Anaerofilum pentosovorans gen. nov., sp. nov., and *Anaerofilum agile* sp. nov., Two New, Strictly Anaerobic, Mesophilic, Acidogenic Bacteria from Anaerobic Bioreactors†

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Strictly anaerobic, gram-positive, nonsporing, thin rod-shaped organisms whose cells were 0.2 to 0.6 by 3 to 6 μm were isolated from a Hoechst Biohochreaktor (strain Fae^T [T = type strain]) and from the biofilm population of a fixed-film reactor treating sour whey (strain F^T). Strain F^T was vigorously motile during early logarithmic growth by means of peritrichously inserted flagella, while strain Fae^T was seldom motile and usually possessed no flagella. During the stationary growth phase both strains formed spheroplasts. The temperature optimum was close to 37°C (temperature range for growth, ≥ 17 to $< 45^\circ\text{C}$) and the pH optimum was 7.0 to 7.4 (pH range, 6.5 to 8.0) for both strains. The two organisms grew chemorganotrophically on a number of mono- and disaccharides, including glucose and xylose; yeast extract was required for growth. The principal fermentation products from glucose included lactate, acetate, ethanol, formate, and CO₂. Hydrogen was not generated. The G+C contents of the DNAs of strains Fae^T and F^T were 55 and 54.5 mol%, respectively. The cell wall architecture was typical of gram-positive bacteria; the cells had an extraordinarily thin type A3 α' peptidoglycan layer containing muramic acid. Analysis of 16S ribosomal DNA sequences of the two new isolates demonstrated that they represent members of a new genus of bacteria in *Clostridium* cluster IV of the domain *Bacteria* and that the misclassified organism *Fusobacterium prausnitzii* and *Clostridium leptum* are among their closest relatives. The names *Anaerofilum pentosovorans* gen. nov., sp. nov. (type strain, strain Fae [= DSM 7168]) and *Anaerofilum agile* sp. nov. (type strain, strain F [= DSM 4272]) are proposed.

The genus *Clostridium* represents a large variety of species that occur ubiquitously in anaerobic habitats, utilize a broad spectrum of substrates (2, 9), and belong to the low-G+C-content subphylum of the phylum of gram-positive *Bacteria* (30). The genus *Clostridium*, however, is not coherent phylogenetically, and representatives of various genera, including both spore-forming and non-spore-forming organisms, are found in the radiation of *Clostridium* species (2, 3, 17, 21, 22). In this paper we report the isolation of two acidogenic, non-sporing rods that have high DNA G+C contents from anaerobic high-rate bioreactor consortia; phylogenetically, these organisms belong to *Clostridium* cluster IV as defined by Collins et al. (3), and we propose the new genus *Anaerofilum* for them.

MATERIALS AND METHODS

Isolation. Strain Fae^T (T = type strain) was isolated from a Biohochreaktor at the Hoechst Chemical Co. in Kelsterbach, Frankfurt, Germany. Sludge from the wastewater reactor was serially diluted in 120-ml serum bottles containing 20 ml of prerduced medium with a gaseous atmosphere consisting of 80% N₂ and 20% CO₂ (300 kPa). The dilutions were streaked onto agar plates containing the same medium supplemented with 0.5% (wt/vol) glucose and 2% (wt/vol) agar (Oxoid, Wesel, Germany) in an anaerobic chamber and were incubated in a stainless steel anaerobic jar (1) at 37°C with a gas atmosphere containing 80% N₂ and 20% CO₂ (200 kPa). After 14 days colonies of strain Fae^T that were 1 to 2 mm in diameter and white with lobate margins and raised centers were picked and transferred into serum bottles. Culture purity was checked microscopically. Strain F^T was isolated from an anaerobic biofilm population in a fixed-film

reactor treating sour whey (32, 33) that had been inoculated with anaerobic sewage sludge from the municipal sewage treatment plant of Regensburg, Germany.

Reference strain. *Bifidobacterium longum* DSM 20219 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

Media. Media were prepared by using the anaerobic technique of Balch et al. (1), and strains were grown in prerduced medium in pressurized serum bottles as described previously (33). *B. longum* was grown in MRS medium (5).

Transmission electron microscopy. Photomicrographs of thin sections and freeze-etched cell preparations were taken with a Philips model EM 301 electron microscope at 80 kV (26).

Cell wall composition. Cell walls were purified and analyzed by the method of Schleifer and Kandler (25). Total cell wall acid hydrolysates (4 N HCl, 100°C, 16 h) were subjected to a quantitative amino acid and amino sugar analysis with a Biotronik model L 6001 amino acid analyzer. The structure of the murein was deduced from the molar ratio of the amino acids and the peptide pattern found in partial acid hydrolysates (4 N HCl, 100°C, 45 min).

Analytical procedures. Growth was determined turbidimetrically at 578 nm. The organic acids, alcohols, acetoin, diacetyl, and hydrogen contents were determined with a gas chromatograph equipped with a flame ionization detector or a thermal conductivity detector (33). The succinate content was determined by gas chromatography after derivatization to the corresponding methyl ester as described by Holdeman et al. (12). The glucose, xylose, and formate contents were determined spectrophotometrically (16, 20). The D-(-)- and L-(+)-lactate contents were determined enzymatically (11). Fructose-6-phosphate phosphoketolase activity was assayed as described previously (24). The presence of catalase was determined by dropping 4% H₂O₂ onto a colony after incubation in air for 0.5 h (12).

DNA base composition and DNA-DNA hybridization. Strain F^T and Fae^T DNAs were isolated, and the G+C contents of these DNAs were determined by the thermal denaturation method and by high-performance liquid chromatography (HPLC) (19, 31). The DNAs used for DNA hybridization experiments were isolated and levels of DNA-DNA homology were calculated from renaturation rates as described previously (4, 13, 14).

Sequence analysis of 16S rDNA and phylogenetic analysis. The 16S ribosomal DNA (rDNA) sequences of the new strains were compared with the sequences in the 16S rDNA database for members of the *Clostridium-Bacillus* subphylum of gram-positive bacteria (18). Similarity values were transformed into phylogenetic

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FIG. 1. Electron micrographs of cells of strains *Fae^T* and *F^T*. (A) Freeze-etched preparation of strain *Fae^T*. Bar = 0.25 μ m. (B) Thin section of strain *F^T*. Bar = 0.2 μ m. (C) Freeze-etched preparation of strain *F^T* showing insertion sites of flagella. Bar = 0.1 μ m.

distance values that compensated for multiple substitutions at any given site in the sequence (15). The least-squares distance method of De Soete (6) and the neighbor-joining and maximum-likelihood programs in the PHYLIP package (8) were used to construct phylogenetic dendrograms (23). To calculate bootstrap values, 300 trees were analyzed by using the NJFIND and NJBOOT programs.

Nucleotide sequence accession numbers. The sequences of the 16S rDNAs of strains *Fae^T* and *F^T* have been deposited in the EMBL database under accession numbers X97852 and X98011, respectively.

RESULTS

Morphology and cell wall. The cells of strains *F^T* and *Fae^T* were gram-positive, straight rods (0.2 to 0.6 by 3 to 6 μ m) that occurred singly or in pairs and occasionally formed filaments up to 30 μ m long. Both strains produced spheroplasts after depletion of the substrate. No S-layer outside the cell wall of strain *Fae^T* was observed on freeze-fracture electron micro-

graphs (Fig. 1a). Cells of strain *Fae^T* were seldom motile. In contrast, strain *F^T* was motile during logarithmic growth. The cell wall architecture of strain *F^T* was gram positive, but the wall had an unusually thin murein sacculus (Fig. 1b). A freeze-etched preparation of intact cells of strain *F^T* revealed highly structured flagella, but no S-layers were observed (Fig. 1c). Neither strain formed spores.

Growth conditions. The organisms did not grow aerobically and did not grow in MRS medium under nitrogen gas. Neither organism formed catalase, and both organisms grew only in prereduced media. Strains *F^T* and *Fae^T* required 0.2% yeast extract for growth, while the presence of Trypticase peptone and 61 mM sodium acetate had no influence on growth. Strain *Fae^T* grew optimally on glucose with a broad temperature optimum of 25 to 40°C and a doubling time of 2.5 to 5 h. The

optimal growth temperature of strain F^T was around 37°C, and the shortest doubling time observed was 16.7 h. Strains Fae^T and F^T did not grow at ≤ 15 and $\geq 45^\circ\text{C}$ (growth temperature range, ≥ 17 to $< 45^\circ\text{C}$). The pH optimum was 7.0 to 7.4 (pH range, 6.5 to 8.0) for both strains. NaCl had no influence on the growth of strain Fae up to a concentration of 400 mM.

Physiology. Strains Fae^T and F^T were grown in 120 ml-serum bottles under a 80% N_2 -20% CO_2 gas phase (300 kPa) at 37°C and 100 rpm with several substrates at a concentration of 0.2 or 0.5% (wt/vol or vol/vol). Both strains grew on cellobiose, fructose, galactose, glucose, maltose, ribose, trehalose, and xylose. In addition, arabinose, mannose, and sorbose were utilized by strain Fae^T , while D-salicin and D-sorbitol were utilized by strain F^T . Neither strain utilized lactose, melibiose, raffinose, rhamnose, sucrose, methanol, ethanol, 1-propanol, citrate, fumarate, malate, succinate, L-(+)-lactate, cellulose, starch, xylan, pectin A, L-glutamate, L-tryptophan, L-phenylalanine, L-alanine, or L-glycine. Growth of strain F^T on all substrates was poor compared with growth of strain Fae^T . Very weak growth of strain F^T occurred on L-valine and L-isoleucine. Furthermore, 2-propanol, 2-butanol, L-aspartate, L-valine, glutaric acid, and monomethylamine were not utilized by strain Fae^T , and 1-butanol, glycerol, and esculin were not utilized by strain F^T . Sulfate was not reduced by either strain.

The principal products of glucose fermentation formed by strain Fae^T were lactic acid [both isomers but the L-(+)-lactate isomer was dominant], ethanol, acetate, formate, and CO_2 , as well as a small amount of 2,3-butanediol. Strain F^T fermented glucose to L-(+)-lactate, acetate, formate, ethanol, and CO_2 . Neither organism produced hydrogen or hydrogen sulfide from sulfate. Traces of isobutyrate and isovalerate were found in culture supernatants of strain F^T , and these compounds presumably were derived from amino acid degradation (degradation of valine and isoleucine, respectively). Propionate, butyrate, succinate, acetoin, diacetyl, methanol, and 2-propanol were not fermentation products of strains Fae^T and F^T . The presence of hydrogen in the gas atmosphere (300 kPa of 80% H_2 -20% CO_2) neither inhibited growth of strain F^T nor altered the pattern of fermentation products (data not shown). Fructose-6-phosphate phosphoketolase activity, the key enzyme activity of the "bifido-shunt," was not detected in crude extracts of strain F^T (with *B. longum* used as a positive control).

Murein composition and peptidoglycan structure. The total acid hydrolysates of the cell walls of strains Fae^T and F^T were identical and contained N-acetylmuramic acid, N-acetylglucosamine, and the amino acids glycine (Gly), glutamic acid (Glu), lysine (Lys), serine (Ser), and alanine (Ala) at a molar ratio of 1:1:1:<1:<1. In partial acid hydrolysates the following peptides were detected by thin-layer chromatography: L-Gly-D-Glu, L-Lys-L-Ser, L-Lys-D-Ala, and L-Ser-D-Ala. The lactyl group of the muramic acid was esterified with glycine. The interpeptide linkage was formed by L-serine, which was identified by chiral chromatography because of the inability of the amino acid analyzer to distinguish between L-Ser and D-Ser. The data obtained are consistent with peptidoglycan type A3 α' .

G+C contents of DNAs. The base composition of the DNA of strain F^T was 54.5 mol% G+C (average of two determinations; thermal denaturation method). The G+C content of the DNA of strain Fae^T was 55 mol% (as determined by HPLC).

Phylogenetic position. The 16S rDNA analysis indicated that there is a high level of relatedness (level of sequence similarity, 99.2%) between strains Fae^T (= DSM 7168 T) and F^T (= DSM 4272 T) (Fig. 1). The corresponding level of DNA-DNA similarity was 48%, as determined by the renaturation method. Phylogenetically, these two strains are members of *Clostridium*

FIG. 2. Phylogenetic positions of strains Fae^T and F^T within the *Clostridium-Bacillus* subphylum (cluster IV). Bar = 5% sequence divergence. C., *Clostridium*. Numbers refer to bootstrap values.

cluster IV, as defined by Collins et al. (3). This cluster contains several *Clostridium* species, including *Clostridium sporosphaeroides*, *Clostridium cellulosi*, and *C. leptum* (levels of 16S rDNA similarity with the new isolates, 88 to 90%), as well as members of the genus *Ruminococcus* (*Ruminococcus albus*, *Ruminococcus flavefaciens*, *Ruminococcus callidus*, and *Ruminococcus bromii*; levels of 16S rDNA similarity with the new isolates, 87 to 89%) and members of the genus *Eubacterium* (*Eubacterium plautii*, *Eubacterium siraeum*, and *Eubacterium desmolans*; levels of 16S rDNA similarity with the new isolates, 85 to 88%) (Fig. 2) (21). The nearest neighbor of the new isolates is the misclassified strain *F. prausnitzii* ATCC 27766, although this organism is only remotely related (level of 16S rDNA similarity, 90%).

DISCUSSION

Phylogenetically, strains F^T and Fae^T belong to the *Clostridium-Bacillus* branch of the *Bacteria*. These two strains are closely related to each other (level of 16S rDNA sequence similarity, 99.2%), but despite the high level of 16S rDNA similarity they may belong to distinct species (27). The level of DNA-DNA homology obtained for strains Fae^T and F^T (48%) is far below the threshold value of 70% recommended for species delineation (28). This justifies the creation of two separate species, because DNA-DNA homology takes precedence over 16S rDNA sequence similarity (27). The closest relatives of the new strains were found to be the misclassified organism *F. prausnitzii* ATCC 27766 and *C. leptum*, both of which have high DNA G+C contents (52 to 57 and 51 to 52 mol%, respectively) (9, 10, 12) and both of which belong to cluster IV defined by Collins et al. (3). The G+C contents of *Fusobacterium* species usually range from 26 to 34 mol% (10). The G+C contents of *R. flavefaciens*, *R. albus*, and *R. callidus* range from 39 to 46 mol% (7), while the G+C content of *E. siraeum* is 45 mol% (12). Phylogenetically, the new organisms were about equally related to *C. leptum* and *C. sporosphaeroides*, both of which are members of "family 3" proposed by Collins et al. (3). However, the latter organism has a DNA G+C content of only 27 mol%. In contrast, *Clostridium quercicolum*, which has a high DNA G+C content (52 to 54 mol%) (9, 12), was phylogenetically more distantly related to the new organisms (data not shown).

Morphologically, strains Fae^T and F^T were thin, straight,

rod-shaped organisms, similar to *C. leptum*, which forms non-motile, slightly curved cells and, rarely, oval spores. In contrast, the new organisms never formed spores, either after starvation or after heat treatment. Freshly isolated strains may not form spores (2). The new strains also exhibited a phylogenetic relationship to some *Ruminococcus* species and *E. siraeum*, which also do not form endospores. Thin sections of strain Fae^T and F^T preparations revealed a gram-positive cell wall profile, but the murein layers were extraordinarily thin, which explains why a positive Gram stain reaction was observed only during the early logarithmic growth phase. Older cultures exhibited gram-negative staining. *Clostridium* spp., as well as *C. sporosphaeroides*, have typical peptidoglycan topology with direct cross-linking of the meso-diaminopimelic acid (25, 29). The cell walls of strains F^T and Fae^T were peptidoglycan type A3a' walls and were similar to the cell walls of *C. leptum*, which contain a lysine-serine-glycine type of peptidoglycan (K-type peptidoglycan containing Lys-Ser-D-Glu) (29).

The fermentation products of strains F^T and Fae^T included lactic acids, ethanol, acetate, formate, and CO₂. Strains Fae^T and F^T had to be differentiated physiologically from *F. prausnitzii*, *R. flavefaciens*, *R. albus*, *R. callidus*, *E. siraeum*, and *C. leptum* of cluster IV. *F. prausnitzii* did not utilize or only weakly utilized mono-, di-, and oligosaccharides, but the fermentation products were butyrate, formate, and lactate, as well as minor amounts of succinate; thus, *F. prausnitzii* is distinct from the new organisms (10). *E. siraeum* produced acetate, ethanol, and large amounts of H₂, as well as traces of lactic, butyric, and succinic acids (12). *C. leptum* produced mainly acetate, as well as some ethanol and large quantities of hydrogen in peptone-yeast extract-maltose medium (12). *C. sporosphaeroides*, however, produced acetate, butyrate, and traces of propionate as fermentation products (9, 12).

The chemotaxonomic and molecular taxonomic features of strains F^T and Fae^T described above justified creation of a novel genus, the genus *Anaerofilum*.

Description of *Anaerofilum* gen. nov. *Anaerofilum* (An.ae.ro' fi. lum. Gr. pref. *an*, not; Gr. n. *aer*, air; Gr. adj. *anaero*, absence of air, referring to the anaerobic mode of living; M.L. n. *filum*, thread, referring to the very thin rod-shaped cells; M.L. n. *Anaerofilum*, anaerobic thin rods). Gram-positive, non-spore-forming, straight, thin rods. The cell wall architecture is typical of gram-positive bacteria. A murein layer is present, but is unusually thin. The peptidoglycan of strain F^T contains L-glycine, D-glutamate, L-lysine, and D-alanine. The interpeptide linkage is formed by L-serine. Cells are fragile and form spheroplasts during the stationary growth phase. Cells are motile and peritrichously flagellated or nonmotile. Obligately anaerobic. No microaerophilic or aerobic growth occurs. Catalase negative. Strains are mesophilic (temperature range, ≥17 to <45°C). Chemoorganotrophic. A variety of mono- and disaccharides are fermented. The major fermentation products from glucose include lactic acid, acetate, ethanol, formate, 2,3-butanediol, and carbon dioxide. Propionate, isobutyrate, isovalerate, succinate, diacetyl, and acetoin are not produced from glucose. Sulfate is not reduced. Hydrogen and hydrogen sulfide are not produced. Isobutyrate and/or isovalerate may be produced by amino acid degradation (isoleucine, valine). The G+C contents of the DNAs of known strains range from 54 to 55 mol%. The habitats are anaerobic sewage sludge of municipal and industrial wastewater treatment plants. Two species are known, *Anaerofilum pentosovorans* and *Anaerofilum agile*; *A. pentosovorans* is designated the type species because it grows better.

Description of *Anaerofilum pentosovorans* sp. nov. *Anaerofilum pentosovorans* (pen.to.so'vo.rans. M.L. n. *pentosum*, sugar

with five carbon atoms; L.v. *vorare*, to eat; L. adj. *pentosovorans*, fermenting pentose). Cells are thin, straight, long rods that are 0.2 to 0.6 by 3 to 6 µm. Cells are seldom motile. Spheroplasts are formed during the stationary growth phase. Colonies on glucose-containing agar plates are white and translucent with lobate margins and raised centers. The G+C content is 55 mol% (as determined by HPLC). The habitat is an industrial wastewater bioreactor at the Hoechst Chemical Co. in Kelsterbach, Frankfurt, Germany. The type strain is strain Fae (= DSM 7168).

Description of *Anaerofilum agile* sp. nov. *Anaerofilum agile* (a'gi.le. M.L. adj. *agile*, rapidly moving). Cells are thin, long rods that are 0.2 to 0.6 by 3 to 6 µm. Cells are motile by means of several peritrichously inserted flagella. Spheroplasts are formed during the stationary growth phase. Colonies on glucose-containing agar plates are circular, slightly convex, white, and translucent. The G+C content is 54.5 mol% (as determined by the thermal denaturation method). The habitat is a methane-producing fixed-film reactor digesting sour whey which was inoculated with sewage sludge from the municipal sewage treatment plant of Regensburg, Germany. The type strain is strain F (= DSM 4272).

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