Growth requirements and fermentation products of *Fusobacterium prausnitzii*, and a proposal to reclassify it as *Faecalibacterium prausnitzii* gen. nov., comb. nov.

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Two newly isolated strains of obligately anaerobic bacteria from human faeces are shown here to be related to *Fusobacterium prausnitzii*, which is regarded as one of the most abundant colonizers of the human colon. These strains, along with *Fusobacterium prausnitzii* ATCC 27768^T and 27766, are non-motile and produce butyrate, formate and lactate, but not hydrogen as fermentation products. A new finding is that all four strains produce D-lactate, but not L-lactate. The strains have a requirement for acetate in the growth medium and this may account for the previously reported requirement for rumen fluid. The DNA G+C content of the four strains is 47–57 mol%. Together with phylogenetic analysis based on 16S rRNA sequencing, this establishes that *Fusobacterium prausnitzii* strains are only distantly related to *Fusobacterium sensu stricto* and are more closely related to members of *Clostridium* cluster IV (the *Clostridium leptum* group). It is proposed that a new genus, *Faecalibacterium* gen. nov. be created; this genus should include *Faecalibacterium prausnitzii* gen. nov., comb. nov. ATCC 27768^T (= NCIMB 13872^T) (formerly *Fusobacterium prausnitzii*) as the type species together with ATCC 27766 and the newly isolated strains A2-165 and L2-6.

Keywords: *Faecalibacterium prausnitzii*, *Fusobacterium*, human faeces, butyrate, D-lactate

INTRODUCTION

Bacteria currently classified as *Fusobacterium prausnitzii* are recognized as being among the most abundant representatives of the human faecal flora (Cato *et al.*, 1974; Holdeman *et al.*, 1976; Moore & Moore, 1995). Their abundance has been further confirmed by recent evidence from 16S rRNA analyses of bacterial diversity in human faeces and colon samples that are independent of cultivation (Wilson & Blitchington, 1996; Wang *et al.*, 1996a; Suau *et al.*, 1999, 2001; Franks *et al.*, 1998; Hold *et al.*, 2002). In addition, bacteria related to *Fusobacterium prausnitzii*

accounted for a significant proportion of randomly isolated anaerobes from human faeces that produce major quantities of butyrate (Barcenilla *et al.*, 2000).

Strains classified phenotypically as *Fusobacterium prausnitzii* are not phylogenetically related to true *Fusobacterium* species, based on the 16S rDNA sequence of *Fusobacterium prausnitzii* ATCC 27766 (Wang *et al.*, 1996a). True representatives of the genus *Fusobacterium* are Gram-negative, non-spore-forming anaerobic rods that show a DNA G+C content between 26 and 34 mol% (Hauduroy *et al.*, 1937; Cato *et al.*, 1974; Moore *et al.*, 1984). Based on 16S rRNA sequencing, typical species (including *Fusobacterium nucleatum* and *Fusobacterium necrophorum*) form a distinct cluster in the eubacterial phylogenetic tree. In marked contrast, *Fusobacterium prausnitzii* strains show a DNA G+C content between 47 and 57 mol% and their 16S rRNA sequences indicate relatedness

Abbreviation: SCFA, short-chain fatty acid(s).

The GenBank accession numbers for the 16S rRNA gene sequences of *Fusobacterium prausnitzii* ATCC 27768^T and from the new isolates L2-6 and A2-165 are AJ413954, AJ270469 and AJ270470, respectively.

with Gram-positive bacteria of the *Clostridium leptum* group (Franks *et al.*, 1998; Suau *et al.*, 2001).

Here we examine the characteristics and growth requirements of two newly isolated *Fusobacterium prausnitzii* strains from human faeces and propose that these strains, along with *Fusobacterium prausnitzii* ATCC 27768^T (= NCIMB 13872^T) and ATCC 27766, should be placed in the new genus *Faecalibacterium*.

METHODS

Bacterial strains and growth conditions. Two strains (A2-165 and L2-6) were isolated from the highest countable dilution of human faecal samples in roll tubes (Hungate, 1966) of anaerobic M2GSC medium (Miyazaki *et al.*, 1997), as described by Barcenilla *et al.* (2000). Anaerobic culture methods were those of Bryant (1972) using Hungate culture tubes, sealed with butyl rubber septa (Bellco Glass). Media were prepared and maintained anaerobically using O₂-free CO₂. The isolates were routinely maintained by growing for 16–18 h at 37 °C in 7.5 ml aliquots of M2GSC medium.

Morphology. The cellular morphology of the new isolates was determined by Gram staining exponential- and stationary-phase cultures as described by Holdeman *et al.* (1977). Sixteen-hour-old cultures were examined by phase-contrast microscopy for motility and observed by scanning electron microscopy for the presence of flagella, following the procedure described by Stewart *et al.* (1990).

Substrate utilization and hydrolysis. Substrate utilization was determined by adding a final concentration of 0.5% stock filter-sterilized sugar solutions (10%, w/v) to YCFA medium dispensed in 7.5 ml aliquots in Hungate tubes. YCFA medium consisted of (per 100 ml): 1 g casitone, 0.25 g yeast extract, 0.4 g NaHCO₃, 0.1 g cysteine, 0.045 g K₂HPO₄, 0.045 g KH₂PO₄, 0.09 g NaCl, 0.009 g MgSO₄·7H₂O, 0.009 g CaCl₂, 0.1 mg resazurin, 1 mg haemin, 1 µg biotin, 1 µg cobalamin, 3 µg *p*-aminobenzoic acid, 5 µg folic acid and 15 µg pyridoxamine. Final concentrations of short-chain fatty acids (SCFA) in the medium were 33 mM acetate, 9 mM propionate and 1 mM each of isobutyrate, isovalerate and valerate. All components were added aseptically while the tubes were flushed with CO₂. Heat labile vitamins were added after the medium was autoclaved to give a final concentration of 0.05 µg thiamine ml⁻¹ and 0.05 µg riboflavin ml⁻¹. YCFA supplemented with 25 mM glucose (YCFAG) provided a convenient alternative to rumen fluid medium for the cultivation of the strains in this study. Growth was measured spectrophotometrically as OD₆₅₀. Twelve different arylamidase activities were tested using API rapid ID-32A test kits (bioMérieux). The activities tested were arginine arylamide, proline arylamide, leucine-glycine arylamide, phenylalanine arylamide, leucine arylamide, pyroglutamic acid arylamide, tyrosine arylamide, alanine arylamide, glycine arylamide, histidine arylamide, serine arylamide and glutamyl glutamic acid arylamide. Aesculin hydrolysis was determined as described by Duncan *et al.* (1998).

Fermentation product analysis. Acid production was determined by capillary GC (Richardson *et al.*, 1989) and the gaseous products were analysed by packed-column GC (Rumney *et al.*, 1995).

DNA preparation and DNA base composition. DNA was extracted from 24-h-old cultures grown on M2GSC medium

following the method of Ausubel *et al.* (1994). Chromosomal DNA was purified by standard methods (Sambrook *et al.*, 1989) and the G+C content was determined using the thermal denaturation procedure as described by Johnson (1981).

16S rDNA sequencing and phylogenetic analysis. For amplification of 16S rDNA, a universal primer set, corresponding to positions 8–27 (forward primer) and 1492–1510 (reverse primer) of the *Escherichia coli* numbering system was used (Weisberg *et al.*, 1991). The PCR conditions were as described by Wood *et al.* (1998). Direct sequencing of the amplified DNA fragments was performed using an automated ABI 377 sequencer as described previously (Hold *et al.*, 2001). Similarity of the 16S rRNA sequences was compared with all sequence data in GenBank and EMBL using the BLAST algorithm (Altschul *et al.*, 1990) and the Ribosomal Database Project (Maidak *et al.*, 2001). Nucleotide sequences were aligned using the PILEUP program (Devereux *et al.*, 1984). The resulting multiple sequence alignment was corrected manually, with approximately 1340 nt being used in the subsequent phylogenetic analysis which corresponded to positions 60–1400 within the 16S rRNA gene (based on *E. coli* numbering). The phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987), via the PHYLIP package (Felsenstein, 1989) using DNADIST for distance analysis (Kimura, 1980). The stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs of the PHYLIP package (Felsenstein, 1989).

RESULTS

Molecular characterization and phylogenetic relationships

The 16S rRNA sequences of the two recently isolated strains, A2-165 and L2-6, were determined and found to be 96% identical to that of *Fusobacterium prausnitzii* strain ATCC 27766 as sequenced by Wang *et al.* (1996a) and 97% identical to the 16S rRNA sequence of *Fusobacterium prausnitzii* ATCC 27768^T obtained during the current investigation (Fig. 1). Comparison of the 16S rRNA gene sequences from *Fusobacterium prausnitzii* with the 16S rRNA gene sequence from the *Fusobacterium* type species, *Fusobacterium nucleatum* ATCC 25586^T, showed that they possessed <77% sequence identity. The G+C content was determined for strains A2-165, L2-6 and ATCC 27766, with all strains giving values between 47 and 49 mol% (Table 1). This is in contrast to G+C content of other *Fusobacterium* strains which have been reported to have values between 26 and 34 mol% (Moore *et al.*, 1984).

Cell morphology

The new strains isolated from human faeces were Gram-negative rods measuring approximately 0.5 × 0.8–2.0 × 9.0 µm and similar data were also obtained from existing strains (Table 1). All four isolates were shown to be non-motile and a scanning electron micrograph of the non-flagellated strain A2-165 is shown in Fig. 2.

Table 1. Phenotypic properties of *Faecalibacterium prausnitzii* strains

Growth was determined by measuring OD₆₅₀ in YCFA medium supplemented with the appropriate substrate (0.5 %, w/v, final concn; see Methods). All strains were non-motile, Gram-negative rods. All strains grew on fructose, fructo-oligosaccharides and glucose, but failed to grow on arabinose, melibiose, raffinose, rhamnose, ribose and xylose. All strains possessed β -galactosidase, α -glucosidase and phosphatase activities in API 32A tests, but did not possess urease, arginine dihydrolase, β -glucosidase and α -fucosidase activities. None of the strains reduced nitrate or produced indole. All strains utilized acetate, but did not produce H₂. The products of fermentation from all four strains were formate, butyrate, D-lactate and CO₂. w, Weak reaction; NA, data not available.

Isolate	A2-165	L2-6	<i>F. prausnitzii</i> ATCC 27766	<i>F. prausnitzii</i> ATCC 27768 ^{T*}
Size (μ m)	0.5–0.8 $\times$ 2.0–9.0	0.5–0.8 $\times$ 2.0–9.0	0.5–0.8 $\times$ 2.0–9.0	0.5–0.9 $\times$ 2.5–14
DNA G + C (mol %)	49	47	47	52–57
Growth on:				
Cellobiose	+	–	–	W
Maltose	+	+	–	W
Mannitol	–	–	–	NA
Melezitose	–	–	+	–
Sucrose	–	W	–	W
Trehalose	–	–	–	W
Starch solution	+	+	+	W
Inulin	+	W	+	+
Hydrolysis of aesculin	W	+	+	+
α -Galactosidase activity [†]	W	–	–	W

* Description as for *Fusobacterium prausnitzii* (Moore *et al.*, 1984).

[†] Determined by an API 32A test kit.

Substrates fermented

Phenotypic characteristics of the four strains are shown in Table 1. All strains possessed the ability to hydrolyse fructose, fructo-oligosaccharide, starch and inulin. None of the strains utilized arabinose,

melibiose, raffinose, rhamnose, ribose and xylose. All strains were net acetate utilizers (Table 2) and produced carbon dioxide, but none produced hydrogen. The four strains differed in their ability to ferment cellobiose, maltose and melezitose.

Fig. 2. Scanning electron micrograph of *F. prausnitzii* strain A2-165. Bar, 1 μ m.

All strains hydrolysed the arginine and histidine arylamide substrates, but failed to hydrolyse the proline, leucine-glycine, phenylalanine, leucine, tyrosine, alanine, serine, pyroglutamic acid, glutamyl glutamic acid and glycine, with the exception of strains A2-165 and L2-6 that possessed weak glycine arylamidase activity, when tested using an API 32A test kit.

Fermentation products, growth requirements and tolerance of air

The major end products of glucose fermentation by the *Fusobacterium prausnitzii* strains were formate, butyrate and D-lactate (Table 2), with L-lactate being undetectable for all four strains. Although first isolated on M2GSC (30% rumen fluid) medium, A2-165 and L2-6 were able to grow on simplified medium containing SCFA (YCFA medium), but showed strong growth stimulation by 33 mM acetate (Table 3). Indeed strains ATCC 27766 and L2-6 failed to grow in the absence of added acetate. No growth of strain A2-165 was detected when yeast extract was omitted from YCFAG medium (data not shown).

Experiments aimed at identifying the susceptibility of A2-165 to air indicated that *Fusobacterium prausnitzii* A2-165 could be cultivated on pre-reduced 1.5% agar plates contained in an anaerobic glove box, rather than using the Hungate technique. However, exposure of inoculated plates to air for more than 2 min was sufficient to prevent subsequent anaerobic growth. *Fusobacterium nucleatum* ATCC 25586^T on the other hand is reported to grow in the presence of 6% oxygen and survives exposure to air for 100 min (Loesche, 1969).

DISCUSSION

It is important that the phylogenetic position of this major species of human colonic anaerobe is now clarified to avoid confusion with the unrelated *Fuso*-

Table 2. Fermentation products formed by *Faecalibacterium prausnitzii* isolates on the rumen fluid containing M2G medium (25 mM glucose final concentration)

Concentrations (mM) are the means of three values $\pm$ SD.

Isolate	Formate	Acetate	Butyrate	D-Lactate
A2-165	17.30 $\pm$ 3.51	-5.31 $\pm$ 2.71	13.79 $\pm$ 1.75	1.70 $\pm$ 0.27
L2-6	7.65 $\pm$ 1.30	-12.29 $\pm$ 1.83	18.64 $\pm$ 1.15	5.33 $\pm$ 0.19
ATCC 27766	4.68 $\pm$ 0.67	-10.25 $\pm$ 1.33	13.18 $\pm$ 0.67	2.56 $\pm$ 0.31
ATCC 27768 ^T	10.25 $\pm$ 0.72	-9.72 $\pm$ 2.10	18.56 $\pm$ 1.57	5.47 $\pm$ 0.83

Table 3. Effects of SCFA on growth rates of *Faecalibacterium prausnitzii* isolates on YCFA medium

Isolate	+ SCFA*		- SCFA		- SCFA + 33 mM acetate	
	Max. OD ₆₅₀	μ (h ⁻¹)†	Max. OD ₆₅₀	μ (h ⁻¹)†	Max. OD ₆₅₀	μ (h ⁻¹)†
A2-165	0.97 $\pm$ 0.03	0.21 $\pm$ 0.03	0.25 $\pm$ 0.05	0.10 $\pm$ 0.01	0.90 $\pm$ 0.00	0.32 $\pm$ 0.04
L2-6	0.39 $\pm$ 0.10	0.15 $\pm$ 0.01	0.10 $\pm$ 0.05	ND	0.39 $\pm$ 0.11	0.38 $\pm$ 0.06
ATCC 27766	0.19 $\pm$ 0.01	0.11 $\pm$ 0.014	0.04 $\pm$ 0.01	ND	0.22 $\pm$ 0.02	0.10 $\pm$ 0.01

* SCFA final concentrations in the medium were 33 mM acetate, 9 mM propionate and 1 mM each of isobutyrate, isovalerate and valerate.

† Growth rates (μ) are the means of three replicate values $\pm$ SD. ND, Growth rates not determined as increase in absorbance was $\leq$ 0.1.

bacterium spp. through the designation of the new genus *Faecalibacterium*. There is only 77% 16S rDNA sequence identity between *Fusobacterium prausnitzii* and the *Fusobacterium* type species *Fusobacterium nucleatum* ATCC 25586^T. Furthermore, the G+C content of *Fusobacterium prausnitzii* is 47–57 mol% compared to 26–34 mol% for true *Fusobacterium* spp., clearly indicating that they are unrelated. Phenotypic characteristics also show that all of the 10 *Fusobacterium* spp. that have been described produced acetate as a fermentation product (Moore *et al.*, 1984) with the exception of *Fusobacterium prausnitzii* which, as confirmed here, is a net acetate utilizer. In addition, *Fusobacterium prausnitzii* is particularly sensitive to exposure to air, surviving for less than 2 min, while *Fusobacterium nucleatum* is reported to be relatively tolerant, retaining viability for nearly 2 h (Loesche, 1969).

The creation of the proposed new genus, *Faecalibacterium*, will help to facilitate future research involving its ecology and physiology in the human colonic ecosystem. The present analyses indicate that *Faecalibacterium* (formerly *Fusobacterium*) *prausnitzii* may make a significant contribution to D-lactate and butyrate formation in the large intestine. The production of butyrate is potentially significant because of the role that butyrate plays as an energy source for the colonic epithelium (Roediger, 1980; Scheppach *et al.*, 1998) and in the prevention of colitis and colorectal cancer (Roediger, 1980; Tran *et al.*, 1998). In contrast, the production of D-lactate may also be highly significant in view of the potentially toxic effects of D-lactate accumulation in the bloodstream (Lalive *et al.*, 2001) as few of the predominant anaerobic bacteria inhabiting the gut have been identified as D-lactate producers.

Description of *Faecalibacterium* gen. nov.

Faecalibacterium (Fae.ca.li.bac.te'ri.um. L. adj. *faecalis* pertaining to faeces; Gr. dim. n. *bakterion* a small rod; N.L. neut. n. *Faecalibacterium* rod from faeces, as this bacterium is present in high numbers in faeces in the colon, its presumed habitat).

Gram-negative, non-spore-forming and strictly anaerobic. The non-motile organism produces butyrate, D-lactate and formate, and utilizes acetate. Genomic DNA G+C content is 47–57 mol% (as determined by thermal denaturation). The type strain, whose characteristics were reported by Cato *et al.* (1974), is *Faecalibacterium prausnitzii* ATCC 27768^T (= NCIMB 13872^T).

Transfer of *Fusobacterium prausnitzii* to the genus *Faecalibacterium* gen. nov. as *Faecalibacterium prausnitzii* gen. nov., comb. nov.

Physiological, phenotypic and phylogenetic data indicate that *Fusobacterium prausnitzii* is not closely related to the type species of the genus *Fusobacterium* within which it is currently held. It is clearly placed

within *Clostridium* cluster IV (described by Collins *et al.*, 1994), although with sufficient distance to merit the creation of a new genus. The original description of the species given by Moore *et al.* (1984) is unchanged.

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