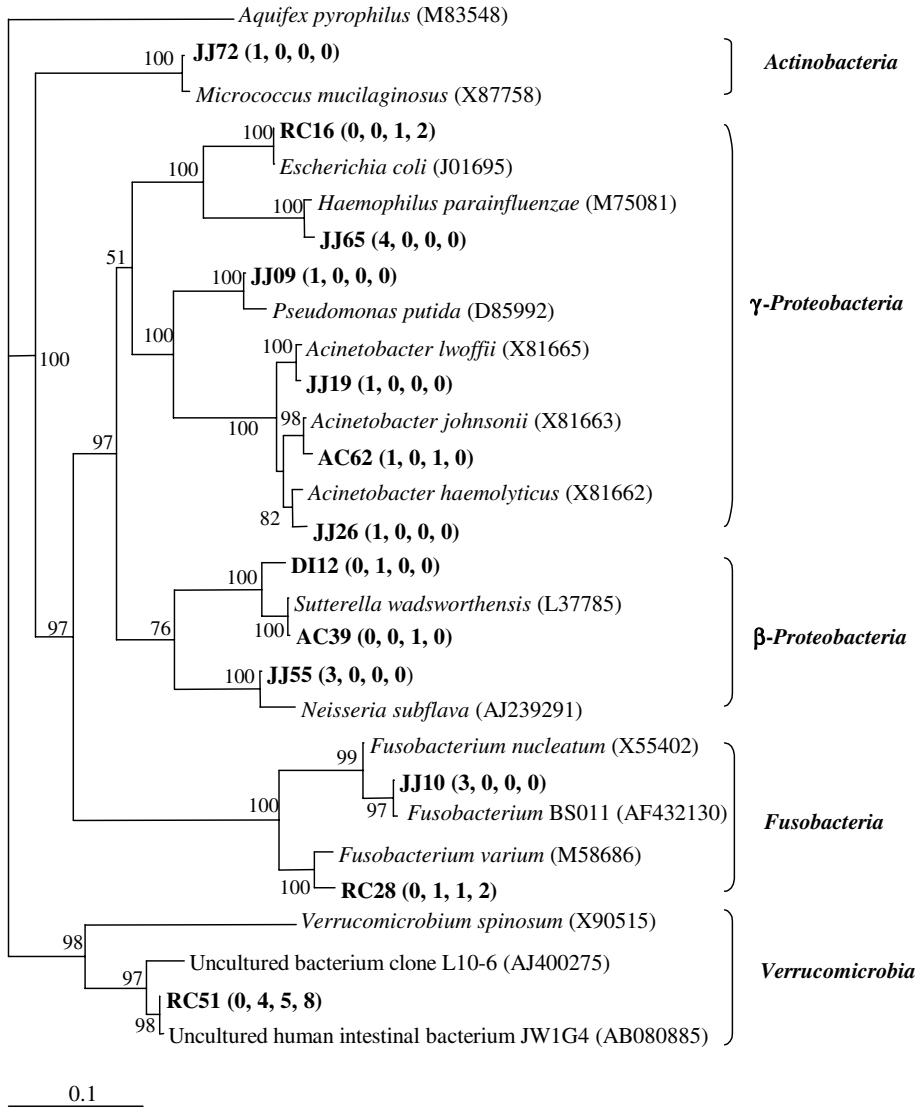

Fig. 6. Phylogenetic tree showing the positions of 16S rDNA phylotypes recovered from mucosa biopsies of human jejunum, distal ileum, ascending colon and rectum for *Acitinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia*. The tree was constructed using neighbor-joining method based on the partial 16S rDNA sequences (*E. coli* positions 32–478). The bootstrap values (expressed as percentages of 100 replications) higher than 50% are shown at branch points. See legend of Fig. 3 for explanation.

bacterial diversity in the human intestinal tract, one great advantage of sequence analysis is that the generation of sequence data can be used to design group-specific probes and primers for further studies. The data derived from jejunal and ileal mucosal biopsies in this study are particularly important since little 16S rDNA sequence information has been previously reported from these two regions. Although many efforts have been made to define the microbial diversity of the intestinal tract in recent years [21–23], the present study still revealed that 9.5% of the clones had less than 97% sequences similarity to any database entry.

One conclusive result of our study is that the bacterial community in jejunum is remarkably different from that in distal ileum, ascending colon and rectum in terms of diversity indices, phylotype composition and phylogenetic distribution of the 16S rDNA clones. The jejunum library was the least diverse (Table 1), had a few or no phylotypes in common with the other libraries (Table 2), and was dominated (67%) by sequences closely related to the genus of *Streptococcus* (Fig. 2). The libraries of distal ileum, ascending colon and rectum were more diverse (Table 1) and dominated by sequences affiliated with *Bacteroidetes* (27–49%), *Clostridium* clusters XIVa (20–34%) and IV (7–13%) (Fig. 2). It is unlikely that the differences found in this study were simply due to PCR and cloning bias, as our findings are consistent with what is know about the physiological environments that the microbes inhabit, and the evidence from previous investigations supports this. Studies using

conventional culture methods have shown that the principal microbial types in the upper two-thirds of small intestine (duodenum and jejunum) are acid-tolerant bacteria such as streptococci and lactobacilli [1]. Hold et al. [22] analysed the 16S rDNA clone libraries obtained from colonic tissue of three elderly subjects and showed that *Clostridium* clusters XIVa was the most dominant group (43–49%), followed by *Bacteroidetes* (20–35%) and *Clostridium* cluster IV (11–18%). A recent investigation performed on mucosal biopsies of a 35-year-old female revealed the most representative groups harboured in human distal ileum and colon were *Bacteroidetes* (38%) and *Clostridium* clusters XIVa (34%) [23]. The study on faecal samples of 27 healthy human adults using oligonucleotide probe hybridization indicated that *Bacteroidetes* in faecal flora could vary from 20% to 52% between individuals [45].

Several studies compared the bacterial communities in different parts of the human intestinal tract. Wang et al. [23], who used 16S rRNA gene cloning and sequencing analysis to compare the microbiota of terminal ileum, proximal colon and distal colon of one person, suggested an internally consistent proximal-to-distal gradient in which the number of operation taxonomic units (OTUs) increased and the shared OTUs were least between most distant sites. Zoetendal et al. [20] used DGGE to compare the bacterial communities of ascending, transverse and descending colon of 10 volunteers, revealing that mucosa-associated bacteria are uniformly distributed along the colon. In our study, the clone libraries of distal ileum, ascending colon and rectum were similar when diversity indices and phylotype compositions were compared, but phylogenetic analysis indicated the sequences affiliated with *Bacteroidetes* phylum were less abundant in the ascending colon library (27%) than in the libraries of distal ileum (43%) and rectum (49%). However, as we only compared the samples from a single person, the general nature of our findings needs to be tested in studies of a larger number of individuals.

PCR-based analysis can introduce different types of biases [25,46,47] and these biases may result in an over-or underestimation of the microbial diversity. Thus, microbial diversity in the 16S rRNA gene clone library does not necessarily reflect the diversity of a natural microbial system. For example, formation of chimera and 16S rRNA gene heterogeneity within one organism could lead to the diversity being overestimated. On the other hand, underestimation of diversity may result from insufficient cell lysis and differential PCR amplification caused by differences in the efficiencies of primer binding [47]. Previous studies showed that high G + C Gram positive bacteria, such as *Bifidobacterium* and *Atopobium* group, were found in significant numbers when FISH with probes targeted to these groups were used [48], but they were hardly detected in 16S rRNA gene clone libraries generated from human faecal or mucosal samples [21–23]. However, all clone libraries were created under identical conditions in our study to ensure that any biases occurred to the same degree across the samples, so statistical analysis could be used to make relative comparisons of bacterial diversity at different parts of intestinal tract. In addition, low template DNA concentrations were added to PCR amplification mixtures and multiple PCR amplifications were combined to minimize PCR selection and drift [49].

In conclusion, we have compared the bacterial diversity on the mucosa samples obtained from different parts of the human intestinal tract by analysis of 16S rDNA clone libraries. Our results revealed that the microbial community in jejunum is significantly different from those in distal ileum, ascending colon and rectum, and that the major phylogenetic groups are similar from distal ileum to rectum. Moreover, we also found significantly less *Bacteroidetes* in ascending colon compared to distal ileum as well as rectum. These findings suggest that to study functions such as fermentation of fibers or involvement of microbiota in diseases, occurring in different regions of the GI-tract, it is of importance to take samples from the actual region. This is the first attempt to compare the microbial communities in mucosal biopsies of jejunum, distal ileum, ascending colon and rectum, and to describe bacterial population in the human jejunum by molecular analysis of 16S rRNA genes.

Acknowledgements

We thank Jonas Björk, biostatistician, at Competence Centre for Clinical Research, Lund University Hospital, for discussions concerning statistical analysis of the data. We also thank the volunteer for kindly providing the mucosal biopsies.

References

[1] Tannock, G.W. (1995) Normal Microflora: An Introduction to Microbes Inhabiting the Human Body. Chapman and Hall, London.
[2] Evaldson, G., Heimdahl, A., Kager, L. and Nord, C.E. (1982) The normal human anaerobic microflora. Scan. J. Dis. 35, 9–15.
[3] Cummings, J.H. and Macfarlane, G.T. (1991) The control and consequences of bacterial fermentation in the human colon. J. Appl. Bacteriol. 70, 443–459.
[4] Conly, J.M., Stein, K., Worobetz, L. and Rutledge-Harding, S. (1994) The contribution of vitamin K2 (metaquinones) produced by the intestinal microflora to human nutritional requirements for vitamin K. Am. J. Gastroenterol. 89, 915–923.
[5] Falk, P.G., Hoopper, L.V., Midtvedt, T. and Gordon, J.I. (1998) Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology?. Microbiol. Mol. Biol. Rev. 62, 1157–1170.
[6] Cebra, J.J. (1999) Influences of microbiota on intestinal immune system development. Am. J. Clin. Nutr. 69, 1046S–1051S.

[7] van der Waaij, D., Berghuis-de Vries, J.M. and Lekkerkerk-vander wees, J.E.C. (1971) Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J. Hyg. 69, 405–411.
[8] Fernandes, C.F., Shahani, K.M. and Amer, M.A. (1987) Therapeutic role of dietary lactobacilli and lactobacilli fermented dairy products. FEMS Microbiol. Rev. 46, 343–356.
[9] Fuller, R. (1991) Probiotics in human medicine. Gut 32, 439–442.
[10] de Roos, N.M. and Katan, M.B. (2000) Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. Am. J. Clin. Nutr. 71, 405–411.
[11] Sartor, R.B. (1997) Review article: role of the enteric microflora in the pathogenesis of intestinal inflammation and arthritis. Aliment. Pharmacol. Ther. 11 (Suppl. 3), 17–23.
[12] Guarner, F., Casellas, F., Borruel, N., Antolín, M., Videla, S., Vilaseca, J. and Malagelada, J.R. (2002) Role of microecology in chronic inflammatory bowel diseases. Eur. J. Clin. Nutr. 56, S34–S38.
[13] Moore, W.E.C. and Moore, L.H. (1995) Intestinal floras of population that have a high risk of colon cancer. Appl. Environ. Microbiol. 61, 3202–3207.
[14] Swidsinski, A., Khilkin, M., Kerjaschki, D., Schreiber, S., Ortner, M., Weber, J. and Lochs, H. (1998) Association between intraepithelial *Escherichia coli* and colorectal cancer. Gastroenterology 115, 281–286.
[15] Lemaire, L.C., van Lanschot, J.J., Stoutenbeek, C.P., van Deventer, S.J., Wells, C.L. and Gouma, D.J. (1997) Bacterial translocation in multiple organ failure: cause or epiphenomenon still unproven. Br. J. Surg. 84, 1340–1350.
[16] Langendijk, P.S., Schut, F., Jansen, G.J., Raangs, G.C., Kamphuis, G.R., Wilkinson, M.H.F. and Welling, G.W. (1995) 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.
[17] O'Sullivan, D.J. (2000) Methods for analysis of the intestinal microflora. Curr. Issues Intest. Microbiol. 1, 39–50.
[18] Wilson, K.H. and Blitchington, R.B. (1996) Human colonic biota studied by ribosomal DNA sequence analysis. Appl. Environ. Microbiol. 62, 2273–2278.
[19] Zoetendal, E.G., Akkermans, A.D.L. and de Vos, W.M. (1998) 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, 3854–3859.
[20] Zoetendal, E.G., von Wright, A., Vilpponen-Salmela, T., Ben-Amor, K., Akkermans, A.D.L. and de Vos, W.M. (2002) 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, 3401–3407.
[21] Suau, A., Bonnet, R., Sutren, M., Godon, J.J., Gibson, G.R., Collins, M.D. and Doré, J. (1999) 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.
[22] Hold, G.L., Pryde, S.E., Russell, V.J., Furrie, E. and Flint, H.J. (2002) Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. FEMS Microbiol. Ecol. 39, 33–39.
[23] Wang, X., Heazlewood, S.P., Krause, D.O. and Florin, T.H. (2003) Molecular characterization of microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. J. Appl. Microbiol. 95, 508–520.
[24] Brosius, J., Palmer, M.L., Kennedy, P.J. and Noller, H.F. (1978) Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA 75, 4801–4805.
[25] Polz, M.F. and Cavanaugh, C.M. (1998) Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. 64, 3724–3730.
[26] Zheng, D., Alm, E.W., Stahl, D.A. and Raskin, L. (1996) Characterization of universal small-subunit rRNA hybridisation probes for quantitative molecular microbial ecology studies. Appl. Environ. Microbiol. 62, 4504–4513.
[27] Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M. and Tiedje, J.M. (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. 31, 442–443.
[28] Huber, T., Faulkner, G. and Hugenholtz, P. (2004) Bellerophon: a program to detect chimeric sequence in multiple sequence alignments. Bioinformatics 20, 2317–2319.
[29] Altschul, S.F., Gish, W., Miller, W., Meyers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
[30] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
[31] Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Window 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.
[32] Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
[33] Page, R.D.M. (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.
[34] Krebs, C.J. (1998) Ecological Methodology, 2nd edn. Benjamin/Cummings, Menlo Park, CA.
[35] Magurran, A.E. (1996) Ecological Diversity and Its Measurement. Chapman and Hall, London.
[36] Chao, A. (1987) Estimating the population size for capture–recapture data with unequal catchability. Biometrics 43, 783–791.
[37] Hughes, J.B., Hellmann, J.J., Ricketts, T.H. and Bohannan, B.J. (2001) Counting the uncountable: statistical approaches to estimating microbial diversity. Appl. Environ. Microbiol. 67, 4399–4406.
[38] Singleton, D.R., Furlong, M.A., Rathbun, S.L. and Whitman, W.B. (2001) Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl. Environ. Microbiol. 67, 4374–4376.
[39] Good, I.J. (1953) The population frequencies of species and the estimation of population parameters. Biometrika 40, 237–264.
[40] Stackebrandt, E. and Goebel, B.M. (1994) 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.
[41] Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. and Farrow, J.A. (1994) The phylogeny of genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. 44, 812–826.
[42] Barcenilla, A., Pryde, S.E., Martin, J.C., Duncan, S.H., Stewart, C.S., Henderson, C. and Flint, H.J. (2002) Phylogenetic relationships of butyrate-producing bacteria from the human gut. Appl. Environ. Microbiol. 66, 1654–1661.
[43] Leser, T.D., Amenuvor, J.Z., Jensen, T.K., Lindecrona, R.H., Boye, M. and Møller, K. (2002) Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl. Environ. Microbiol. 68, 673–690.

[44] Bonnet, R., Suau, A., Doré, J., Gibson, G.R. and Collins, M.D. (2002) Differences in rDNA libraries of faecal bacteria derived from 10- and 25-cycle PCRs. Int. J. Syst. Evol. Microbiol. 52, 757–763.

[45] Sghir, A., Gramet, G., Suau, A., Rochet, V., Pochart, P. and Doré, J. (2000) Quantification of bacterial groups within human faecal flora by oligonucleotide probe hybridisation. Appl. Environ. Microbiol. 66, 2263–2266.

[46] Farrelly, V., Rainey, F.A. and Stackebrandt, E. (1995) Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl. Environ. Microbiol. 61, 2798–2801.

[47] Wintzingerod, F.V., Hoebel, U.B. and Stackebrandt, E. (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. 21, 213–229.

[48] Harmsen, H.J.M., Raangs, G.C., He, T., Degener, J.E. and Welling, G.W. (2002) Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. Appl. Environ. Microbiol. 68, 2982–2990.

[49] Wagner, A., Blackstone, N., Cartwright, P., Dick, M., Misof, B., Snow, P., Wagner, G.P., Bartels, J., Murtha, M. and Pendleton, J. (1994) Surveys of gene families using polymerase chain reaction: PCR selection and PCR drift. Syst. Biol. 43, 250–261.