Molecular inventory of faecal microflora in patients with Crohn’s disease

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Received 25 February 2004; received in revised form 13 May 2004; accepted 17 May 2004

First published online 2 June 2004

Abstract

Intestinal microbial community is involved in the pathogenesis of Crohn’s disease, but knowledge of its potential abnormalities has been limited by the impossibility to grow many dominant intestinal bacteria. Using sequence analysis of randomly cloned bacterial 16S ribosomal DNA, the dominant faecal species from four Crohn’s disease patients and four controls were compared. Whereas marked inter-individual differences were observed in the faecal microflora of patients, three remained distantly related to controls on the basis of their operational taxonomic unit composition. Bacteroides vulgatus and closely related organisms represented the only molecular species shared by all patients and exhibited an unusually high rate of occurrence. Escherichia coli clones were isolated only in two patients with ileocolonic Crohn’s disease. Moreover, numerous clones belonged to phylogenetic groups or species that are commonly not dominant in the faecal microflora of healthy subjects: Pectinatus, Sutterella, Verrucomicrobium, Fusobacterium, Clostridium disporicum, Clostridium glycolicum, Clostridium ramosum, Clostridium innocuum and Clostridium perfringens.

Keywords: Crohn’s disease; 16S rRNA gene sequencing; Faecal bacterial diversity

1. Introduction

The aetiology of Crohn’s disease (CD) remains unclear. Microbial community of the bowel, the genetic susceptibility of the host and mucosal immunity seem to be involved in the development and recurrence of this inflammatory bowel disease (IBD) [1,2]. Mutations of the CARD15/NOD2 protein, which is involved in the recognition of bacterial cell wall components by human cells, are identified in about 30% of the patients with CD [3,4]. Although there is evidence concerning the role of intestinal bacteria, only few studies have looked for possible modifications of the microbial community during Crohn’s disease. Most of our current knowledge on the intestinal microflora is based on culturing and isolations of microorganisms from faecal material. These studies suggest higher levels of Bacteroides and enterobacteria in patients than in controls [5,6]. Interestingly, several animal models of inflammatory bowel disease have shown that Bacteroides are endowed with proinflammatory properties [7,8]. Culture-dependent methods to assess the colonic microflora suffer major limitations since they only enable the detection of
25–50% of dominant colonic bacterial populations [9,10]. Culture-independent techniques, based on sequence analysis of ribosomal RNA genes, have allowed considerable progress [10]. In a previous study using rRNA dot-blot hybridization method, we confirmed increased level of faecal enterobacteria in patients with colonic Crohn’s disease, and observed that more than 30% of the microflora did not hybridize with the probes used for healthy controls [11]. Phylogenetic analysis of bacterial 16S rRNA genes amplified directly from complex communities has proven to be an efficient strategy for exploring biodiversity of numerous environments [10,12,13]. Although time-consuming, this strategy has enabled the retrieval of non-cultivated intestinal species [10,14–16]. It also contributed to the design of oligonucleotide probes for monitoring and quantifying specific microorganisms that may not have been cultured yet. The aim of the present study was to identify potential primary modifications of the faecal microflora in patients with Crohn’s disease, which can be independent of treatment or status of the disease.

2. Materials and methods

2.1. Subjects

One faecal sample from four subjects with Crohn’s disease was collected into a clean container. Each sample was aliquoted in 125 mg amounts into 2 ml sterile screw-cap tubes and frozen at −20 °C within 4 h of defecation. The characteristics of the patients are presented in Table 1. All subjects had colonic involvement and none of them had received antibiotics for at least 1 month prior to faecal sampling. The published microflora of a 40-year healthy adult was used as control-1 [10] and the microflora of three other healthy adults (26-, 25- and 56 years) were analysed as controls 2, 3 and 4 [17].

2.2. Extraction of total DNA and PCR amplification

Total DNA was extracted from 125 mg of frozen faecal sample aliquots and purified as described by Suau et al. [10]. PCR amplifications were carried out with forward primer S-*Eub-0339-a-A-20 (5’-CTC CTA CGG GAG GCA GCA GT-3’) and reverse primer S-*Univ-1385-b-A-18 (5’-GCG GTG TGT ACA AGR CCC-3’) using the following conditions: initial denaturation of template DNA at 94 °C for 10 min; 25 cycles of denaturation (1 min at 97 °C), annealing (1 min at 59 °C), extension (1 min 30 s at 72 °C) and a final extension at 72 °C for 10 min.

2.3. Cloning, sequencing and analysis of sequence data

PCR products were purified as described by Suau et al. [10] and ligated into the pGEM-T vector (Promega) as specified by the manufacturer. Competent Escherichia coli was transformed.

Table 1

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* Crohn’s disease activity index.
Fig. 1. Phylogenetic tree derived from partial sequence data for the dominant faecal microflora of patient 1. Aligned bases corresponding to *E. coli* positions 561–1048 were used to construct this tree. Bar represents 1% sequence divergence. Designation of clones are in boldface type. The tree was constructed with the Neighbor-Joining program (with Olsen correction). Bootstrap values are based on 1000 replications. Bars on the right point out the phylogenetic groups according to the RDP [19] and Collins [24] nomenclatures. Strains used for phylogenetic analysis and not reported in Suau et al. [10] are listed below with their GenBank accession numbers:

- *Bacteroides acidofaciens* (AB021165)
- *Bacteroides fragilis* (X83937)
- *Bacteroides putredinis* (L16497)
- *Bacteroides thetaiotaomicron* (L16489)
- *Bacteroides uniformis* (AB050110)
- *Bifidobacterium dentium* (D86183)
- *Bifidobacterium longum* (M58739)
- *Bifidobacterium pseudocatenulatum* (AF028352)
- *Clostridium difficile* (AF072474)
- *Clostridium disporicum* (Y18176)
- *Clostridium faecalis* (AF070224)
- *Clostridium glycolicum* (AF028350)
- *Clostridium perfringens* (Y12669)
- *Clostridium ramosum* (M23731)
- *Dialister sp.* (AF287788)
- *Enterococcus faecalis* (AF070224)
- *Escherichia coli* (AF233451)
- *Fusobacterium naviforme* (AJ006965)
- *Herbaspirillum seropedicae* (Y10146)
- *Lactococcus lactis* (M58837)
- *Neisseria meningitidis* (Y17657)
- *Oxalobacter formigenes* (U49749)
- *Pectinatus frisingensis* (AF028352)
- *Peptostreptococcus anaerobius* (L04168)
- *Peptostreptococcus ramosum* (U25260)
- *Prosthecobacter fusiformis* (U60015)
- *Ruminococcus bromii* (X85099)
- *Selenomonas sp.* (AF385495)
- *Sporobacter termitidis* (Z49863)
- *Streptococcus pneumoniae* (AF385525)
- *Sutterella wadsworthensis* (L38011)
- *Veillonella atypica* (X84007)
- *Veillonella dispar* (X84006)
- *Veillonella ratti* (AF186071)
- *Verrucomicrobium sp.* VeGl2 (X93990)

coli DH10B cells (Life Technologies) were transformed with ligation products by heat shock (40 s at 42 °C) and recombinant cells were selected. Two plasmid-targeted primers, T7 (5'-TAA TAC GAC TCA CTA TAG GGC GA-3') and SP6 (5'-ATT TAG GTG ACA CTA TAG AAT AC-3') were used to amplify each insert using 35-cycle colony PCR. Sequencing reactions were prepared as described by Suau et al. [10]. Clones from patient 1 were sequenced with primer 1100R (5'-AGG GTT GCG CTC GTT G/A-3') and from patient 4 with primer 947R (5'-TTC GAA TTA AAC CAC ATG C-3') in our laboratory. Clones from patients 2 and 3 were entirely sequenced using SP6 and T7 primers by the Genoscope, Evry, France.

The 16S rRNA gene sequences we determined and the sequences of reference strains (obtained from the EMBL Data Library [18] and the Ribosomal Database Project [19]) were aligned using the PILEUP program in the GCG software package (EGCG version 8.00; Peter Rice, The Sanger Centre, Cambridge, UK) and the

Fig. 2. Phylogenetic tree derived from partial sequence data for the dominant faecal microflora of patient 2. Aligned bases corresponding to E. coli positions 339–1307 were used to construct this tree. For legend, see Fig. 1.
alignment was manually refined in the Seqlab Editor of GCG. Chimeric sequences were detected using the RDP CHECK_CHIMERA program [19]. Phylogenetic reconstructions were performed with Neighbor Joining method [20] using the Olsen correction of Seqlab software. The stability of relationships was assessed with the Bootstrap N-J program of ClustalX software (Version 1.64B). One thousand bootstrap trees were generated for each data set.

An operational taxonomic unit (OTU), or molecular species, contained all sequences from faecal clones and reference strains with less than 2% divergence [10,21]. In phylogenetic trees, each OTU is represented by a single representative faecal clone. This clone was used as a

Fig. 3. Phylogenetic tree derived from partial sequence data for the dominant faecal microflora of patient 3. Aligned bases corresponding to E. coli positions 381–1113 were used to construct this tree. For legend, see Fig. 1.
reference sequence for calculating phylogenetic distances from other aligned sequences, according to the Jukes–Cantor model on masked (unambiguously aligned) positions [22]. Coverage was calculated using Good’s method [23], according to which the percentage of coverage is 

\[
\frac{1}{2} \left(1 - \left(\frac{n}{N}\right)\right) \times 100
\]

where \(n\) is the number of molecular species represented by only one cloned sequence (single-clone OTUs) and \(N\) is the total number of cloned sequences. Phylogenetic groups and subgroups were defined according to the RDP [19] and Collins [24] nomenclatures. Algorithmic analyses using the numerical taxonomy and multivariate analysis system (NTSYS-pc Version 2.1, Exeter Software, Setauket, NY) were carried out to compare the different microflora. Strains used for phylogenetic analysis and not reported in Suau et al. [10] are shown in the legend of Fig. 1.

### 2.4. Sequence accession numbers

Partial sequences of representatives of each OTU observed in the Crohn’s disease or control microflora were deposited in GenBank with the acronym Cadhufec for Crohn Adult Human Faeces (Accession Nos.)

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**Fig. 4.** Phylogenetic tree derived from partial sequence data for the dominant faecal microflora of patient 4. Aligned bases corresponding to \(E. coli\) positions 362–841 were used to construct this tree. For legend, see Fig. 1.
AF530292 to AF530382) or Adhufec for Adult Human Faeces (Accession Nos.: AY471651 to AY471720).

3. Results

The average determined length of the ribosomal DNA sequences was 500 bases for patient 4, 580 bases for patient 1, and 1000 bases (Genoscope, Evry, France) for patients 2 and 3. About 20% of sequences were identified as possible chimera and were abandoned from further analyses. Phylogenetic analyses using the Neighbor-Joining method [20] with the Olsen correction were based on 420–860 unambiguously aligned homologous nucleotides. Among 359 clones analysed in Crohn’s disease microflora, 79 distinct OTUs were detected and 112 additional OTUs were defined for the controls.

3.1. Microflora of patient 1

Fig. 1 depicts the phylogenetic relationships among bacterial clones, which belong to the dominant microflora of patient 1. Ninety-seven clones (19 OTUs) clustered into seven subgroups. The Bacteroides group represented 37% of all clones with a high percentage of Bacteroides vulgatus OTU (27%). The E. coli OTU was represented by six clones. An OTU represented by three clones exhibited 8.1% divergence from the gram-negative species Sutterella wadsworthensis that belongs to the Bacteroides group. Thirteen clones (five OTUs) were included in the Clostridium coccoides group, another major phylogenetic group of the human microflora, which equates to Clostridium rRNA Cluster XIVa [24].

3.2. Microflora of patient 2

Fig. 2 shows the phylogenetic relationships among bacterial clones of the microflora of patient 2. One hundred and nine clones (18 OTUs) clustered into 10 subgroups. 42% of the sequences were included in the Bacteroides group, with a high occurrence for the B. vulgatus OTU (29%). Enterobacteria were represented by 11 clones (two OTUs); five in E. coli OTU and six in Klebsiella pneumoniae OTU. One clone branched within the gram-negative Fusobacterium group (Cluster XIX), in the Fusobacterium naviforme OTU. In the Clostridium coccoides group (3 OTUs), members of the Ruminococ- cus gnavus OTU were the most abundant with 32 clones. The Clostridium leptum subgroup was represented only by two clones. Two clones branched with Clostridium aff. innocuum, which belongs to the Eubacterium cylindroides subgroup equating to Clostridium innocuum rRNA Cluster XVI. Two other clones were included in the Clostridium ramosum rRNA Cluster XVIII. The Sporomusa group was represented by five clones.
included in the *Veillonella dispar* OTU. There were only six one-clone OTUs within this microflora and the coverage was 94.5%. Only two out of 18 OTUs (3/109 clones) had no cultured representatives.

### 3.3. Microflora of patient 3

The phylogenetic distribution of clones from the microflora of patient 3 is shown in Fig. 3. Seventy-two clones (29 OTUs) clustered into nine subgroups. In the *Bacteroides* group, seven clones were most closely related to the *B. vulgatus* OTU, but with 2.9% sequence divergence. One clone was included in the pathogenic *Clostridium perfringens* OTU. Twenty-five clones (seven OTUs) branched with the *Clostridium leptum* subgroup. *E. coli* 

### 3.4. Microflora of patient 4

Fig. 4 shows the phylogenetic relationships among bacterial clones of the dominant microflora in patient 4.
OTU closely related to *B. vulgatus* and with only 2.9% sequence divergence was identified in the remaining patient (patient 3) and represented 10% of its clones. This OTU formed a monophyletic group with the previously described *B. vulgatus* OTU. High bootstrap values also supported this branching. Some OTUs were shared by two microflora. Patients 1 and 2 shared six OTUs, gathering a large proportion of their clones (47.4% and 72.2%, respectively): *B. vulgatus*, *B. stercoris*, *B. distasonis*, *E. coli*, *R. gnarus* and an OTU presenting 5% divergence from *E. celerecrescens*. Patients 3 and 4 shared 5 OTUs: *B. vulgatus*, an OTU close to *B. putredinis*, another close to *Faecalibacterium praunitzii* (formerly named *Fusobacterium praunitzii*), *R. bromii* and HucB7. Patients 2 and 4 shared 2 OTUs: *B. vulgatus* and *E. faecalis*. This last OTU was not detected in the dominant microflora of controls. Finally, patients 2 and 3 shared 2 OTUs: *B. vulgatus* and an OTU close to *F. praunitzii*. Interestingly, an OTU close to *E. formicigenerans* was detected in all four control microflora and in patient 4. Similarly, *E. halii* and close relatives were present in all control microflora but not in any patient microflora (data not shown).

Algorithmic analysis using numerical taxonomy and multivariate analysis system (NTSYS) was performed to compare the four CD microflora and the controls using cluster analysis on the basis of their OTU composition and the relative abundance of each OTU (percentage of clones). A dendrogram illustrating the relationships among different microflora is presented in Fig. 5. In the consensus tree, the microflora of patients 1 and 2 clustered together, while those of patients 3 and 4 remained highly specific and distantly related. The microflora of patient 3 was more closely related to controls 1, 2 and 4 compared to the other Crohn’s disease patients. Marked inter-individual differences were also observed in the faecal microflora of controls, but they clustered together, except for control 3 (particularly due to a very high level of *Bifidobacterium* clones).

### 4. Discussion

The molecular inventory method eliminates dependence upon the isolation of pure cultures as a means of studying the diversity of microbial communities, and enables the description of species that have not yet been cultivated. We used this method to describe with high resolution the dominant faecal microflora of four patients with Crohn’s disease and to identify changes in the microbial community that could be either causal or consequential to this inflammatory bowel disease. These data could serve as reference for further studies.

We observed wide inter-individual differences in the faecal microflora composition of CD patients and specificities not evidenced in healthy volunteers. The
first concerned *B. vulgatus* and its close relatives. Not only was it the only species shared by all four patients, but was also represented by a particularly high percentage of clones (7–29%). For comparison, the *B. vulgatus* OTU represented only 2%, 1.1%, 0% and 1.5% of the clones in the molecular inventories of controls 1–4. Similarly, it also represented 2% for another 40-year healthy adult [25]. Previous data obtained using culture techniques showed that *B. vulgatus* was detected in the majority of human faecal microflora and accounted for about 10^10 CFU g^-1 [26]. Ruseler-van Embden reported that this species accounted for 40% of the total cultivated flora in a group of patients with Crohn’s disease and hypothesised that this microorganism might play a role in the pathogenesis of CD by increasing breakdown of the mucus layer [5,27]. In line with these results, *B. vulgatus* also plays a key role in initiating spontaneous colitis in HLA-B27 transgenic rats [8].

The second distinctiveness in microflora of our CD patients concerned enterobacteria. Algorithmic analyses using NTSYS provided a dendrogram clustering the microflora from patients 1 and 2. In addition to their very high count in *B. vulgatus* clones, the *E. coli* OTU represented 6% and 5% of all clones in patients 1 and 2, respectively. For comparison, *E. coli* clones were never recovered among the dominant microflora of healthy controls [10,17]. Using culture techniques, enterobacteria were more often isolated as dominant microorganisms in CD patients than in controls [28]. In a study using quantitative dot-blot hybridization, a signal for enterobacteria was detected in faecal samples from patients with Crohn’s disease, indicating that they belonged to the dominant microflora, whereas no signal was detected in the dominant faecal flora of 16 healthy controls [11]. In addition, some invasive *E. coli* strains with particular adhesion properties are more likely than others to be associated with the ileal mucosa of patients with CD [29]. Clones from *K. pneumoniae*, another species of enterobacteria known to increase intestinal permeability, were also recovered from the microflora of patient 2. Swidsinski and colleagues [30] analysed the mucosal microflora of biopsies from CD patients and also found a predominance of *Bacteroides* and *Enterobacteriaceae* as they were isolated from 95% and 82% of 340 patients. In agreement with these results, Neut and colleagues [31] recovered high counts of *Bacteroides* and *E. coli* in the mucosal flora, often accompanied by *Fusobacteria* during an early recurrence of Crohn’s disease after ileocolonic resection. Potentially, the analysis of more than 100 clones would make it possible to obtain enterobacteria from patients 3 and 4. However, the increase in facultative anaerobes in patients with ileocolonic Crohn’s disease accompanied the decrease in the strictly anaerobic *Clostridium leptum* group, since only 1% and 1.8% of all clones from the microflora of patients 1 and 2, respectively, branched within this group.

*Enterococcus faecalis* was recovered from two microflora. This is consistent with the recovery of streptococcal antigens from the faeces of CD patients [32]. Moreover, *E. faecalis* has been shown to induce inflammatory bowel disease in interleukin-10 knockout mice [33]. This genus was also recovered from the mucosal flora of CD patients after ileocolonic resection [31].

Numerous uncommon clones were recovered from the dominant faecal microflora of patients in this study. Some of these have not been cultivated yet. In our previous study using dot-blot hybridization, we pointed out that many bacteria in the dominant microflora of patients with colonic Crohn’s disease were different from bacteria in healthy subjects and remained unidentified [11]. The present study elucidates this phylogenetic gap and shows that these unusual species do not belong to a single group but to diverse phylogenetic groups or species, uncommon in dominance: *Pectinatus*, *Sutterella*, *Verrucomicrobiurn*, *Fusobacterium*, *Clostridium dispersicum*, *Clostridium perfringens*, *Clostridium glycolicum*, *Clostridium innocua*, and *Clostridium ramosum*. As stools were directly collected into clean containers, these unusual OTUs should not come from environmental contamination. Firstly, clones whose closest relative was the gram-negative species *S. wadsworthensis* were recovered from the microflora of patient 1. This microaerophilic *Campylobacter gracilis*-like microorganism has already been associated with infections in humans [34]. Similarly, 12 clones recovered from the dominant microflora of patient 4 (15%) were linked to one OTU detected in microflora of control 1 (0.35%), whose closest relative was the facultative anaerobic prosthecate bacterium, *Verrucomicrobium spinosum*. Verrucomicrobia constitute a newly identified gram-negative bacterial division that is widely distributed in aquatic and terrestrial ecosystems, but for which only few organisms have been isolated and none from the gastrointestinal tract [35]. In addition, one clone branched with the gram-negative genus *Fusobacterium* in the microflora of patient 2. The species *F. necrophorum* of these strongly proteolytic bacteria is readily involved in human intestinal diseases [36]. The faecal microflora of patient 1 also contained large numbers of clones (31%) exhibiting 5.1% rDNA sequence divergence with the strictly anaerobic beer-spoilage bacterium *P. frisingensis*. Interestingly, *P. frisingensis* is phylogenetically ranked in the *Sporomusa* “low G+C gram-positive phylum”, members of which have a gram-negative cell wall. One clone from the microflora of patient 3 corresponded to *Clostridium perfringens*, another to *Clostridium disporicum*, and a third clone was included in *Clostridium coelestum*, close to *C. ramosum*. *C. ramosum* was also recovered from the microflora of patient 2. In a previous study, a high agglutination titre against *C. ramosum* was detected in patients with ulcerative colitis [37].

With respect to the *Bifidobacterium* genus, which is considered as beneficial to the host, only two clones of *B.
Acknowledgements

This work was supported by grants from Association François Aupertit, Paris, Société Nationale Française de Gastroentérologie, Paris, Institut de Recherche sur les Maladies de l’Appareil Digestif (IRMAD), Laboratoires AstraZeneca, Rueil Malmaison, France, and the European Research Project FLAIR CT97-3035. We acknowledge Genoscope, Evry, France, for part of the sequencing.

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