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The bacteriology of biopsies differs between newly diagnosed, untreated, Crohn's disease and ulcerative colitis patients

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The bacterial community (microbiota) that inhabits the gut of humans appears to be an important source of antigens that drive the chronic immunological processes characteristic of Crohn's disease (CD) and ulcerative colitis (UC). Most of the members of the microbiota have not yet been cultured, but nucleic-acid-based methods of detection and enumeration can provide information about the community. This investigation used these methods to obtain information about the bacteria associated with mucosal surfaces in the gut of 20 CD and 15 UC patients. Biopsies were collected from inflamed and non-inflamed sites in the intestines of newly diagnosed, untreated patients. Biopsies were also collected from several intestinal sites of 14 healthy subjects. The bacterial collections associated with the biopsies were analysed by generating PCR/denaturing gradient gel electrophoresis (DGGE) profiles, the preparation of 16S rRNA gene clone libraries, and qualitative PCR to detect specific groups of bacteria. The total numbers of bacteria associated with the biopsies were determined by real-time quantitative PCR. DGGE profiles generated from the terminal ileum and various colonic regions were characteristic of each individual but differed between subjects. DGGE profiles and 16S rRNA gene libraries showed that the bacteria associated with inflamed and non-inflamed tissues did not differ. UC patients had more bacteria associated with biopsies than did CD patients ($P<0{\cdot}01$). Statistical analysis of the composition of 16S rRNA gene libraries showed that the bacterial collections in UC and CD patients differed ($P<0{\cdot}05$). Unclassified members of the phylum Bacteroidetes were more prevalent in CD than in UC patients. Therefore, the types and numbers of bacteria associated with biopsy samples were distinctly different for UC and CD patients. The observations made in this study should permit targeting of specific bacteriological abnormalities in investigations of the pathogenesis of inflammatory bowel diseases and provide targets for medical interventions.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are considered to be distinct clinical entities that have in common an inappropriate, exaggerated and ongoing activation of the mucosal immune system that is fuelled by the presence of the bacterial collection (gut microbiota) normally resident in the intestinal tract. This pathological response is likely to be associated with defects in epithelial barrier function and of the immune system, which are, in part, genetically determined (Podolsky, 2002; Bouma & Strober, 2003). Several bacterial and viral pathogens have been suggested as causes of inflammatory bowel diseases (IBDs), but none has gained general acceptance. Rather than specific pathogens, current views favour members of the gut microbiota as the source of antigens with which the dysfunctional immune system reacts (Schultz & Rath, 2002). Determining whether certain members of the microbiota, or the microbial collection *en masse*, are responsible for the abnormal microbe–immune system interplay is not a simple process. The analysis of the gut microbiota of humans has been fraught with difficulties due to an inability to culture most of the bacterial inhabitants of the gut. Perhaps as much as 60 % of the gut microbiota has not yet been cultivated in the laboratory, even when state-of-the-art bacteriological methods have been used (Tannock *et al.*, 2000). Analysis of the faecal microbiota in relation to IBDs has been attempted (Ruseler-Van Embden & Both-Patoir, 1983; Seksik *et al.*, 2003), which although representative of the microbiota in

Abbreviations: CD, Crohn's disease; DGGE, denaturing gradient gel electrophoresis; IBD, inflammatory bowel disease; OTU, operational taxonomic unit; UC, ulcerative colitis.

the distal colon (Moore *et al.*, 1978), does not necessarily provide information that is relevant to the region of the bowel where disease is present (Zoetendal *et al.*, 2002). Biopsies collected at endoscopy provide samples pertinent to the study of the diseased site. They are not perfect for microbiological analysis because they consist of only a few milligrams of tissue and have been collected from subjects that have undergone bowel cleansing prior to endoscopy. Nevertheless, as has been demonstrated by others, the presence of small numbers of bacteria associated with biopsies can be detected by nucleic-acid-based analytical methods and useful contributions to knowledge can be obtained (Schultsz *et al.*, 1999; Swidsinski *et al.*, 2002; Ott *et al.*, 2004; Prindiville *et al.*, 2004; Lepage *et al.*, 2005). Usually, however, the human subjects that have been studied have already received treatment for their medical conditions, which might have influenced the analytical outcomes.

We have studied the numbers and types of bacteria associated with biopsies collected from intestinal sites of newly diagnosed and untreated CD and UC patients. Nucleic-acid-based methods of analysis were used in order to overcome the problem of non-cultivability of bacterial members of the microbiota. The results of the analyses show that UC and CD are bacteriologically distinct diseases.

METHODS

Sample collection. Intestinal biopsies were obtained from the Canadian IBD Network Tissue Bank established by the Crohn's and Colitis Foundation of Canada (Collins *et al.*, 2003). The biopsies were collected during endoscopy, after standard bowel cleansing methods, of patients at Canadian institutions and were stored at −70 °C until diagnosis was made. The samples were curated by Gamma Dynacare Medical Laboratories before shipment on dry ice to the analytical laboratory at the University of Alberta, Edmonton. The approval of ethics committees at institutions involved in collecting samples was obtained and the laboratory manipulations were approved by the Agriculture, Forestry and Home Economics Research Ethics Board (permit 0302) at the University of Alberta.

Patients were included in the study if they had clinically active UC or CD. Diagnosis was confirmed by endoscopy and histology by pathologists at the participating institutions. The patients were newly diagnosed and none had received antibiotics, immunosuppressives or glucocorticosteroids prior to endoscopy. Biopsies were obtained during endoscopy from inflamed and non-inflamed gut regions of 15 patients with UC and 20 patients with CD. Tissue was considered normal if there was an absence of macroscopic or histological evidence of inflammation. Three biopsy pieces taken from each site (range 3·2–30·5 mg; mean 14·3 mg) were pooled for analysis. Three biopsy pieces (range 4·6–25·0 mg; mean 11·6 mg) were also collected from five intestinal sites (terminal ileum, right colon, transverse colon, left colon, rectum) of 14 non-matched patients, subsequently termed healthy subjects, who were undergoing routine diagnostic workup or had a family history of colon cancer. They did not have mucosal abnormalities of the terminal ileum, colon or rectum. Relevant details of patients and healthy subjects are summarized in Table 1, and the sites from which biopsies were collected are shown in Table 2.

Bacterial strains. DNA extracted from the following bacterial strains was used as positive controls in PCR detection of specific bacterial groups: *Bacteroides vulgatus* ATCC 29327, *Bifidobacterium adolescentis* DSM 20083^T, *Clostridium difficile* DSM 1296^T, *Clostridium coccoides* ATCC 29236^T, *Helicobacter pylori* DSM 7492, *Lactobacillus ruminis* ATCC 27780^T and *Desulfovibrio desulfuricans* isolated from the faeces of an ankylosing spondylitis patient (Stebbings *et al.*, 2002). *Mycobacterium avium* subsp. *paratuberculosis* 316f (Thorel *et al.*, 1990) DNA was a gift from the Disease Research Laboratory, University of Otago.

Table 1. Human subjects

	Healthy subjects	CD patients	UC patients
Males/females	3/11	12/8	7/8
Age (years)	47 (12–73)*	22 (12–56)	24 (11–53)
Smokers†	2/4/8	3/3/14	0/2/13
Birth control pill†	1/4/9	1/2/17	3/1/11
Aspirin†	4/2/8	0/1/19	0/3/12
NSAID†‡	1/3/10	1/2/17	1/3/11

*Mean (range).
†Currently/previously/never. Previously indicates former use but ceased at least 1 year previously.
‡Non-steroidal anti-inflammatory drugs (ibuprofen, naproxen or similar).

Nucleic acid extraction. In the case of biopsies, DNA was extracted from the samples using the Qiagen DNA/RNA Extraction kit. Biopsies were not washed prior to analysis. The three biopsy pieces from each site were pooled and placed in 300 µl lysozyme solution (30 mg ml^{-1}) and vortexed every 10 min during a 30 min period at room temperature. After the addition of 500 µl QRL1 buffer supplemented with 2-mercaptoethanol, samples were shaken at 5000 r.p.m. in a bead-beater for 2 min. QRV1 buffer (300 µl) was added to the tubes, and samples were vortexed and centrifuged at 15 000 ***g*** for 20 min. The supernatant was recovered, 800 µl isopropanol was added, and the supernatant was stored overnight at

Table 2. Sites from which biopsies were collected

Abbreviations: TI, terminal ileum; RC, right colon; TC, transverse colon; LC, left colon; REC, rectum. Biopsies were obtained from all five sites from healthy subjects. Values show the number of patients.

Patient group	Site sampled	Inflamed mucosa	Non-inflamed mucosa
CD	TI	4	2
	RC	7	6
	TC	0	1
	LC	0	2
	REC	9	9
UC	TI	0	2
	RC	0	10
	TC	2	2
	LC	0	1
	REC	13	0

−20 °C. DNA pellets were recovered by centrifugation at 15 000 ***g*** for 30 min and dissolved in 150 µl QRL1 buffer (supplemented with 2-mercaptoethanol) at 60 °C. After the addition of 1·35 ml QRV2 buffer, the preparation was centrifuged at 5000 ***g*** for 5 min at 4 °C, then the supernatant was loaded onto a separation column equilibrated with QRE solution. The flow-through was recovered and reloaded onto the column and drained by gravity. The column was washed three times with QW buffer, and DNA was eluted with QF buffer at 45 °C and precipitated with 700 µl isopropanol at −20 °C. The DNA preparations were centrifuged at 15 000 ***g*** for 30 min at 4 °C, and the pellets were washed twice with 500 µl 80 % cold ethanol (−20 °C). The DNA extracts were stored at −80 °C until further analysis. DNA integrity was assessed by electrophoresis of each sample in a 1·2 % agarose gel that was stained with ethidium bromide solution (5 µg ml^{-1}) and viewed by UV transillumination.

DNA was extracted from PCR control bacteria by harvesting cells grown on agar plates, suspension in sterile deionized water, and centrifugation at 10 000 ***g*** for 15 min. The cells were washed twice with TN150 buffer (10 mM Tris/HCl, pH 8, 150 mM NaCl) and resuspended in 1 ml of the same buffer. Samples were disrupted in a bead-beater for 3 min at 5000 r.p.m., then centrifuged at 14 000 ***g*** for 5 min at 5 °C. DNA was purified from supernatants by phenol/chloroform extraction and sodium acetate/ethanol precipitation, as described previously (Tannock *et al.*, 2000).

Denaturing gradient gel electrophoresis (DGGE) profiles of biopsy-associated bacteria. Bacterial DNA was amplified by PCR targeting the V3 region of the 16S rRNA gene using the universal bacterial primers HDA1-GC and HDA2, and a previously described programme (Tannock *et al.*, 2000). PCR products were checked before DGGE analysis by electrophoresis in a 2 % agarose gel stained with ethidium bromide (5 µg ml^{-1}) and viewed by UV transillumination. Electrophoresis was performed using a DCode apparatus (Bio-Rad) and 6 % polyacrylamide gels with a 30–55 % gradient of 7·0 M urea and 40 % (v/v) formamide that increased in the direction of electrophoresis. Electrophoretic runs were in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) at 130 V and 60 °C for 275 min. Gels were stained with ethidium bromide solution (5 µg ml^{-1}) for 20 min, washed with deionized water for 20 min, and viewed by UV transillumination. DGGE profiles were compared by determining the Dice similarity coefficient (D_{sc}) using the Bionumerics software package (version 3.0, Applied Maths) at a sensitivity of 2–3 %.

Detection of selected bacterial groups by PCR. PCR detection of selected bacterial groups utilized a GeneAmp PCR System 9700 thermocycler (Applied Biosystems). The PCR reaction mixture (50 µl volume) contained 1·5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris/HCl (pH 8·4), deoxynucleoside triphosphates at a concentration of 200 µM each, 20 pM each primer (Table 3), 2·5 U *Taq* DNA polymerase (Invitrogen) and 2 µl biopsy DNA. Five-microlitre aliquots of the PCR product were electrophoresed in a 2 % agarose gel, which was stained with ethidium bromide and viewed by UV transillumination. The size of the products was confirmed by reference to a DNA molecular mass ladder (Invitrogen) and by cloning and sequencing.

Preparation of 16S rRNA gene clone libraries. Universal bacterial primers, *Sac*I-POmod (*Escherichia coli* position 11) and HDA2 (*E. coli* position 539) (Rodtong & Tannock, 1993; Tannock *et al.*, 2000) were used to generate a partial sequence of 16S rRNA genes (528 bp). PCR was performed using the reaction mixture described above for other PCR analyses, and the following programme: 95 °C for 4 min, followed by 25 cycles 1 min at 95 °C, 1 min at 58 °C, 2 min at 72 °C, then 72 °C for 7 min. The least number of cycles was used to minimize preferential amplification of sequences that would create bias. The 16S rRNA gene amplicons were cloned in the pCR2.1 TOPO plasmid vector (Invitrogen), and One Shot Top10 (Invitrogen) competent *E. coli* cells were chemically transformed as described by the supplier. Recombinant cells were cultured on Luria–Bertani (LB; Becton Dickinson) agar plates containing 100 µg ampicillin ml^{-1} (Sigma) and 40 µg X-Gal ml^{-1} (Sigma). Sixty white colonies from each transformation reaction were picked randomly with sterile toothpicks and transferred to a second plate of LB agar plus ampicillin/X-Gal to confirm the colour selection. Forty-eight white colonies from the second plate were each transferred to individual wells of a 96-well microtitre tray, each well containing 200 µl LB freezing buffer [36 mM K_2HPO_4, 13·2 mM KH_2PO_4, 1·7 mM sodium citrate, 0·4 mM $MgSO_4$, 6·8 mM $(NH_4)_2SO_4$, 4·4 % (v/v) glycerol, LB broth] (Zimmer & Gibbins, 1997). The plates were incubated at 37 °C overnight to allow proliferation of the cells, then duplicated and stored at −80 °C until further analysis. Cloned 16S rRNA gene sequences were amplified using plasmid-targeted primers (M13 forward, 5′-GTAAAACGACGGCCAG-3′, and M13 reverse, 5′-CAGGAAACAGCTATGAC-3′) and the following PCR programme: 94 °C for 4 min, followed by 25 cycles 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min, then 72 °C for 7 min. The size of the PCR products was confirmed by agarose gel electrophoresis with molecular mass marker, as described above. Amplified DNA (4 µl) was used as template for sequencing. The dideoxy chain-termination reaction was conducted using the M13 reverse primer, the CEQ DTCS kit (Beckman Coulter) and the CEQ8000 Genetic Analyser (Beckman Coulter) following the manufacturer's instructions. Sequences were edited to 515 bp using EditSeq software (DNASTAR). Each biopsy sample contributed, on average, 27 clones per library, which approximately matched the average number of intensely stained 16S rRNA gene fragments per DGGE profile. The library prepared from healthy subjects was composed of sequences generated from biopsies collected from the right colon.

Comparison of 16S rRNA gene clone libraries. The libraries prepared from 16S rRNA genes of bacteria associated with inflamed and non-inflamed biopsies obtained from CD and UC patients, and biopsies of healthy subjects, were compared using webLIBSHUFF version 0.95 (http://libshuff.mib.uga.edu), the web interface for the LIBSHUFF (LIBrary SHUFFling) program version 1.22. The program provides a statistical test for the null hypothesis that two 16S rRNA gene libraries are samples of the same prokaryotic community. Significantly different libraries are assumed to have been derived from communities of different composition. The program is based on the work of Singleton *et al.* (2001).

Assessment of the richness of the collection of bacteria associated with biopsy samples obtained from CD, UC and healthy subjects was made using the DOTUR (distance-based OTU and richness) program (http://www.plantpath.wisc.edu/fac/joh/DOTUR.html), which assigns sequences to operational taxonomic units (OTUs, molecular species). OTUs are defined as containing sequences that are more than 3 % different from each other. The Shannon index is a sensitive measure of community richness and can be calculated using the DOTUR software (Hughes & Bohannan, 2004). The program is based on the work of Schloss and Handelsman (2005).

Coverage of the bacterial collections associated with biopsies was calculated by the method of Good (1953), according to which the percentage of coverage was calculated using the formula $[1-(n/N)]\times 100$, where n is the number of molecular species represented by one clone (single-clone OTU) and N is the total number of sequences in the library.

Library sequences were classified using the Library Compare Tool provided by the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/comparison/comp.jsp). The tool uses the RDP naïve Bayesian classifier, 2003, version 1.0 to provide rapid classification of library

Table 3. Detection of specific bacteria by PCR

Bacterial group	Primer and sequence (5′→3′)	Amplicon size	PCR programme reference
Bacteroides/Prevotella group	Bac32F: AACGCTAGCTACAGGCTT Bac303R: CAAATGTGGGGGACCTTC	276 bp	Bernhard & Field (2000)
Bifidobacterium sp.	Bif164: GGGTGGTAATGCCGGATG Bif662: CCACCGTTACACCGGGAA	523 bp	Kok *et al.* (1996)
C. coccoides group	gCcoc-F: AAATGACGGTACCTGACTAA gCcoc-R: CTTTGAGTTTCATTCTTGCGAA	438–441 bp	Matsuki *et al.* (2002)
C. difficile	KAG1: CTCGCATATAGCATTAGACCA KAG2: CATGGGATAGATATCAGGGC KAG4: ATTTCCCAACGGTCTAGTCC	KAG1/KAG4, 905 bp; KAG2/KAG4, 236 bp	Dove *et al.* (1990)
Helicobacter sp.	H276F: TATGACGGGTATCCGGC H676R: ATTCCACCTACCTCTCCCA	375 bp	Beckwith *et al.* (1997)
Lactic acid bacteria	Lac1: AGCAGTAGGGAATCTTCCA Lac2: ATTYCACCGCTACACATG	340 bp	Walter *et al.* (2001)
M. avium subsp. *paratuberculosis*	p89: CGTCGGGTATGGCTTTCATGTGGTTGCTGTG p92: CGTCGTTGGCCACCCGCTGCGAGAGCAAT	284 bp	Sechi *et al.* (2001)
	p90: GTTCGGGGCCGTCGCTTAGG p91: GAGGTCGATCGCCCACGTGA AV1: ATGTGGTTGCTGTGTTGGATGG AV2: CCGCCGCAATCAACTCCAG	p90/p91, 398 bp; AV1/AV2, 298 bp	Naser *et al.* (2004)
Sulfate-reducing bacteria	APS-FW: TGGCAGATMATGATYMACGGG APS-RV: GGGCCGTAACCGTCCTTGAA	396 bp	Deplancke *et al.* (2000)
Universal bacterial primers, real-time quantitative PCR	UniBac1 for: TCCTACGGGAGGCAGCAGT UniBac2 rev: GGACTACCAGGGTATCTAATCCTGTT	466 bp	Nadkarni *et al.* (2002)

sequences into the bacterial taxonomy. The classifier is trained mostly on known type-strain rRNA sequences. Each library sequence is assigned to a set of hierarchical taxa from phylum to genus rank, along with a confidence estimate for each assignment. The current hierarchy model used by the naïve Bayesian rRNA classifier comes from that proposed in the 2004 release of Bergey's Manual of Systematic Bacteriology (http://bergeysoutline.com).

Measurement of bacterial cell numbers associated with biopsies by real-time quantitative PCR. Because of the variation in biopsy size and weight, the unknown amounts of mucus associated with each biopsy, and the preponderance of human relative to bacterial nucleic acid in DNA extracts of biopsies, we standardized comparison of the numbers of bacteria associated with biopsies by determining the ratio of *E. coli* equivalents to the number of β-actin equivalents (human cells). The number of bacteria (*E. coli* equivalents) associated per human cell (human β-actin equivalents) was measured by reference to standard DNA curves generated from known numbers of *E. coli* DH5α and HEp-2 cells, respectively. Cells in an *E. coli* culture in LB broth were enumerated by plating aliquots of 10-fold dilutions on agar plates, which were incubated for 24 h at 37 °C, after which the remainder of the cells in the culture were used to extract DNA, as described above. This provided a DNA sample which was then subjected to 10-fold dilutions to generate samples equivalent to $4{\cdot}5\times10^{7}$–$4{\cdot}5\times10^{1}$ *E. coli* cells ml^{-1}. HEp-2 cells were harvested from cultures prepared in RPMI 1640 medium (GibcoBRL), and counted microscopically. DNA was extracted from the cells as described for *E. coli*, and dilutions were prepared equivalent to $2{\cdot}1\times10^{6}$–$2{\cdot}1\times10^{1}$ cells. Real-time quantitative PCR was performed using an Applied Biosystems 7900HT Fast Real-Time PCR system. Each PCR reaction, in duplicate, consisted of 12·5 µl SYBR Green PCR Master Mix (Applied Biosystems), 300 nM each primer in the case of bacterial detection (Table 3) or 0·5 µM of primers for the detection of the human β-actin gene (Stratagene), and 1 µl template DNA, brought to a final volume of 25 µl with sterile deionized water. The amplification programme was 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 60 s, and a final stage of 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s. Numerical values of *E. coli* equivalents and human β-actin equivalents were used to plot standard curves, from which the relative number of bacteria per human cell associated with biopsy samples was calculated.

Statistical tests. Tests used in the comparison of 16S rRNA gene clone libraries are given above. Other statistical analyses were Fisher's exact test, the Mann–Whitney non-parametric test, and Kruskal–Wallis non-parametric ANOVA.

RESULTS

Comparison of PCR/DGGE profiles

The 16S rRNA gene profiles of the bacterial collections associated with biopsies were generated by PCR coupled with DGGE. Examples of profiles are shown in Fig. 1. There was an average of 25 intensely stained DNA fragments per profile, regardless of intestinal region or subject group. To gain insight into the constancy of biopsy-associated bacterial profiles in the distal intestinal tract, the Dice similarity coefficient (tolerance 2–3 %) was determined from comparison of four to five regions of the intestine of 12 healthy subjects. The biopsy-associated bacterial profiles were very similar within individual subjects (mean 85·0 %, SEM 2·4 %). Comparisons made between individuals showed that the profiles were much less similar (terminal ileum 71·0 %, right colon 74·1 %, transverse colon 66·5 %, left colon 72·8 %, rectum 74·8 %, $n=12$), as has been reported for human faeces (Zoetendal *et al.*, 1998). Non-inflamed and inflamed biopsy samples from the same patient gave profiles that were highly similar (UC, mean 91·1 %, SEM 1·4 %; CD, mean 90·2 %, SEM 1·7 %).

Prevalence of selected bacterial groups detected by group-specific PCR

DNA sequences originating from sulfate-reducing bacteria, *Helicobacter* species, or *M. avium* subsp. *paratuberculosis* were not detected in biopsy samples collected from healthy subjects or patients. One healthy transverse colon biopsy harboured *C. difficile*. This result was confirmed by elution and sequencing of the PCR product (100 % identity, accession number X92982 *C. difficile*). Members of the *Bacteroides/Prevotella* group were detected in association with biopsies from 93 % of healthy subjects and 92 % of patients. Similarly, members of the *C. coccoides* group were commonly detected in healthy subjects (92 %) and IBD

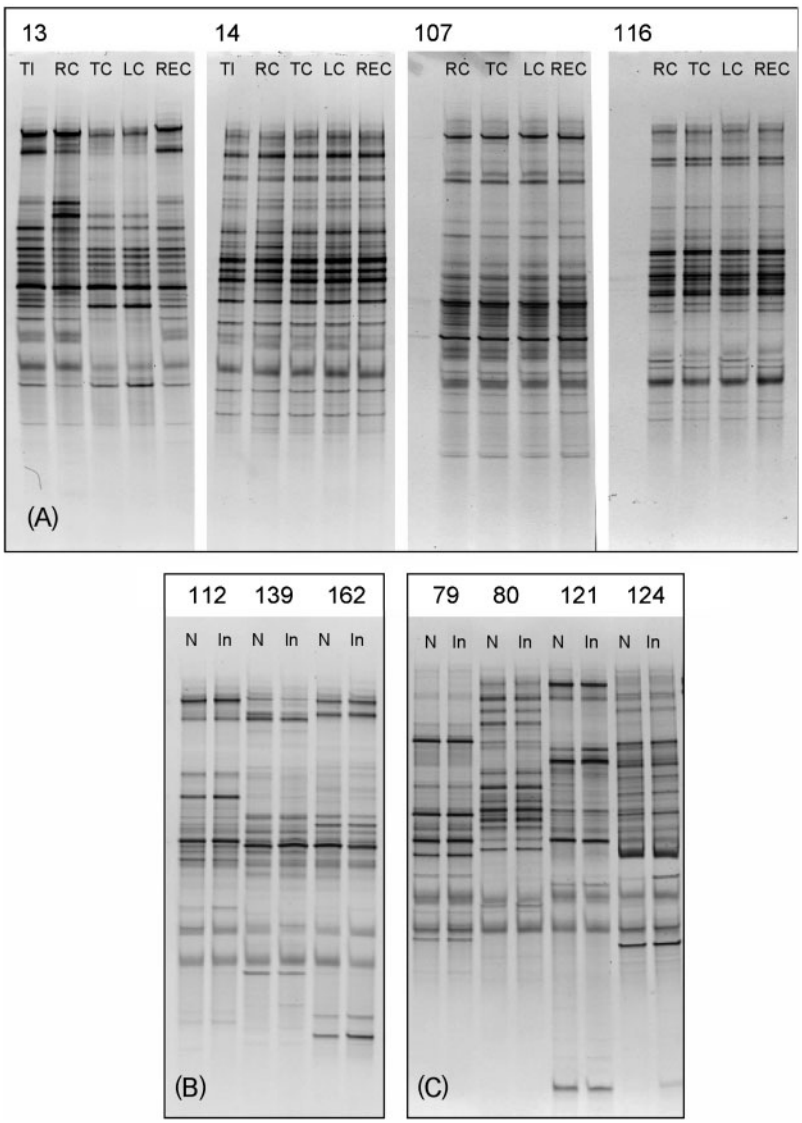

Fig. 1. Examples of PCR/DGGE profiles of bacterial collections associated with biopsies. These examples show the similarity of profiles obtained from different intestinal sites within individual subjects (A) and between inflamed and non-inflamed mucosa (B, C). (A) Healthy subjects; (B) CD patients; (C) UC patients. Abbreviations: TI, terminal ileum; RC, right colon; TC, transverse colon; LC, left colon; REC, rectum; N, non-inflamed mucosa; In, inflamed mucosa. Subject numbers are shown.

Table 4. OTU per library (3 % distance level) and coverage

Library	Total clones	OTU	Single-clone OTU	Coverage (%)	Shannon index
Healthy	235	99	50	78·7	4·3
Non-inflamed CD	374	132	80	78·6	4·2
Inflamed CD	435	153	91	79·0	4·4
Non-inflamed UC	305	102	58	80·9	4·1
Inflamed UC	386	138	68	82·3	4·4

patients (UC, 77 %; CD, 90 %). Bifidobacteria were detected in 17 % of healthy subjects and 26 % of patients. Lactic acid bacteria seemed to be more prevalent in patients (UC, 97 %; CD, 90 %) compared to healthy subjects (69 %), but these results were not statistically significant (Fisher's exact test $P>0{\cdot}05$).

Enumeration of bacteria associated with biopsies

UC patients had higher numbers of bacteria associated with biopsies than healthy subjects and CD patients (Fig. 2; Kruskal–Wallis non-parametric ANOVA, $P<0{\cdot}01$). The results obtained from CD and healthy subject samples did not differ ($P>0{\cdot}05$). Bacterial numbers associated with non-inflamed and inflamed mucosa within CD and UC groups did not differ ($P>0{\cdot}05$).

Comparison of 16S rRNA gene clone libraries

Coverage of the bacterial collections was similar for each library and averaged 80 % (Table 4), thus providing a valid basis for inter-group library comparisons. LIBSHUFF analysis showed that the libraries prepared from inflamed or non-inflamed mucosa of CD patients did not differ, nor did those generated from inflamed or non-inflamed mucosa of UC patients ($P>0{\cdot}05$). In contrast, the libraries prepared from CD and UC patients, and libraries prepared from healthy subjects differed from each other ($P=0{\cdot}05$).

DOTUR analysis indicated that the richness (bacterial diversity) tended to be similar for all groups (Shannon index; Table 4). Details of the classification of sequences in each library to bacterial phyla are given in Table 5. There was a trend for clones representing members of the phylum Bacteroidetes to be more prevalent in the libraries prepared from UC and CD patients compared to that of healthy subjects, but it was not statistically significant (Fishers exact test, $P>0{\cdot}05$). Comparison of the prevalence of genera within the phylum Bacteroidetes, however, showed that unclassified Bacteroidetes were more prevalent in samples collected from CD patients than in those from healthy subjects or UC patients (Table 6; Fishers exact test, $P<0{\cdot}01$). Unclassified members of the phylum Verrucomicrobia were only detected in biopsies from CD patients (Table 5), and unclassified *Porphyromonadaceae* from UC patients (Table 6).

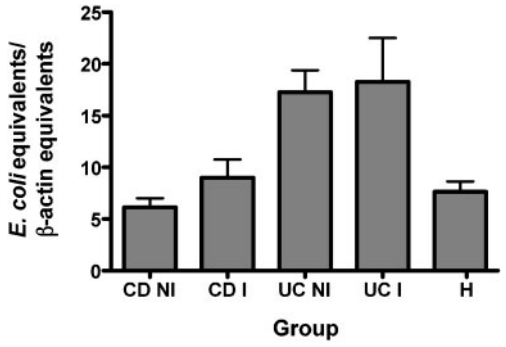

Fig. 2. Comparison of numbers of bacteria (*E. coli* equivalents) associated with biopsies. Mean values and standard errors (bars) are shown. Abbreviations: CD, Crohn's disease patients; UC, ulcerative colitis patients; H, healthy subjects (all sites combined); NI, non-inflamed mucosa; I, inflamed mucosa. Fourteen, 11 and 33 biopsies were examined for the CD, UC and H groups, respectively.

DISCUSSION

A considerable amount of information about genetic and immunological aspects of CD and UC has been obtained in recent years (Podolsky, 2002; Bouma & Strober, 2003). Yet knowledge of the microbiota–human interplay is incomplete, even though the bacterial community that inhabits the gut seems to play an important role in fuelling the chronic inflammation characteristic of IBDs (Macdonald & Monteleone, 2005). The patients investigated in our study were newly diagnosed and had not yet received treatment for their disease. The bacteriological results that we have obtained therefore represent the bacteriology of untreated disease, whether CD or UC. Three methodical approaches were used to compare the nature of the collections of biopsy-associated bacteria: creating DGGE profiles of 16S rRNA gene sequences amplified from bacterial DNA extracted from the biopsies, preparing 16S rRNA gene clone libraries whose sequences were compared, and PCR detection of selected bacterial groups known to be common in the human gut, reported to be aetiological agents of IBDs or putatively 'beneficial' bacteria.

Table 5. Representation of bacterial phyla in 16S rRNA gene clone libraries

Phylum	Healthy (235)*	UC inflamed (386)	UC non-inflamed (305)	CD inflamed (435)	CD non-inflamed (374)
Actinobacteria	3 (1·3)†	2 (0·5)	0·0	3 (0·2)	3 (0·8)
Bacteroidetes	63 (26·8)	134 (35·0)	100 (32·8)	138 (31·7)	138 (36·9)
Firmicutes	121 (52·8)	164 (42·2)	166 (54·4)	143 (42·8)	142 (38·2)
Fusobacteria	0·0	0·0	1 (0·3)	4 (0·5)	4 (1·1)
Genera *incertae sedis* TM7	0·0	0·0	1 (0·3)	0·0	0·0
Proteobacteria	15 (6·4)	34 (8·8)	17 (5·6)	32 (3·4)	32 (8·6)
Unclassified Bacteria	32 (12·8)	42 (12·7)	20 (6·6)	52 (20·9)	53 (14·2)
Verrucomicrobia	0	0	0	1 (0·2)	2 (0·5)

*Numbers in parentheses in the headings show the total number of clones.
†Values show the number of clones (percentage of total clones).

The numbers of bacteria associated with biopsies obtained from UC patients were approximately double those associated with samples from CD patients. This difference may reflect the altered nature of the mucus present on the mucosal surface of the colon of UC patients. The mucus is thinner and less sulphated than that of healthy subjects (Pullan *et al.*, 1994; Corfield *et al.*, 1996). The thinner layer may provide a more secure habitat for bacterial proliferation because the bacteria could be less likely to be dislodged by mucus flow. Alternatively or additionally, the bacteria might be more numerous because the non-sulphated mucins in the mucus are more easily degraded by bacterial cells and therefore provide an improved nutritional milieu (Roberton & Corfield, 1999). A thin mucus layer containing larger than normal numbers of bacteria might facilitate contact between bacterial antigens and the mucosal immune system.

Although there was inter-subject diversity among the bacterial profiles associated with biopsies, just as there is in the case of the faecal microbiota (Zoetendal *et al.*, 1998), pooling of 16S rRNA gene clones from individuals of the same group provided an overview of the bacterial collections associated with each disease. The compositions of the bacterial collections associated with biopsies were different in each group of subjects: the 16S rRNA gene clone library of healthy subjects was different in composition from that of CD and UC patients. In turn, the libraries from CD patients differed in composition from those of UC subjects. These results clearly indicated that the bacteria associated with the mucosal surface were characteristic of a specific disease. These differences in bacterial composition were not due to the inflamed condition of the tissue, which might have altered the microbial ecology of the site, because bacterial collections associated with biopsies collected from inflamed and non-inflamed regions of the same gut did not differ in composition. Further, the biopsy-associated bacterial profile was highly conserved within subjects from whom biopsies were obtained from different regions of the intestinal tract. The differences between the bacterial collections were therefore real and not due to sampling artifacts. Pin-pointing bacterial species that are exclusively associated with CD or UC is still difficult, given the incomplete knowledge of the taxonomy of gut bacteria. Our study shows, however, that the altered prevalence of unclassified members of the

Table 6. Prevalence of groups detected within the phylum Bacteroidetes

Phylogenetic group	Healthy (63)*	UC (234)†	CD (276)†
Genus *Bacteroides*	49 (78·0)‡	186 (79·5)	208 (75·4)
Genus *Chryseobacterium*	0	1 (0·4)	0
Genus *Prevotella*	7 (11·0)	7 (3·0)	9 (3·3)
Genus *Rikenella*	1 (1·5)	9 (3·8)	5 (1·8)
Unclassified order Bacteroidales	5 (8·0)	21 (9·0)	26 (9·4)
Unclassified class Bacteroidetes	1 (1·5)	8 (3·4)	28 (10·1)
Unclassified family *Porphyromonadaceae*	0	2 (0·9)	0

*Total number of clones within phylum Bacteroidetes.
†Combined clones from inflamed and non-inflamed samples.
‡Values show the number of clones detected (percentage of total clones).

phylum Bacteroidetes set the CD patients apart from other subjects. These bacteria therefore provide a target for future microbiological research concerning this disease. The qualitative PCR screen for *M. avium* subsp. *paratuberculosis*, *Helicobacter* species, *C. difficile* and sulfate-reducing bacteria did not support reports of their possible association with IBDs (Meyers *et al.*, 1981; Gibson *et al.*, 1991; Thompson, 1994; Ward *et al.*, 1996; Tiveljung *et al.*, 1999), nor did we detect a deficiency of lactic acid bacteria in patients relative to healthy subjects which might have indicated a need for 'probiotic' therapy (Macfarlane & Cummings, 1999).

There is now considerable agreement between studies that UC is characterized by increased numbers of mucosa-associated bacteria (Schultsz *et al.*, 1999; Kleessen *et al.*, 2002; Swidsinski *et al.*, 2002). Antibiotics are considered to have very limited usefulness in the treatment of UC (Podolsky, 2002), but, perhaps in the light of several bacteriological observations, the use of antibiotic preparations with pharmacological properties that target the mucosal surface of the colon would now be worthy of investigation. Specific bacterial targets for therapy of UC have not been identified by studies to date. LIBSHUFF analysis showed that the composition of the UC-derived library was different from that of healthy subjects, therefore further investigations may reveal suitable antimicrobial targets.

CD studies have produced variable reports, in which there was a lack of consistent association between particular bacteria and CD lesions (Prindiville *et al.*, 2004), the loss of certain anaerobic bacteria present in control samples (Ott *et al.*, 2004), and/or an increased number of mucosa-associated bacteria (Schultsz *et al.*, 1999; Kleessen *et al.*, 2002; Swidsinski *et al.*, 2002). Our results from newly diagnosed patients show that the bacteriology of CD and UC is different, and that unclassified members of the phylum Bacteroidetes have a higher prevalence in CD. There is a need to move now to culture-based studies that specifically target these bacteria in order to study their antigenicity in relation to the immune systems of CD patients. If these bacteria continue to be refractory to cultivation, the application of metagenomics methodology will be appropriate, because this approach provides access to the genetics of complex bacterial communities of the gut even when most members are non-cultivable (Walter *et al.*, 2005). While metagenomics has already been used to assess the biochemistry of bacterial communities (Handelsman, 2004), the gene pool that encodes the antigens to which the dysfunctional immune systems of IBD patients respond could equally well be investigated.

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