Phylogenetic Analysis of *Fusobacterium prausnitzii* Based upon the 16S rRNA Gene Sequence and PCR Confirmation

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In order to develop a PCR method to detect *Fusobacterium prausnitzii* in human feces and to clarify the phylogenetic position of this species, its 16S rRNA gene sequence was determined. The sequence described in this paper is different from the 16S rRNA gene sequence of *F. prausnitzii* in the GenBank database (accession number M58682). A PCR assay based on the new sequence is specific for *F. prausnitzii*, and the results of this assay confirmed that *F. prausnitzii* is the most common species in human feces. However, a PCR assay based on the original GenBank sequence was negative when it was performed with two strains of *F. prausnitzii* obtained from the American Type Culture Collection. A phylogenetic tree based on the new 16S rRNA gene sequence was constructed. On this tree *F. prausnitzii* was not a member of the *Fusobacterium* group but was closer to some *Eubacterium* spp. and located between *Clostridium* "clusters III and IV" (M. D. Collins, P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. E. Farrow, *Int. J. Syst. Bacteriol.* 44:812–826, 1994).

Fusobacterium prausnitzii is one of the most common organisms in human gastrointestinal tracts. In previous studies it was found that *F. prausnitzii* accounted for more than 7% of the human fecal microflora and ranked second in relative frequency among bacterial species isolated from human feces (6, 7).

Fusobacterium spp. are anaerobic, gram-negative, straight, curved, or helical rod-shaped organisms which belong to the family *Bacteroidaceae* (7). Lawson et al. (4) investigated the phylogenetic relationships of 14 members of the genus *Fusobacterium*, but *F. prausnitzii* was not included in this study. Neefs et al. (8) described a taxonomic classification of all *Fusobacterium* species based on the results of a comparison of their small-subunit rRNA sequences. These authors found that on their bacterial subtree the *Fusobacterium* spp. did not belong to any cluster, and therefore these organisms were listed separately in a group designated "Fusobacterium and relatives." All *Fusobacterium* species except *F. prausnitzii* were listed as members of this group, and *F. prausnitzii* was listed as a member of a group that contained gram-positive organisms and their relatives which have high G+C contents (8). Nicholson et al. (9) reported that the results of an analysis of 16S rRNA sequences indicated that *F. prausnitzii* is more closely related to the gram-positive organisms *Propionibacterium acnes* and *Actinomyces israelii* than to *Fusobacterium* spp. These authors pointed out that this independent finding is consistent with the findings of Neefs et al.; the 16S rRNA sequence of *F. prausnitzii* used in this study was the sequence deposited in the GenBank database under accession number M58682.

We have been developing molecular methods to monitor intestinal microflora to study the effects of food additives, xenobiotic compounds, and pharmaceutical drugs on the human colonic microflora (10). In a study to develop a PCR method for detection of *F. prausnitzii* in human feces, the 16S rRNA gene sequence deposited in the GenBank database (accession number M58682) was used first. We designed a pair of PCR primers on the basis of this sequence. The sequence of forward primer oFPR-1 is GCTTTTGTGGGGGCTGAGT

(base positions 82 to 100), and the sequence of reverse primer oFPR-2 is CTGATAAGCCGCGAGTCCA (base positions 246 to 228). When we used this primer set, we did not obtain positive PCR results with either *F. prausnitzii* ATCC 27766 or *F. prausnitzii* ATCC 27768. We wondered if the sequence deposited in the GenBank database might be incorrect; therefore, we partially sequenced the 16S rRNA gene of *F. prausnitzii* ATCC 27768 and designed a new set of PCR primers on the basis of the resulting sequence information. The sequence of forward primer FPR-1 was AGATGGCCTCGCGTCCGA (base positions 222 to 239), and the sequence of reverse primer FPR-2 was CCGAAGACCTTCTTCCTCC (base positions 420 to 402). PCR assays in which we used this primer set gave positive results with *F. prausnitzii* ATCC 27766 and ATCC 27768. In addition, after reviewing the results of the studies of Neefs et al. (8) and Nicholson et al. (9), we decided that the entire 16S rRNA gene sequence of *F. prausnitzii* ATCC 27766 should be determined.

Amplification and sequencing of the 16S rRNA gene. A 1.5-ml pure culture of *F. prausnitzii* was centrifuged to pellet the bacterial cells. The cells were washed twice with phosphate-buffered saline (PBS) and once with autoclaved distilled water and then resuspended in 0.1 ml of distilled water. The cells (10 μ l) were diluted 1:10 with 90 μ l of 1% Triton X-100, heated at 100°C for 5 min, immediately cooled in ice water, and then subjected to PCR amplification without isolating the DNA. A 3.5- μ l portion of each sample was added to 45 μ l of a PCR mixture containing 50 mM Tris-HCl (pH 8.5), 20 mM KCl, 3 mM MgCl₂, 0.05% bovine serum albumin (catalog no. A-4378; Sigma Chemical Co., St. Louis, Mo.), 0.25 mM dATP, 0.25 mM dTTP, 0.25 mM dCTP, 0.25 mM dGTP, each primer at a concentration of 0.25 μ M, and 1.8 U of *Taq* polymerase. The PCR was performed in a Perkin-Elmer model 480 thermal cycler. The following program was used: one cycle consisting of 95°C for 3 min, followed by 35 cycles consisting of 95°C for 20 s, 55°C for 20 s, and 74°C for 60 s, and finally one cycle consisting of 74°C for 3 min and 25°C for 5 s. The PCR products were separated by electrophoresis in a 1% agarose gel containing ethidium bromide. The DNA band (about 1.5 kb) was excised from the agarose gel under a long-wave UV lamp. The Glassmilk method (Geneclean II kit; Bio 101, Inc., La Jolla, Calif.) was used to recover the DNA as recommended by

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the manufacturer. The sequences of the PCR products were determined directly by using a SequiTherm cycle sequencing kit (Epicentre Technologies, Madison, Wis.) with ³⁵S-dATP as recommended by the manufacturer.

The amplification primers used were primers Amp-1 and Amp-2, which were located at the 5' and 3' ends of 16S rRNA gene sequence. These two primers were also used as sequencing primers with the following eight other sequencing primers: primers U2, RU2, U3, RU3, U6, RU6, U7, and RU7, which are located in the internal conserved regions of the 16S rRNA gene sequence. The sequences of the primers were as follows: primer Amp-1 (base positions 7 to 27 in the *Escherichia coli* numbering system [1]), GAGAGTTTGATYCTGGCTCAG; primer Amp-2 (base positions 1538 to 1519), AAGGAGGT GATCCARCCGCA; primer U2 (base positions 355 to 338), TGCTGCCTCCCGTAGGAG; primer RU2 (base positions 338 to 355), CTCCTACGGGAGGCAGCA; primer U3 (base positions 535 to 518), GWATTACCGCGGCKGCTG; primer RU3 (base positions 518 to 535), CAGCAGCCGCGGTAA TAC; primer U6 (base positions 1072 to 1054), ACGAGCT GACGACAGCCAT; primer RU6 (base positions 1054 to 1072), ATGGCTGTCGTCAGCTCGT; primer U7 (base positions 1405 to 1387), ACGGGCGGTGTGTACAAGG; and primer RU7 (base positions 1391 to 1407), GTACACACCGC CCGTCA.

First, part of the 16S rRNA gene of *F. prausnitzii* ATCC 27768 was sequenced by using primer U3. The entire 16S rRNA gene sequence of *F. prausnitzii* ATCC 27766 was then determined on both strands. The partial sequence of *F. prausnitzii* ATCC 27768 which we determined was exactly the same as the partial sequence of *F. prausnitzii* ATCC 27766.

PCR detection. PCR detection was performed with a model 1605 air thermal cycler (Idaho Technology, Idaho Falls, Idaho). The following conditions were used: one cycle consisting of 94°C for 15 s, followed by 35 cycles consisting of 94°C for 3 s, 50°C for 10 s, and 74°C for 35 s at transition speed S-9, and finally one cycle consisting of 74°C for 2 min and 45°C for 2 s. The results of our PCR detection analysis performed with selected bacterial species are shown in Table 1. The primer set consisting of primers oFPR-1 and oFPR-2, which were originally designed by using GenBank sequence M58682, gave negative PCR results with both *F. prausnitzii* ATCC 27766 and *F. prausnitzii* ATCC 27768. However, the primer set consisting of primers FPR-1 and FPR-2, which were designed by using the 16S rRNA sequence of *F. prausnitzii* determined in this study, gave positive PCR results with both *F. prausnitzii* ATCC 27766 and *F. prausnitzii* ATCC 27768 but negative results with all of the other bacterial strains tested (Table 1). As few as four cells of *F. prausnitzii* in pure culture could be detected by this PCR method (data not shown).

The American Type Culture Collection has only two strains of *F. prausnitzii* (ATCC 27766 and ATCC 27768) which could be used as positive controls. However, the PCR method described above was used to detect *F. prausnitzii* in nine human fecal samples and several animal fecal samples. For each fecal sample preparation, 1 g (wet weight) of feces was mixed with 9 ml of PBS (0.05 M; pH 7.4) by inverting and vortexing for 5 to 10 min. The mixed samples were then centrifuged at a low speed (200 × g) for 5 min to remove the debris, and this procedure was repeated three times. The upper phase was then centrifuged at 9,000 × g for 3 min to collect the bacterial cells. The cells were washed four times in PBS and once in water, resuspended in 0.1 ml of distilled water, and serially diluted in 100 µl of 1% Triton X-100 for a semiquantitative detection analysis. The samples were heated at 100°C for 5 min and

TABLE 1. PCR results for bacterial strains obtained by using primers based on the new and original sequences

Strain	PCR results obtained with ^a :	
	Primers FPR-1 and FPR-2 ^b	Primers oFPR-1 and oFPR-2 ^c
<i>Fusobacterium prausnitzii</i> ATCC 27768	+	—
<i>Fusobacterium prausnitzii</i> ATCC 27766	+	—
<i>Fusobacterium nucleatum</i> ATCC 25586	—	—
<i>Fusobacterium mortiferum</i> ATCC 9817	—	—
<i>Peptostreptococcus productus</i> ATCC 27340	?	—
<i>Bifidobacterium adolescentis</i> ATCC 15703	—	ND
<i>Bifidobacterium longum</i> ATCC 15707	—	ND
<i>Eubacterium lentum</i> ATCC 25559	—	—
<i>Eubacterium hadrum</i> ATCC 29173	—	—
<i>Bacteroides distasonis</i> ATCC 8503	—	ND
<i>Bacteroides thetaiotaomicron</i> ATCC 29148	—	ND
<i>Bacteroides vulgatus</i> ATCC 8482	—	—
<i>Clostridium clostridioforme</i> ATCC 25537	—	—
<i>Clostridium perfringens</i> ATCC 13124	—	ND
<i>Salmonella typhimurium</i> ATCC 14028	—	—
<i>Shigella flexneri</i> ATCC 12022	—	—
<i>Yersinia enterocolitica</i> ATCC 27729	—	ND
<i>Lactobacillus acidophilus</i> ATCC 332	—	ND
<i>Propionibacterium acnes</i> ATCC 6919	—	ND
<i>Escherichia coli</i> ATCC 25922	—	—
<i>Actinomyces naeslundii</i> ATCC 12104	—	ND
<i>Klebsiella pneumoniae</i> ATCC 13883	—	—
<i>Pseudomonas aeruginosa</i> ATCC 27853	—	ND
<i>Citrobacter freundii</i> ATCC 8090	—	ND
<i>Enterococcus faecalis</i> ATCC 19433	—	—
<i>Corynebacterium kutscheri</i> ATCC 15677	—	ND
<i>Bacillus subtilis</i> ATCC 6051	—	ND
<i>Streptococcus equi</i> ATCC 9528	—	ND
<i>Proteus vulgaris</i> ATCC 13315	—	ND
<i>Listeria monocytogenes</i> ATCC 35152	—	ND
<i>Vibrio parahaemolyticus</i> ATCC 17802	—	ND
<i>Pasteurella pneumotropica</i> ATCC 35149	—	ND

^a The PCR product was 199 bp long. Approximately 10⁵ cells of each strain were used for the PCR assay. +, positive; —, negative; ?, a nonspecific reaction occurred, but the size of the PCR product was different from the size of the *F. prausnitzii* product; ND, not determined.

^b Primers based on the new 16S rRNA sequence (the sequence determined in this study).

^c Primers based on the original 16S rRNA sequence (the sequence whose GenBank accession number is M58682).

immediately cooled in ice water, and 2 µl of each sample was directly subjected to the PCR assay without isolating the DNA.

Figure 1 shows the results of a PCR analysis to detect *F. prausnitzii* in a fecal sample, in which PCR-amplified DNA bands were obtained from 10⁻¹ to 10⁻⁶ dilutions of the sample. We also detected 11 other common human intestinal bacterial species in fecal samples by using 11 other specific PCR methods. Our results confirmed that *F. prausnitzii* is one of the most common species in human feces and that the number of cells of this bacterium in feces is 100 to 100,000 times greater than the number of *E. coli* cells (data not shown).

Phylogenetic analysis. The sequence which we determined was sent to the Ribosomal Database Project (RDP) at the University of Illinois for phylogenetic analysis (5). An unrooted tree that included all *Fusobacterium* species for which 16S rRNA gene sequences were available in the RDP database and other closely related bacterial species was produced by the RDP (data not shown). A rooted tree which included some representatives of related taxa was also produced by using the program Lasergene (DANSTAR, Inc., Madison, Wis.) (Fig. 2).

FIG. 1. PCR detection of *F. prausnitzii* in a human fecal sample. The PCR products (199 bp) were separated in a 2% agarose gel. Lane m, molecular size marker; lanes 1 through 7, PCR performed with 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} dilutions, respectively.

On both phylogenetic trees, *F. prausnitzii* was not a member of the *Fusobacterium* group but was closer to some *Eubacterium* spp. and was located between *Clostridium* "clusters III and IV" (2). The levels of similarity between the 16S rRNA sequence of *F. prausnitzii* determined in this study and the sequences of other *Fusobacterium* spp. were only $76 \pm 4\%$. However, the levels of sequence similarity with *Eubacterium siraeum*, *Eubacterium desmolans*, and *Clostridium cellulosi* were 86, 84, and 84%, respectively. These similarity values are not as high as the values obtained for *Listeria* species (3) and *Mycobacterium* species (11). The 16S rRNA sequences of members of the genera *Eubacterium* and *Clostridium* are not as similar as the sequences of members of many better-defined genera, and the taxonomy of these groups is being revised (2). These findings

FIG. 2. Phylogenetic tree for *F. prausnitzii* and related species. The tree was constructed by using the Clustal method (DANSTAR program). Clusters I through IV were the clusters described by Collins et al. (2).

suggested that *F. prausnitzii* is not affiliated with the genus *Fusobacterium*. However, formal reclassification of *F. prausnitzii* is not proposed at this time since there is uncertainty concerning the classification of *Clostridium* spp. and *Eubacterium* spp. (2).

Nucleotide sequence accession number. The 16S rRNA gene sequence of *F. prausnitzii* ATCC 27766 has been deposited in the EMBL database under accession number X85022.

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ADDENDUM

We have received information from the RDP that the sequence whose accession number is M58682 was deleted from the RDP database as of 1 August 1993 because it was determined that this sequence is not an *F. prausnitzii* sequence but is probably the sequence of a contaminant. However, the sequence is still in the GenBank database, and other researchers used it after 1993 (8, 9).

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