

Molecular Profiling of the *Clostridium leptum* Subgroup in Human Fecal Microflora by PCR-Denaturing Gradient Gel Electrophoresis and Clone Library Analysis

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Shen, J., Zhang, B., Wei, G., Pang, X., Wei, H., Li, M., Zhang, Y., Jia, W., & Zhao, L. (2006). Molecular profiling of the *Clostridium leptum* subgroup in human fecal microflora by PCR-denaturing gradient gel electrophoresis and clone library analysis. *Applied and Environmental Microbiology*, 72(8), 5232–5238.

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Abstract:

A group-specific PCR-based denaturing gradient gel electrophoresis (DGGE) method was developed and combined with group-specific clone library analysis to investigate the diversity of the *Clostridium leptum* subgroup in human feces. PCR products (length, 239 bp) were amplified using *C. leptum* cluster-specific primers and were well separated by DGGE. The DGGE patterns of fecal amplicons from 11 human individuals revealed host-specific profiles; the patterns for fecal samples collected from a child for 3 years demonstrated the structural succession of the population in the first 2 years and its stability in the third year. A clone library was constructed with 100 clones consisting of 1,143-bp inserts of 16S rRNA gene fragments that were amplified from one adult fecal DNA with one forward universal bacterial primer and one reverse group-specific primer. Eighty-six of the clones produced the 239-bp *C. leptum* cluster-specific amplicons, and the remaining 14 clones did not produce these amplicons but still phylogenetically belong to the subgroup. Sixty-four percent of the clones were related to *Faecalibacterium prausnitzii* (similarity, 97 to 99%), 6% were related to *Subdoligranulum variabile* (similarity, ~99%), 2% were related to butyrate-producing bacterium A2-207 (similarity, 99%), and 28% were not identified at the species level. The identities of most bands in the DGGE profiles for the same adult were determined by comigration analysis with the 86 clones that harbored the 239-bp group-specific fragments. Our results suggest that DGGE combined with clone library analysis is an effective technique for monitoring and analyzing the composition of this important population in the human gut flora.

Article:

The human gastrointestinal tract harbors a highly diverse microbial community, which plays important roles in host nutrition, immunology, health, and disease. In addition to *Bacteroides* spp. and the *Clostridium coccoides* cluster, the *Clostridium leptum* cluster (3) is one of the most predominant populations in the human fecal microflora (2, 6, 11, 12, 30). Including species that

belong to the genera *Clostridium*, *Eubacterium*, and *Ruminococcus*, the *C. leptum* cluster contains numerous butyrate-producing and fibrolytic species (6, 22). The metabolic activities of these organisms have a significant effect on the health of the human colon. Members of this cluster are highly oxygen sensitive (22) and difficult to culture (9), so the development of molecular methods to specifically study the diversity of this population and to monitor changes in the cluster after intervention is critical.

Probes (17, 27, 29) and primers (18) specific for the *C. leptum* group have been designed and used to enumerate this population by fluorescent in situ hybridization (FISH), dot blot hybridization, and real-time PCR. However, these group-specific methods can provide information only on the abundance of the whole population in the human fecal microflora, and they provide few details concerning the composition of the population at the species level.

Another way to study the diversity of this group is to use fingerprinting approaches, such as PCR-based denaturing/temperature gradient gel electrophoresis (DGGE/TGGE). Due to their obvious strengths, including high sensitivity, high resolution, and high throughput, group-specific PCR-based DGGE-TGGE techniques have been developed for *Bacteroides* spp. (21), *Bifidobacterium* spp. (23, 26), and bacteria related to *Lactobacillus* (13, 34) in the human gut microbial community. However, to the best of our knowledge, no study has used fingerprinting techniques to determine the composition of the *C. leptum* subgroup in the human fecal flora despite its abundance and important functions.

In the present study, we developed a PCR-DGGE method using a validated *C. leptum* cluster-specific primer set (18) to rapidly profile the human fecal *C. leptum* subgroup. A clone library was constructed to study the structure of one adult's fecal *C. leptum* cluster and to determine the identities of the majority of bands in the DGGE pattern. Our results suggest that a combination of *C. leptum* group-specific PCR-DGGE and clone library analysis can be used to analyze and monitor the diversity of this important population in the human gut flora effectively.

MATERIALS AND METHODS

Collection of fecal samples and DNA extraction

Fresh fecal samples were collected from six adults (individuals A to F) (individuals A to D and F were 24 to 28 years old; individual E was 43 years old) and five children (individuals G to K) (individual G was 10 years old, and individuals H to K were 5.5 years old) of both sexes (individuals A, C, and D were females; the other individuals were males). These individuals were genetically unrelated, healthy subjects who lived on a Chinese diet. No volunteer had received antibiotics, probiotics, and prebiotics during the 2 months before sampling. Seven samples were obtained from individual G in a 3-year period. DNA was extracted with a QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany) used according to the manufacturer's protocol. The amount of DNA was determined by using DyNA quant 200 (Hoefer, San Francisco, Calif.), and the integrity of DNA was checked by using 0.8% agarose gel electrophoresis gels stained with ethidium bromide.

C. leptum cluster-specific PCR amplification

C. leptum cluster-specific primers sg-Clept-F and sg-Clept-R3 were used (18); these primers target nucleotides 933 to 948 and 1164 to 1184 of the 16S rRNA gene (*Escherichia coli*

numbering), respectively, and produce 239-bp PCR products. For DGGE analysis, a 40-bp GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) (19) was attached to the 5' end of either sg-Clept-F (5'-GCACAAGCAGTGGAGT) or sg-Clept-R3 (5'-CTTCCTCCGTTTTGTCAA). The PCR mixture (25 μ l) contained 1 U of TaKaRa (Takara, Dalian, China) rTaq polymerase, 1 \times PCR buffer (Mg^{2+} free), 2 mM $MgCl_2$, 6.25 pmol of each primer, each deoxynucleoside triphosphate at a concentration of 200 μ M, and 40 ng of extracted fecal DNA. The samples were amplified using a PCR Express system (Thermo Hybaid, Middlesex, United Kingdom) and the following program: 94°C for 5 min; 30 cycles of 94°C for 20 s, 50°C for 20 s, and 72°C for 30 s; and finally 72°C for 5 min. The sizes of PCR products were assessed by electrophoresis on a 1.5% (wt/vol) agarose gel.

DGGE analysis of PCR amplicons

DGGE was performed using a Dcode System apparatus (Bio-Rad, Hercules, Calif.). PCR products (250 ng) were separated on 8% (wt/vol) polyacrylamide gels with a 30% to 52.5% denaturant gradient (100% denaturant was 7 M urea and 40% deionized formamide). Electrophoresis was performed in 1 \times Tris-acetate-EDTA buffer at 200 V (constant voltage) and 60°C for 4 h. The gels were stained with SYBR Green I (Amresco, Solon, Ohio) and were photographed with a UVI gel documentation system (UVItec, Cambridge, United Kingdom). A dendrogram of the DGGE profiles was constructed based on the Dice similarity coefficient using unweighted pair group method clustering with the UVI band/map software (UVItec).

Cloning of the PCR products and clone library analysis

A PCR was performed with one universal primer, 27f (5'-GAGAGTTTGATCCTGGCTCAG), and one specific primer, sg-Clept-R3, using a 25- μ l mixture that contained 0.625 U of TaKaRa rTaq polymerase (Takara, Dalian, China), 1 \times PCR buffer (Mg^{2+} free), 2 mM $MgCl_2$, 10 pmol of each primer, each deoxynucleoside triphosphate at a concentration of 200 μ M, and 20 ng of extracted fecal DNA from individual F. The thermal cycling conditions were as follows: 94°C for 5 min; 25 cycles of 94°C for 20 s, 50°C for 20 s, and 72°C for 3 min; and finally 72°C for 5 min. PCR products that were approximately 1,100 bp long were excised from the 1.0% agarose gel and purified using a DNA gel extraction kit (V-gene, Hangzhou, China) as recommended by the manufacturer. The purified PCR products were cloned into *E. coli* DH5 α using the pGEM-T Easy vector system (Promega, Madison, Wis.). One hundred recombinant clones were randomly selected for analysis.

The plasmids were extracted using the alkaline lysis method and were used as templates for amplification of the 239-bp *C. leptum* cluster-specific 16S rRNA gene fragment with primers sg-Clept-F and sg-Clept-GC-R3. The amplicons were subjected to DGGE analysis. Clones that migrated the same distance in the gel were temporarily grouped into one operational taxonomic unit (OTU). For multiple clones in the same OTU, inserts were reamplified using primers 27f and sg-Clept-R3 and then digested with restriction enzymes Csp6I and HinfI (MBI Fermentas, Hanover, MD) in order to determine the number of sequence types present in the OTU. A representative clone was selected for each restriction pattern and sequenced (Invitrogen, Shanghai, China). If clones exhibited $\geq 99\%$ sequence similarity, they were classified as the same sequence type and remained in the same OTU. If not, they were classified in different OTUs.

Inserts of clones that did not produce the 239-bp amplicon were also digested with restriction enzymes Csp6I and HinfI (MBI Fermentas, Hanover, MD). Clones that had the same restriction pattern were classified in one OTU, and a representative clone from each OTU was sequenced (Invitrogen, Shanghai, China).

Identification of the phylogeny of the DGGE bands

To determine the identity of each band in the DGGE profile of individual F, the migration positions of the library clones that harbored the 239-bp fragment were compared to the DGGE profile. The sequence of each DGGE band was represented by the sequence of the clones having the same migration behavior.

DGGE bands for which there were no matching clones in the library were excised from the gel and eluted by incubation in 50 µl sterile distilled water at 4°C overnight. A 2.5-µl aliquot of the resulting preparation was used as a template for reamplification of the sequence with primers sg-Clept-F and sg-Clept-R3. The PCR products were purified using an UltraClean 15 DNA purification kit (MO BIO, Solana Beach, CA), ligated into the pGEM-T Easy vector (Promega, Madison, Wis.), and transformed into competent *E. coli* DH5α cells. Positive clones were picked randomly, and inserts were amplified and screened to determine their migration positions by DGGE. Clones that migrated to the same positions as the original DGGE bands were sequenced (Invitrogen, Shanghai, China).

Sequence analysis

Sequences obtained in our study were aligned with the sequences in the Ribosomal Database Project (release 9) database to determine their closest relatives. The levels of similarity between sequences and their relatives were calculated using the Jukes-Cantor model. A neighbor-joining phylogenetic tree was constructed with the Molecular Evolutionary Genetics Analysis package (MEGA3) (16) with the Jukes-Cantor algorithm. The phylogenetic robustness was assessed by bootstrap analysis with 100 replicates using the same software.

Nucleotide sequence accession numbers

The sequences in this study have been deposited in the GenBank database under accession numbers DQ350780 to DQ350810.

RESULTS

*Development of the PCR-DGGE method for profiling the *C. leptum* cluster of bacteria in the fecal microflora*

For the PCR-DGGE method for profiling bacteria belonging to the *C. leptum* cluster, we chose the *C. leptum* cluster-specific primers sg-Clept-F and sg-Clept-R3, which produce amplicons that are 239 bp long (18). To resolve the sequences by DGGE, a GC clamp was attached to each primer. Eight clones in our library were used as references to study the effects of the location of the GC clamp on the separation of different sequences in the gel. These clones were used as templates to amplify the 239-bp group-specific fragments with the GC clamp attached to either sg-Clept-F or sg-Clept-R3, and the products were subjected to DGGE. When the GC clamp was added to the forward primer (sg-Clept-F), amplicons from each recombinant plasmid tested appeared as double bands in gels (data not shown). This may have resulted from the GC clamp (hairpin formation) causing incomplete elongation in PCR and producing DNA molecules with

slightly different melting behaviors (20). When the GC clamp was attached to the reverse primer (sg-Clept-R3), each clone produced a single band that was well separated from the other bands in the gel with a 30% to 52.5% denaturant gradient. Thus, sg-Clept-F and sg-Clept-GC-R3 were used to generate human fecal *C. leptum* cluster-specific DGGE patterns.

Use of DGGE to profile the human fecal C. leptum cluster and monitor the succession over time

The *C. leptum* cluster-specific amplicons derived from fecal samples from 11 healthy individuals were analyzed by DGGE. Both the number of bands (range, 5 bands for individual C to 22 bands for individual A) and the positions of specific bands differed for the different individuals (Fig. 1a), and all subjects were separated from each other by cluster analysis (Fig. 1b), suggesting that the fecal *C. leptum* cluster is host specific. However, there were four bands that were produced by most of the 11 volunteers (at least 8 of 11 people) (Fig. 1a).

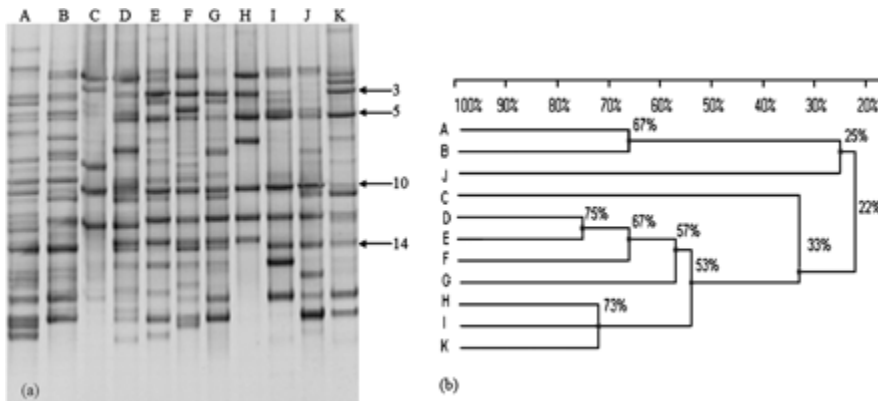


Figure 1: (a) DGGE profiles of the fecal *C. leptum* group for 11 healthy individuals. Bands produced by most volunteers (at least 8 of 11 volunteers) are indicated by arrows, and the numbers correspond to band numbers in the DGGE profile of individual F shown in Fig. 3. (b) Dendrogram of the DGGE profiles shown in panel a.

The composition of the fecal *C. leptum* cluster of individual G was monitored by DGGE over a 3-year period (Fig. 2). There were fluctuations in the structure of the population from June 2001 to March 2003 (Fig. 2a, lanes 1 to 5), and a major change that occurred between early and late August 2001 (Fig. 2a, lanes 3 and 4) was detected based on the similarity-based dendrogram (Fig. 2b). However, on average the variations in this individual over time were smaller than the variations between different individuals (Fig. 1b and 2b). From April 2003 to June 2004 (Fig. 2a, lanes 6 and 7), the structure of the fecal *C. leptum* cluster of this child remained stable for 1 year.

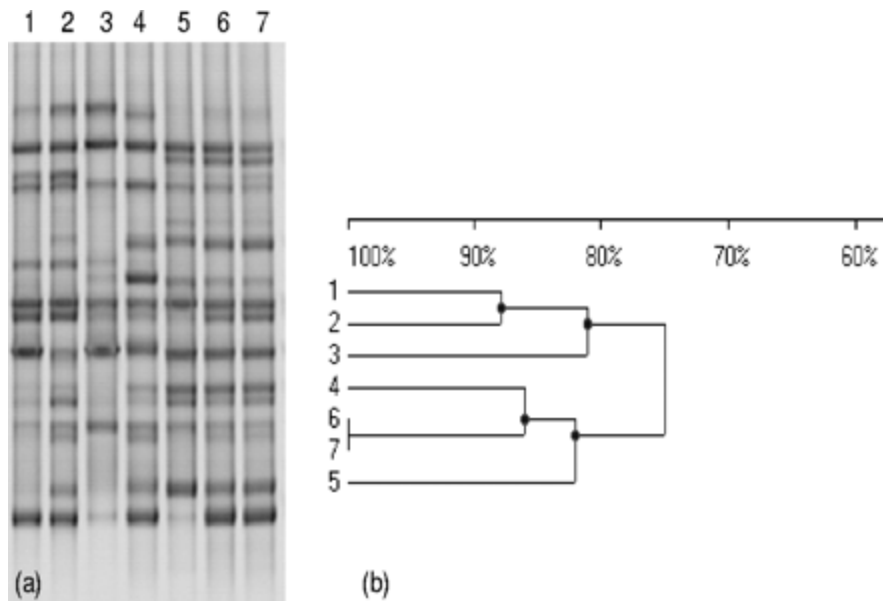


Figure 2: Monitoring of the fecal *C. leptum* subgroup of a child (individual G) over time. (a) DGGE profiles of the *C. leptum* group for seven fecal samples collected from the healthy child during a 3-year period. Lanes 1 to 7 contained fecal samples taken on 3 June 2001, 15 July 2001, 5 August 2001, 23 August 2001, 20 March 2003, 14 April 2003, and 14 June 2004, respectively. (b) Dendrogram of the DGGE profiles shown in panel a.

Clone library analysis of the *C. leptum* cluster for individual F

A clone library was constructed with 100 clones consisting of 1,143-bp inserts of 16S rRNA gene fragments that were amplified from the fecal DNA of individual F with one forward universal bacterial primer (27f) and one reverse group-specific primer (sg-Clept-R3). The recombinant clones in the library were amplified using primers sg-Clept-F and sg-Clept-GC-R3 in order to determine their migration positions in a DGGE gel. Eighty-six of the 100 clones yielded the expected 239-bp PCR products, while 14 clones did not.

Based on their migration behavior and restriction patterns, the 86 clones were classified into 24 OTUs (Fig. 3) either matching specific bands in the DGGE profile or designated OL 1 to OL 5. Representative clones are shown in Fig. 3. Twenty-six percent of the clones (six OTUs) exhibited the highest levels of similarity (98.4 to 99.2%) to *Faecalibacterium prausnitzii* L2-6, 19% of the clones (seven OTUs) exhibited the highest levels of identity (97.3 to 99.6%) to *F. prausnitzii* A2-165, 19% of the clones (four OTUs) were very similar (98.7 to 99.3%) to butyrate-producing bacterium M21/2, whose 16S rRNA gene sequence is closely related to the sequence of *F. prausnitzii* (Fig. 4), 6% of the clones (three OTUs) were most closely related to *Subdoligranulum variabile* (similarity, 98.6 to 99.5%), 2% of the clones (two OTUs) were very similar (~99%) to butyrate-producing bacterium A2-207, an unclassified member of the *Clostridiales* that is distantly related to *Eubacterium desmolans* (similarity, 96%) (1), and 14% of the clones (two OTUs) were remotely related to *Ruminococcus flavefaciens* (similarity, ~92%) and thus may represent unknown species. In a comparison of the migration distances of the 86 clones, 8 clones (belonging to eight OTUs) appeared to be unique as their migration positions were different from those of all other OTUs (data not shown). Therefore, the “coverage” of the library analysis in terms of unique band positions in the gel was 91%.

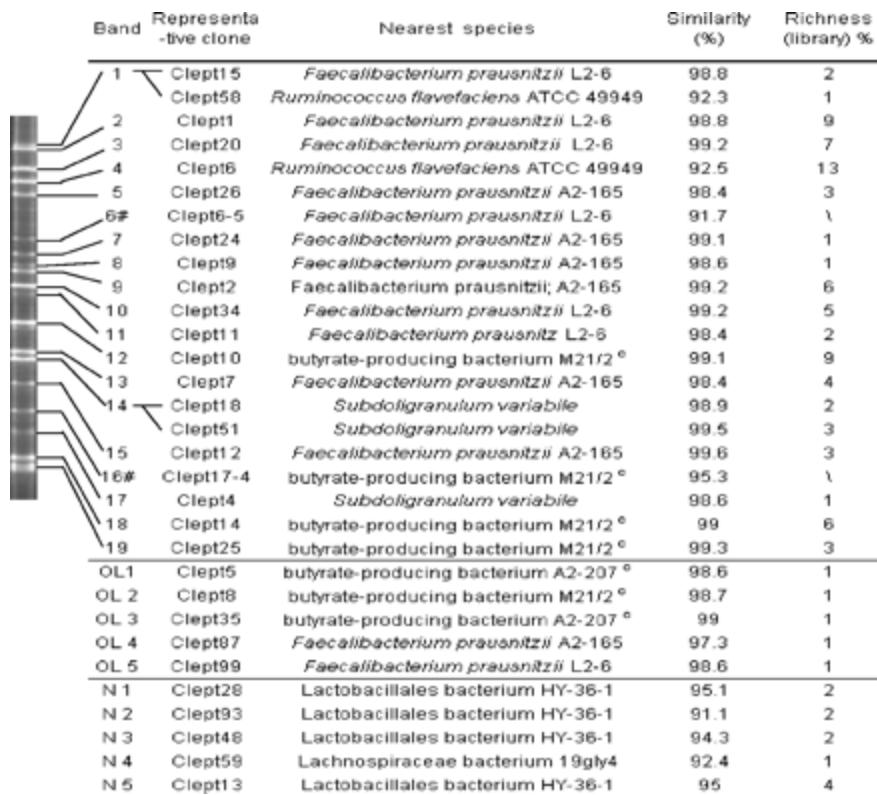


Figure 3: Sequence analysis of representative clones belonging to different OTUs in the library and identification of dominant bands in the DGGE pattern of individual F by a comparison of the migration positions of clones to the DGGE profile. Representative clones, the species most closely related to the clones, the levels of similarity, and the richness in the clone library are indicated. A number sign indicates a DGGE band with no corresponding clone in the library. OL, clones in the library with no matching DGGE bands; c, unclassified *Clostridiales*; N, clones producing no amplicons when they were amplified with primers sg-Clept-F and sg-Clept-R3.

The remaining 14 clones that did not produce the 239-bp fragment were classified into five OTUs based on their restriction patterns. Their closest relatives were either *Lactobacillales* bacterium HY-36-1 or *Lachnospiraceae* bacterium 19gly4; the levels of similarity were low (91 to 95%) (Fig. 3), and the clones potentially belonged to unidentified species. Sequence analysis revealed that the sequences all had a “T” in place of the second base, “G,” at the 3’ end of primer sg-Clept-F, which prevented annealing to the primer and thus yielded no amplicons with the group-specific primer pair.

The phylogenetic relationships of all representative clones in the library were assessed by constructing a phylogenetic tree (Fig. 4). Clones with or without the 239-bp cluster-specific amplicons all grouped with sequences from pure-culture species belonging to the *C. leptum* cluster and fell into four groups: 64% were related to *F. prausnitzii*, 6% grouped closely with *S. variabile*, 2% grouped with butyrate-producing bacterium A2-207, and 28% were not identified at the species level (14% were distantly related to *R. flavefaciens*, and the other 14% were remotely related to *Sporobacter termitidis* and *Papillibacter cinnamivorans*) (4).

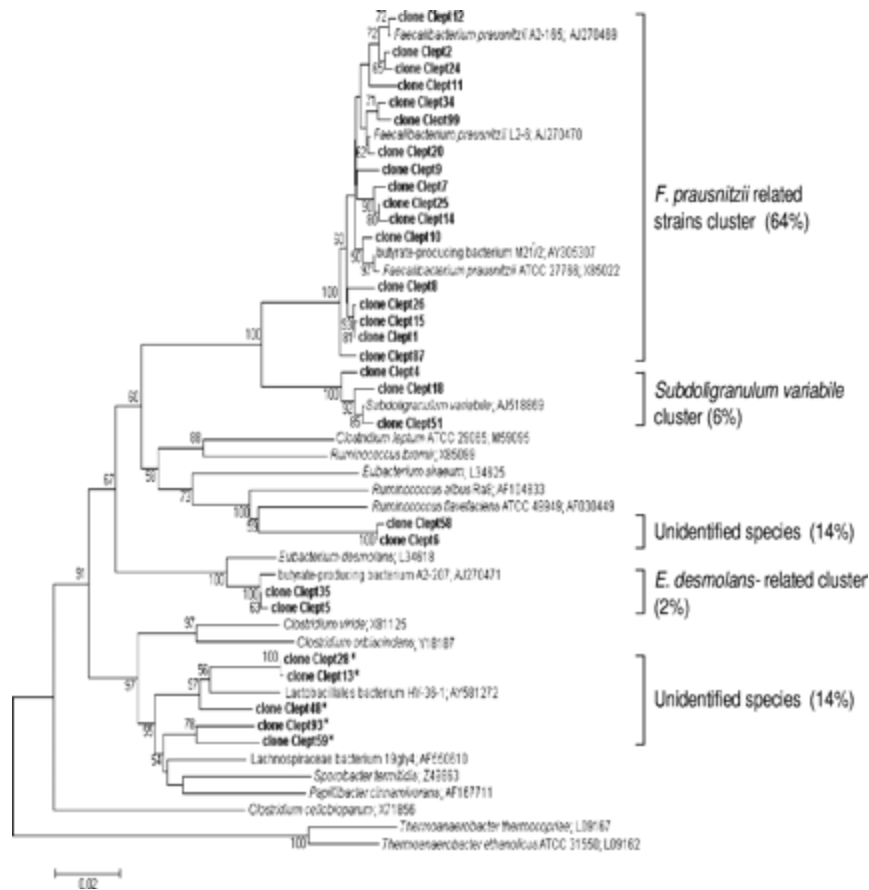


Figure 4: Phylogenetic tree showing the relationships between representative clones in our study and species belonging to the *C. leptum* cluster. The tree was constructed based on the region from base 27 to base 1181 of the 16S rRNA genes. Representative clones in the library are indicated by boldface type; clones retrieved from the GenBank database are indicated by italics, and their accession numbers are given. Bootstrap values greater than 50% are indicated at the nodes. *Clostridium cellobioparum* (a member of *Clostridium* cluster III) and *Thermoanaerobacter thermocopriae* and *Thermoanaerobacter ethanolicus* (members of *Clostridium* cluster V) are included as outgroups. All clones sequenced fell into the four groups indicated on the right, and the values in parentheses are the percentages of clones in the groups (a total of 100 clones). Asterisks indicate clones that do not harbor the 239-bp group-specific fragment.

Phylogenetic analysis of dominant bands in the DGGE pattern of individual F

To determine the identity of each band in the DGGE profile of individual F, the mobilities of *C. leptum* subgroup-specific fragments of the 86 clones were compared to the DGGE pattern of individual F. Eighty-one clones were assigned to 17 of 19 bands, while five clones did not match any detectable bands (Fig. 3).

Bands related to *F. prausnitzii* strains (*F. prausnitzii* A2-165, *F. prausnitzii* L2-6, and the butyrate-producing bacterium M21/2) were most frequently detected in the DGGE pattern. Sequences derived from *S. variabile* and an unidentified species related to *R. flavefaciens* were also detected. Bands 1 and 14 may each harbor two sequences (Fig. 3), and the levels of similarity between the two sequences in each band were 85% and 98.6%, respectively.

For two bands (bands 6 and 16 in Fig. 3) in the DGGE profile of volunteer F there were no matching clones in the library. These bands were excised from the gel, cloned, and sequenced, and they exhibited relatively low levels of similarity to *F. prausnitzii* L2-6 (91.7%) and butyrate-producing bacterium M21/2 (95.3%) based on the 239-bp sequences. These bands may have originated from uncultured members of this cluster.

DISCUSSION

Butyrate is the preferred energy source in the colonic mucosa (25), protecting against colitis and colorectal cancer, and it is important for the normal development of colonic epithelial cells (10, 22). Numerous members of the *C. leptum* cluster can produce butyrate, so in our study we developed the group-specific PCR-DGGE and clone library methods to study the diversity of this important population of the human gut microflora.

Researchers have used nested PCR (cluster-specific primers in the first round and universal primers in the second round)-based temporal thermal gel electrophoresis to study the *C. leptum* cluster in municipal landfill sites (32). However, this type of nested PCR has inherent disadvantages, including increased bias (31) and possible contamination of genomic DNA in the second round of PCR (13). In our DGGE study, we used primers sg-Clept-F and sg-Clept-R3 (18) so that the length of the PCR products (239 bp) was amenable to DGGE separation, so only one round of PCR was needed prior to DGGE analysis, which eliminated potential biases caused by extra PCR cycles (31). The specificity of the primers was confirmed by the observation that all DGGE bands detected by this primer set and all clones producing the 239-bp amplicons were grouped phylogenetically in the *C. leptum* cluster. Although the primer set was determined to underestimate the number of *C. leptum*, *Clostridium viride*, and *Ruminococcus albus* strains (18), it can still be used for DGGE. Both the study of Lay et al. (17) and our study showed that these three species represent only small proportions of the human fecal *C. leptum* cluster, and they were not detected by our DGGE or clone library analysis; in the study of Lay et al. based on FISH, *R. albus* together with *R. flavefaciens* contributed only 1.8% of the total population, and *C. viride* together with *Eubacterium plautii* contributed only 3.1%. Furthermore, according to our investigation, the primer pair which we used could amplify as many as 86% of all the sequences in the clone library, so the DGGE profiles generated represent the majority of the members of the population. However, further improvement of this primer set is needed to include more bacteria belonging to this group. When we monitored the *C. leptum* cluster of individual G over a 3-year period, a major change was detected during early to late August 2001. In our previous investigation, the fecal *Bacteroides* group of this subject also showed a shift in more or less the same time period (21). The consistency suggested that our PCR-DGGE method with the *C. leptum* cluster-specific primer set can be used to monitor succession of the population over time.

In the DGGE pattern of individual F, we observed that multiple bands exhibited high levels of identity (>98%) with sequences of the same species (e.g., 10 bands that corresponded to *F. prausnitzii*), which may have been due to either the heterogeneity of different rRNA operons in one cell or the heterogeneity of different strains of the same species (20, 36). Similar observations have been reported for *Bifidobacterium* species (26) and *Bacillus benzoevorans*-like sequences (8). This suggested that our DGGE method allows differentiation of sequences at the subspecies level and that defined strains in the population can be identified or monitored

conveniently with DGGE using their amplicons as markers. We also observed that two bands harbored two different types of sequences (Fig. 3). It is known that sequences that differ by multiple bases may exhibit the same melting behavior in DGGE gels (15, 21, 28). In general, this problem did not hamper our DGGE analysis, as the identities of most bands were unambiguous.

Usually, the phylogenetic information for the DGGE bands was obtained by excising and sequencing the bands. Sequences obtained in this way may be too short to provide sufficient genetic information for the corresponding microbial species since the bands contain less than 500 bp of DNA sequence (239 bp in our study), making detailed phylogenetic analyses difficult. We prepared a clone library that harbored longer 16S rRNA gene fragments (1,143 bp). Most DGGE bands were identified by comparing the amplicons of the cloned inserts to the DGGE fingerprint and sequencing the matching clones. Strains related to *F. prausnitzii*, whose corresponding bands appeared most frequently in the DGGE pattern, were also the most abundant group in the library, indicating that there was a good correlation between the DGGE fingerprinting and clone library results.

For five clones there were no matching DGGE bands. These clones may have represented less dominant species that did not form visible bands in the DGGE patterns but were randomly picked out by cloning (7, 36). Meanwhile, for two bands (bands 6 and 16 in Fig. 3) in the DGGE pattern there were no corresponding clones in the library. As the “coverage” of the library in terms of band position in the DGGE gel was 91%, we assumed that the corresponding clones of the two bands might have fallen into the remaining 9% and thus were not selected. Another possible reason for the lack of corresponding clones was PCR and/or cloning biases (24, 33, 35).

Our group-specific clone library also provided information about the structure of individual F's fecal *C. leptum* cluster, which was partially consistent with the study of Lay et al. (17). Both studies demonstrated that strains related to *F. prausnitzii* were the most predominant component of the population, and about 20% of the population was not identified at the species level. However, unlike Lay et al., we did not detect *Ruminococcus bromii*, *R. flavefaciens*, or *Ruminococcus callidus*. Instead, 6% and 2% of our clones were affiliated with *S. variable* and butyrate-producing bacterium A2-207, respectively. These inconsistencies may have been due to differences in the compositions of the gut microflora of different individuals or due to the different techniques used (i.e., FISH versus PCR-based clone library). Probes specific for *S. variable* and butyrate-producing bacterium A2-207 were not designed or used in the study of Lay et al., so whether these two bacteria were present in fecal samples obtained in their study is unknown. Our results suggest that the combination of the 27f and sg-Clept-R3 primers is useful for studying the diversity of the *C. leptum* group.

DGGE analysis of 11 different human fecal samples revealed that bands that correspond to *F. prausnitzii* and *S. variable* were produced by most subjects. *F. prausnitzii* has previously been found to be common in different humans (36). The wide distribution of these bacteria suggests that they are functionally important in the human colon. They are known to produce butyrate as a fermentative product (5, 14).

In conclusion, we developed a group-specific PCR-DGGE method to rapidly profile the human fecal *C. leptum* subgroup and a group-specific clone library approach to analyze the diversity of

the population in detail. A combination of the two techniques could be used to monitor the effects of probiotics, prebiotics, and drugs on the composition of the *C. leptum* subgroup in the human gut microflora, as well as to study the relationships between bacterial composition and host age, genetic background, and digestive disease.

ACKNOWLEDGMENTS

This work was supported by grant 30370031 from the National Natural Science Foundation of China and by grant 2001AA214131 from the High Tech Development Program of China (863 Project).

We are grateful to Sunil Kochhar for his help, and we thank Rodrigo Bibiloni for his constructive suggestions on revision of the paper and Alastair Ross for his advice on language.

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