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Differences between Tissue-Associated Intestinal Microfloras of Patients with Crohn's Disease and Ulcerative Colitis[▽][‡]

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A leading hypothesis for the role of bacteria in inflammatory bowel diseases is that an imbalance in normal gut flora is a prerequisite for inflammation. Testing this hypothesis requires comparisons between the microbiota compositions of ulcerative colitis and Crohn's disease patients and those of healthy individuals. In this study, we obtained biopsy samples from patients with Crohn's disease and ulcerative colitis and from healthy controls. Bacterial DNA was extracted from the tissue samples, amplified using universal bacterial 16S rRNA gene primers, and cloned into a plasmid vector. Insert-containing colonies were picked for high-throughput sequencing, and sequence data were analyzed, yielding species-level phylogenetic data. The clone libraries yielded 3,305 sequenced clones, representing 151 operational taxonomical units. There was no significant difference between floras from inflamed and healthy tissues from within the same individual. *Proteobacteria* were significantly ($P = 0.0007$) increased in Crohn's disease patients, as were *Bacteroidetes* ($P < 0.0001$), while *Clostridia* were decreased in that group ($P < 0.0001$) in comparison with the healthy and ulcerative colitis groups, which displayed no significant differences. Thus, the bacterial flora composition of Crohn's patients appears to be significantly altered from that of healthy controls, unlike that of ulcerative colitis patients. Imbalance in flora in Crohn's disease is probably not sufficient to cause inflammation, since microbiotas from inflamed and noninflamed tissues were of similar compositions within the same individual.

The mammalian intestinal flora is a complex ecosystem containing hundreds of microbial species, with a cell density often exceeding 10^{12} viable bacteria per gram in the colon. It is well established that the microflora not only contributes to the host's nutrition and gut development but is also required for development of a healthy immune response. The microbiota was shown to be strongly linked to genotype, as the flora profiles of monozygotic twins were significantly more similar than those of unrelated individuals whereas profiles of marital partners were not (28).

It is increasingly evident that intestinal flora plays a role in the pathogenesis of inflammatory bowel diseases (IBDs). Support for microbial involvement in IBD comes not only from animal models of ulcerative colitis (UC) and Crohn's disease (CD) but also from genetic studies of humans showing disease susceptibility genes, such as NOD2/CARD15 and Toll-like receptors, which are thought to be involved in recognition of bacterial products (5–7). It has been suggested that inflammation may not be due to a specific pathogen but rather created by dysbiosis, a shift in the balance of healthy flora in favor of proinflammatory microbial species, which can lead to intestinal inflammation (16, 23).

In recent years, several culture-independent studies of gut flora attempted to determine whether dysbiosis exists in IBD. A study utilizing DNA hybridization demonstrated previously that fecal microbiota is altered in both active and quiescent CD (21) and that there are differences between UC and CD in composition of both mucosa-adherent and invasive microbiota (9). Ott and colleagues demonstrated a reduction in obligate anaerobe clades in IBD patients, especially in CD patients, by using single-strand conformation polymorphism and real-time PCR (12). A recent report using sequencing of 16S rRNA genes from a metagenomic library showed a reduction in diversity, especially of clostridia, in fecal flora of CD patients compared with that of healthy controls (10).

Here we present a study comparing tissue-associated intestinal microflora by amplification, cloning, and sequencing of 16S rRNA genes from six CD patients, five UC patients, and five healthy (noninflamed) controls. We present a phylogenetic analysis at the species level, investigating whether major differences exist between IBD patients and healthy controls.

MATERIALS AND METHODS

Patients. The protocol was approved by the Research Review Board of the QEII Health Sciences Centre, Halifax, Nova Scotia, Canada, and informed consent was obtained from each participating patient. Table 1 shows a summary of the patients and tissues used in the study. All subjects were recruited by two gastroenterologists and included the following: (i) patients with Crohn's disease, (ii) patients with ulcerative colitis, and (iii) the control group, i.e., patients coming for colonoscopy because of a family history of colon cancer or symptoms which do not involve inflammation, such as polyps. IBD patients included indi-

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TABLE 1. Subjects and tissues used for the generation of 16S rRNA libraries

Subject designation[a]	Tissue(s)[b]	Subject sex	Subject age (yr)	Remark(s)	No. of PCR cycles
CD2	Terminal ileum, right colon, transverse colon, **descending colon**	Male	38	Mild inflammation in descending colon	35
CD3	Neoterminal ileum, **ascending colon**, rectum	Female	22	Mild inflammation and a few ulcers in ascending colon; had past resection in terminal ileum	35
CD5	Neoterminal ileum, rectum	Female	38	Had past resection in terminal ileum; moderate inflammation in ascending colon	30
CD6	Right colon	Female	35	Moderate inflammation in ileum	30
CD8	**Neoterminal ileum**, right colon, sigmoid colon, rectum	Female	46	Had past resection in terminal ileum; mild inflammation in neoterminal ileum	30
CD9	**Neoterminal ileum**, right colon, sigmoid colon, rectum	Female	39	Had past resection in terminal ileum; moderate to severe inflammation in neoterminal ileum	30
UC2	*Sigmoid colon*	Female	28	Mild inflammation in sigmoid colon	30
UC3	Sigmoid colon	Female	53	Remission	34
UC4	**Sigmoid colon**	Male	71	Mild inflammation in sigmoid colon	34
UC6	Right colon normal, **sigmoid colon, rectum**	Male	36	Mild inflammation in sigmoid colon and rectum	34
UC7	**Sigmoid colon**	Male	28	Mild to moderate inflammation in sigmoid colon	34
H2	Sigmoid colon	Female	46	Colonic polyp	27
H3	Sigmoid colon	Male	54	Colon cancer screening	34
H5	Colonic polyps	Male	48	Colonic polyps; previous rectal cancer	34
H12	Right colon, transverse colon, sigmoid colon, rectum	Female	49	Adenoma; healthy mucosa biopsy samples	30
H13	Right colon, transverse colon, sigmoid colon, rectum	Female	41	Healthy; colon cancer screening	30

[a] CD designations indicate patients with Chron's disease, UC designations indicate patients with ulcerative colitis, and H designations indicate healthy controls.
[b] A DNA clone library was obtained from each of the tissues according to the procedure detailed in Materials and Methods. Bold type indicates inflamed tissue. Italic type indicates tissue with patchy inflammation, where biopsy samples were taken from both healthy and inflamed sections.

viduals who were diagnosed for the first time during colonoscopy or patients for whom a recent small bowel follow-up X ray or barium enema showed IBD. Some of the IBD patients had been diagnosed previously with Crohn's disease or ulcerative colitis and underwent colonoscopy because of symptoms suggestive of a flare-up. None of the patients were on any active medication, such as corticosteroids or immunomodulators, with the exception of patient UC6, who was treated with azathioprine and prednisone, and the use of antibiotics in the 4 weeks preceding colonoscopy was an exclusion criterion. Finally, some IBD patients underwent colonoscopy for colon cancer surveillance because of a history of >10 years of having the disease. During colonoscopy, patients received intravenous sedation as required. For each patient, a minimum of eight biopsy samples were obtained. For IBD patients, where possible, biopsy samples were obtained from areas of active disease and of normal mucosa. For patients with CD in the terminal ileum, biopsy samples were also taken from that region of the small intestine. For the control group, biopsy samples were taken from rectum, sigmoid and transverse colons, and cecum. All patients underwent a 1-day bowel cleansing preparation, consisting of 4 liters of polyethylene glycol electrolyte solution (Colyte), prior to colonoscopy.

Biopsies and DNA extraction. Tissue samples of roughly 3 by 3 mm were suspended in saline and kept on ice for no longer than 2 hours, and DNA was extracted using a Bio101 FP120 Fastprep instrument and DNA extraction kit according to the manufacturer's protocol with the following adjustment: after the final elution step, the eluted DNA was centrifuged for 1 minute and the supernatant transferred to a fresh microcentrifuge tube to remove residual binding matrix. For each sample, two DNA extractions were performed, one using the biopsy samples washed five times in commercial sterile phosphate-buffered saline, pH 7.2, and one using 200 μl from the first of these washes. This was done so that both invasive, stronger adherent and weaker adherent bacteria could be represented. In several cases, only one of these fractions yielded bacterial DNA that could be amplified by PCR. Samples in which neither fraction had bacterial DNA detectable by PCR were excluded from the study.

PCR. Purified DNA was diluted 10-fold, and 1 μl was used as a template for a 25-μl PCR. PCR was performed with 1 U of *Taq* DNA polymerase (Invitrogen), 1.5 mM MgCl$_2$, 2.5 μl 10× PCR buffer, 0.2 mM of each deoxynucleoside triphosphate, high-pressure liquid chromatography-grade water, and 10 pmol of primers 63F and 1387R (11). The reaction was performed as follows: 3 min at 94°C; *x* cycles of 45 s at 94°C, 45 s at 53°C, and 2 min at 72°C; and 30 min at 72°C.

Since bacterial DNA concentration was unknown, the number of cycles (*x*) had to be optimized for each individual sample so that PCR amplicons could be observed by gel electrophoresis (Table 1).

Cloning and sequencing. Specific PCR products were extracted from a crystal violet-stained agarose gel by use of a MinElute gel extraction kit (QIAGEN) and cloned into Topo-TA pCR 2.1 (Invitrogen) plasmid vector according to the manufacturer's protocols. Ninety-six clones from each sample were miniprepped using alkaline lysis with GeneMachine robotics (Genomic Solutions). Sequencing of clones was performed using a DYEnamic ET dye terminator kit (Mega-BACE) and a MegaBACE 4000 (Amersham) sequencer with standard M13 forward and reverse primers (IDT).

Sequence analysis. Sequences were analyzed by PhredPhrap to remove low-quality and vector sequence regions. All sequences of 400 nucleotides or shorter were removed. Two sequence sets were obtained for each sample, representing the 5′ and 3′ regions of the rRNA gene fragments of about 1,320 nucleotides. Where possible, contigs were assembled from both ends of each clone by using ContigExpress (Invitrogen) and identified by BLASTN comparison against the National Center for Biotechnology Information nucleotide database, recording best hit and best cultured organism hit. Clones were assigned according to contigs or by 99% or better nucleotide identity of both reads to a contig by local BLASTN, excluding gaps. Clones where one of the two reads lacked sufficient length or quality but its partner's read was of high quality were assigned according to the match of the better read. Sequences, both the single reads and contigs, were scanned for chimeras by use of the now defunct Bellerophon server (http://foo.maths.uq.edu.au/~huber/bellerophon.pl) (the same service is available on the Greengenes website [http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi]), using the Huber-Hugenholtz correction and a window size of 200 nucleotides. Suspected sequences detected by the server, as well as all sequences with less than 95% identity to previous entries in the databases, were checked manually for chimeras and all chimeras were removed (about 10 to 15% of sequences). Overall, we sequenced over 4,000 clones, out of which 3,305 could be reliably assigned to operational taxonomical units (OTUs). Contigs were aligned using CLUSTALX (24), a Windows version of the CLUSTALW algorithm (25), generating a distance matrix and correcting for multiple substitution (8). Analysis by DOTUR (17) grouped sequences by 99% identity threshold to form 151 OTUs. To test which libraries were significantly different from one another, distance matrices were similarly generated from all sequences longer than 500
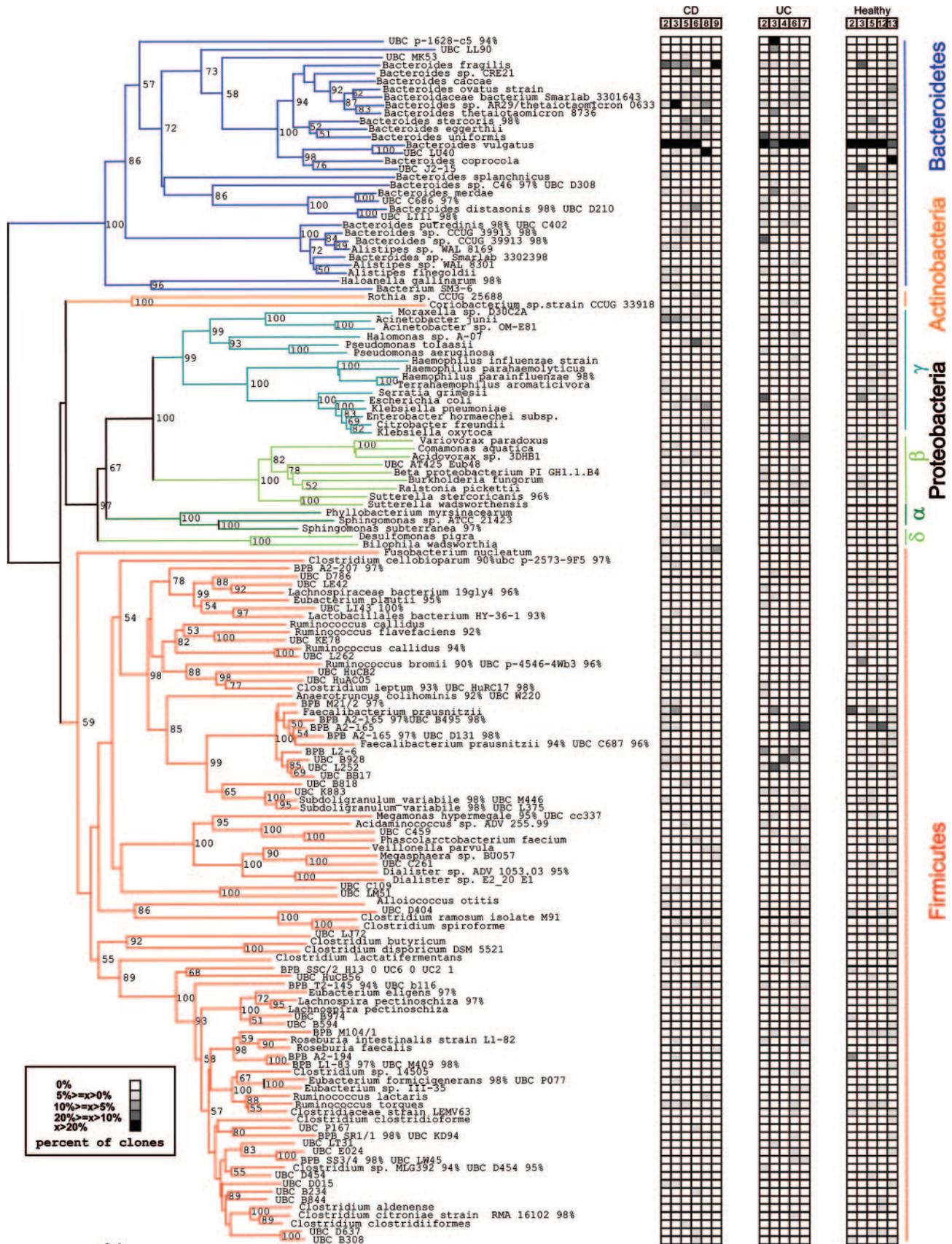
TABLE 2. Phylum level distribution of 16S clones from different study groups

Phylum	No. (%) of clones assigned to phylum		
	CD patients	UC patients	Healthy controls
Bacteroidetes	1,192 (74.97)	436 (64.31)	699 (67.41)
Firmicutes	160 (10.06)	175 (25.81)	250 (24.11)
Fusobacteria	31 (1.95)	0 (0.00)	0 (0.00)
Proteobacteria	206 (12.96)	64 (9.44)	88 (8.49)
Actinobacteria	1 (0.06)	3 (0.44)	0 (0.00)
Total	1,590 (100.00)	678 (100.00)	1,037 (100.00)

nucleotides from the 3′ region (nucleotides 800 to 1339 of the 16S rRNA gene) and compared using ∫-libshuff (18). Statistical comparisons of clone counts in different groups were performed using the chi-square test. Average individual library coverage values, defined as the percentage of sequences in a library that is not comprised of singletons, were 97%, 92%, and 96% for the CD, UC, and healthy control groups, respectively. The Jaccard similarity index was calculated using the formula $S_{12}/(S_1 + S_2 - S_{12})$, as defined in reference 2.

Nucleotide sequence accession numbers. 16S gene sequences were deposited in GenBank under accession numbers DQ441252 to DQ441402.

RESULTS AND DISCUSSION

The 16S clone libraries combined yielded 1,590, 678, and 1,037 sequences for CD, UC, and healthy samples, respectively, which could be grouped to 151 OTUs at 99% identity. Since library sequences generated from different sites in the same individual were not significantly different by ∫-libshuff analysis, all sequences from the same individual were pooled (see below). A phylogenetic reconstruction of the relationships between the observed phylotypes is presented in Fig. 1. Although PCR can introduce certain biases and clone abundance is only partly quantitative, comparisons between libraries obtained using the same procedure can be drawn. The vast majority of clones sequenced belonged to one of three phyla: *Firmicutes*, *Bacteroidetes*, or *Proteobacteria*. Pairwise comparisons of OTUs using the Jaccard similarity index demonstrated that the UC samples were more similar in OTU membership to healthy samples than either UC or healthy samples were to CD samples, with values of 1.03, 0.54, and 0.58, respectively.

Proteobacteria **are increased in Crohn's disease patients.** *Proteobacteria* (gram-negative facultative anaerobes and sulfate reducers) were significantly ($P = 0.0007$) increased in CD patients versus UC patients or healthy subjects (Table 2). This corroborates previous findings by Seksik and colleagues obtained using DNA-DNA hybridization, which compared microbiotas of CD patients with those of healthy individuals (21). Most of the *Proteobacteria* identified were of the class γ-*Proteobacteria*, and *Escherichia coli* was the most common phylotype in that group, represented by over one-third of the clones, with no significant difference in prevalence between the groups. In contrast, about 10% of the proteobacterial clones

were identified as *Acinetobacter junii*, which were significantly increased in the CD group (31 clones, versus two clones in the UC group and six in the healthy group [$P = 0.0004$]). We also detected 43 *Klebsiella pneumoniae* 16S clones in only two CD patients and none of the other groups ($P < 0.0001$). Since antibody levels against *K. pneumoniae* were shown previously to be increased in CD patients (26, 27), this finding may warrant a larger study using *Klebsiella*-specific primers or probes. Sulfate-reducing δ-*Proteobacteria* were rare and encountered only in the CD group.

The phylum *Bacteroidetes* **is the most dominant in tissue-associated bacteria and is increased in Crohn's disease patients.** *Bacteroidetes* (gram-negative obligate anaerobes) were far less diverse than *Firmicutes*, containing only 32 phylotypes, versus 87 OTUs in the latter phylum, but were nevertheless the most abundant, representing over 70% of total clones. *Bacteroidetes* were significantly increased in CD patients versus UC patients or healthy subjects (74.97% versus 64.31% and 67.41%, respectively [Table 2]) ($P < 0.0001$). The most abundant phylotype overall was *Bacteroides vulgatus*, present in 14 subjects and the most common clone in 11 of them. Since *B. vulgatus* was also the most common OTU identified both in CD patients and in healthy controls in a recent fecal metagenomic survey (10) and the most common member of *Bacteroidetes* in another large PCR-based 16S study of both stool and intestinal biopsy samples (3), it may be the most common bacterial species in the human intestine. Levels of diversity, as represented by the number of different OTUs within *Bacteroidetes*, were similar in the three groups. *Bacteroides fragilis* was significantly more abundant in CD patients than in either UC patients or healthy subjects ($P < 0.0001$), while differences in its levels of abundance between UC patients and healthy subjects were not significant. Furthermore, *B. fragilis* was the most common phylotype recovered from the DNA extracted from the biopsy samples of subject CD9, but even after all CD9 sequences were removed from the calculation, significance remained very high ($P < 0.0001$). It therefore seems possible that a subset of CD patients is associated with the presence of *B. fragilis*, as indicated by a recent study (13). In three other samples, CD8, UC3, and H13, different members of the *Bacteroidetes* replaced *B. vulgatus* as the dominant OTU recovered. In sample UC3, the unusual abundant species is a phylotype distant from *B. vulgatus*, whereas in samples CD8 and H13, the OTUs appear to be closely related to it. Thus, it appears that tissue-associated microflora may typically possess one dominant species from the *B. vulgatus* clade, but in a few individuals, some distantly related member of *Bacteroidetes* may occupy this niche due to yet-unknown genetic and/or environmental factors.

Reduction in *Clostridia* **in Crohn's disease patients and implications for butyrate availability.** The increase in *Bacteroidetes* and *Proteobacteria* was accompanied by a significant ($P < 0.0001$) decrease in *Firmicutes*, all belonging to the class

FIG. 1. Phylogenetic relationships of phylotypes of tissue-associated bacteria from the colon. OTUs were named according to their best BLASTN match with the National Center for Biotechnology Information database. Where a hit to an uncultured bacterial clone (UBC) is 2% or greater than the hit from a cultured organism, that hit is provided. Where similarity to the database entry is less than 99%, percent similarity is shown. Numbers near nodes represent percent bootstrap support. The scale bar represents substitutions per site.

Clostridia (gram-positive obligate anaerobes), in the CD group. A recent report based on sequence data from a fecal metagenomic library has also demonstrated a reduction in diversity and abundance of *Firmicutes* in CD patients in remission compared with healthy individuals (10). Taken together, it appears that at least some CD patients have an inherent clostridium deficiency in both luminal and mucosa-associated microfloras, which is not a by-product of inflammation. Many clostridial phylotypes recovered in this study, as well as other members of the class *Clostridia*, can ferment dietary carbohydrates to butyrate (1, 14). This short-chain fatty acid is not only an important energy source for colonocytes (15) but also possesses anti-inflammatory properties, including the ability to reduce lipopolysaccharide-induced cytokine response (19). Thus, clostridium deficiency could predispose some individuals to subsequent intestinal inflammation. Whether this alteration in flora is the result of host genotype, environmental factors (such as nutrition), or both requires further investigation.

Apparent absence of archaea (methanogens) from tissue-associated intestinal flora. It was hypothesized previously that archaea may play a role in IBD (4). Using archaeon-specific universal PCR primers, we could not detect the presence of archaea in any of our samples, even when an increased number of cycles (35 cycles) was used. It therefore appears that although they are known to be found in some individuals, methanogenic archaea were either absent from or unable to adhere to the tissues of all of our research subjects, both healthy subjects and those with IBD. This means that while a possible protective role for archaea in maintaining intestinal health cannot be rejected, their involvement as causative agents of mucosal inflammation in IBD appears unlikely.

Flora of inflamed tissue is similar to that of healthy tissue from the same individual. We compared different clone libraries by using the ∫-libshuff algorithm (18). Either the libraries derived from different sites within the intestine of the same individual, including terminal ileum biopsy samples, were not significantly different, or one was a subset of the other (see Table S1 in the supplemental material). This is in agreement with the recent findings of Eckburg and colleagues (3) but is somewhat surprising since several of our analyses compared inflamed and healthy tissues. A previous study of CD patients by use of temporal temperature gradient gel electrophoresis has also shown that ulcerated and nonulcerated tissues are similar in dominant-species composition (20). These findings suggest that it is unlikely that inflammation is directly caused by a mucosa-associated pathogen, a conclusion also reached by another group using a different methodology (22). Our data show that even in mucosa-associated flora, which is presumably less diverse than the fecal microbiota (3), there is great variation across individuals. Within the three volunteer groups, most individual floras were all significantly different from one another, according to ∫-libshuff analysis, with the exception of sample UC7, which was not significantly different from samples UC6 and UC2 (see Table S2 in the supplemental material).

Concluding remarks. The finding that tissue-associated flora of UC patients is broadly similar to that of healthy individuals and significantly different from that of CD patients may indicate that, beyond its obvious role in fuelling inflammation, the commensal flora has profoundly different roles in the etiologies of these diseases. Furthermore, since the differences in microflora between CD and UC patients appear relatively large and well-defined, this study should be followed by quantitative phylum level assessment in tissue-associated and fecal microbiotas of a large group of patients to establish their generality in multiple ethnic backgrounds and nutritional regimens. These differences could be used to diagnose cases of indeterminate colitis and perhaps even identify people with potential CD susceptibility before they become symptomatic, as well as to enable the design of future means of prevention.

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