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Proteiniphilum acetatigenes gen. nov., sp. nov., from a UASB reactor treating brewery wastewater

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Two proteolytic, strictly anaerobic bacterial strains (TB107^T and TB6-6) were isolated from the granule sludge of an upflow anaerobic sludge blanket reactor treating brewery wastewater. The strains were Gram-negative, non-spore-forming and motile. Cells were rod-shaped (0·6–0·9 × 1·9–2·2 μm). Growth of the strains was observed at 20–45 °C and pH 6·0–9·7. The strains were proteolytic. Yeast extract, peptone, pyruvate, glycine and L-arginine could be used as carbon and energy sources. Weak growth was also observed with tryptone, L-serine, L-threonine and L-alanine as carbon and energy sources. Both strains did not use any of the tested carbohydrates, alcohols and fatty acids except pyruvate. Acetic acid and NH_3 were produced from yeast extract, peptone and L-arginine, and propionic acid was also produced from yeast extract. Pyruvate was converted to acetic acid and CO_2. Gelatin was not hydrolysed. Indole and H_2S were not produced. The two strains did not grow in medium containing 20 % bile. Addition of strain TB107^T to a syntrophic propionate-degrading co-culture accelerated the propionate-degradation rate. The predominant cellular fatty acid was the branched-chain fatty acid anteiso-$C_{15:0}$ (46·21 %). The genomic DNA G + C contents of strains TB107^T and TB6-6 were 46·6 and 48·9 mol%, respectively. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the two strains represent a new phyletic sublineage within the *Cytophaga–Flavobacterium–Bacteroides* (CFB) group, with < 91 % 16S rRNA gene sequence similarity to the closest species with validly published names. On the basis of polyphasic evidence from this study, a new genus and species, *Proteiniphilum acetatigenes* gen. nov., sp. nov., is proposed, with strain TB107^T (= JCM 12891^T = AS 1.5024^T) as the type strain.

In methanogenic environments, propionate, one of the central intermediates in the degradation of complex organic matter to CO_2 and methane, can be converted to methane and acetate only by the concerted action of syntrophic bacteria and methanogens, because of energetics difficulties (Schink, 1997). Neither syntrophic propionate-degrading bacteria nor methanogens utilize saccharides and proteinaceous substances, but can utilize short-chain organic acids or inorganics such as CO_2. Pyruvate is an important intermediate not only in the syntrophic degradation of propionate, but also in the dissimilation of glucose and some amino acids. During an investigation of syntrophic propionate-degrading bacteria from methanogenic environments, some propionate-degrading tricultures were isolated. These tricultures degraded propionate to acetate and methane, and included three different micro-organisms; firstly, an egg-shaped, syntrophic, propionate-degrading bacterium (Chen *et al.*, 2005); secondly, *Methanobacterium formicicum*-like cells; and finally, an unidentified rod-shaped bacterium. The rod-shaped bacterium on its own did not consume propionate or synthesize methane; however, it accelerated the propionate-degradation rate of the triculture. The purified rod-shaped bacterial strains utilized pyruvate and produced acetate and propionate from proteinaceous materials, but not glucose or other sugars. Phylogenetically the strains were affiliated to the *Cytophaga–Flavobacterium–Bacteroides* (CFB) group; however, they were only distantly related to recognized genera in this cluster. Hence, a new genus was designated based on the distinctive phenotypic and phylogenetic characteristics of the two strains.

Strains TB107^T and TB6-6 were isolated in pre-reduced peptone-yeast extract (PY) medium (Holdeman *et al.*, 1977)

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Abbreviations: CFB, *Cytophaga–Flavobacterium–Bacteroides*; UASB, upflow anaerobic sludge blanket.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Proteiniphilum acetatigenes* TB107^T is AY742226.

A comparison of the cellular fatty acid compositions of *Proteiniphilum acetatigenes* TB107^T and its phylogenetic relatives is available as a supplementary table in IJSEM Online.

from methanogenic propionate-degrading mixtures by serial dilution and the Hungate roll-tube technique (Hungate, 1969). Single colonies were picked, and transferred to the same broth and incubated at 37 °C for 2 days. The roll-tube procedure was repeated several times until a pure culture was obtained. Culture purity was also checked by microscopic examination. Routine cultivation was in PY broth in anaerobic tubes (18 × 180 mm) sealed with butyl rubber stoppers under a gaseous atmosphere of 100 % N_2 (100 kPa) at 37 °C.

Substrate utilization studies were performed in a basal medium containing various substrates: peptone, yeast extract and tryptone at 0·2 % (final concentration); sugars, fatty acids and alcohols at 20 mM (final concentration); and amino acids at 10 mM (final concentration). The basal medium contained (l^{-1}): 1 g NH_4Cl, 0·3 g K_2HPO_4, 0·3 g KH_2PO_4, 0·6 g NaCl, 0·1 g $CaCl_2.2H_2O$, 0·2 g $MgCl_2$, 0·1 g KCl and 1 mg resazurin. The pH was adjusted to 7 with 1 M NaOH and cultivation conditions were as described above.

Cell morphology was examined under a light microscope (Olympus BH-2) and an electron microscope (Hitachi H-600A). For electron microscopy studies, bacterial cells grown in PY at 37 °C for 2 days were negatively stained with uranyl acetate. For ultrathin-section examination of the cell wall, bacterial cells were fixed with osmic acid and embedded in araldite; the samples were then sliced and stained with lead citrate (Reynolds, 1963).

The generation time of the strains was determined by monitoring the OD_{600} of the PY culture at 37 °C at 1 h intervals up to 72 h. Temperature profiles were determined in PY broth by using a water bath (Guangming medical instrument plant, Beijing) at temperatures of 15 to 55 °C, at 1 °C intervals. The pH range for growth was determined in PY broth at various pH values adjusted with HCl or NaOH (1 mol l^{-1}). Growth was determined by measuring the OD_{600} of cultures at 1, 3 and 7 days. Biochemical traits were determined using both conventional methods (Holdeman *et al.*, 1977) and the API 50 CH system (bioMérieux). All of the tests were performed in duplicate. Short-chain fatty acids and gases of fermentation were detected by using a gas chromatograph (GC-14B; Shimadzu), as described previously (Chen & Dong, 2004).

Genomic DNA was extracted and purified by using the method of Marmur (1961). The G+C content of the DNA was determined by the thermal denaturation method (Marmur & Doty, 1962) using a DU800 spectrophotometer (Beckman) with *Escherichia coli* K-12 as the reference. The 16S rRNA gene was amplified and sequenced according to Chen & Dong (2004). Sequencing was performed by Sangon Biological Engineering Technology Service, Shanghai, China, using ABI PRISM Big Dye Terminator cycle sequencing ready reaction kits (Perkin Elmer) and an ABI PRISM 377XL DNA sequencer. The 16S rRNA gene sequence of strain TB107^T was submitted to GenBank and EMBL to search for similar sequences using the BLAST algorithm. The best matching sequences were retrieved from the database and aligned, and similarity analysis was performed using the program CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic trees were constructed by using neighbour-joining, maximum-likelihood and maximum-parsimony methods implemented in the program MEGA2 (Kumar *et al.*, 2001) and the PHYLIP package (Felsenstein, 1993). The resultant tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings.

Cellular fatty acids were extracted, methylated and analysed using the standard MIDI (Microbial Identification) system (Miller, 1982; Sasser, 1990).

Cells of strains TB107^T and TB6-6 were rod-shaped (0·6–0·9 × 1·9–2·2 μm), occurring singly or in pairs, and were motile by means of two peritrichous flagella (Fig. 1a). The cell wall was Gram-negative, as confirmed by the KOH lysis test (Smibert & Krieg, 1994) and ultrathin-section electron microscopy of strain TB107^T (Fig. 1b). No spores were observed and resistance to treatment at 80 °C for 10 min was not observed. Colonies on PY agar were white, smooth, circular, entire, translucent and slightly convex, reaching 1·5 mm in diameter after cultivation at 37 °C for 72 h.

Strains TB107^T and TB6-6 grew exclusively in pre-reduced media and growth was completely inhibited by air. Both strains grew at 20–45 °C and pH 6·0–9·7, with optimum growth occurring at 37 °C and approximately pH 7·5–8·0. The two strains could grow in the presence of 0–5 % (w/v) NaCl. The mean generation time of strain TB107^T was 11·2 h when grown in PY at 37 °C. The strains were proteolytic. Yeast extract, peptone, pyruvate, glycine and L-arginine could be used as carbon and energy sources. Weak growth was also observed with tryptone, L-serine, L-threonine and L-alanine. The two strains did not use any of the tested carbohydrates, alcohols and fatty acids except pyruvate (detailed data are given in the species description) and did not grow in medium containing 20 % bile. Acetic acid (18 mM) and propionate (10 mM) were produced from 1 % yeast extract, and 10 mM acetic acid and trace amounts of propionate were also produced from 0·5 % peptone. Pyruvate was converted to acetic acid and CO_2. In addition to acetic acid and propionic acid, a trace amount of succinic acid was occasionally detected in PY medium.

The two strains exhibited almost identical physiological and biochemical profiles determined using conventional methods as well as the API 50 CH system. Both isolates hydrolysed aesculin and starch, but not gelatin. NH_3 was produced from yeast extract, peptone and L-arginine, but indole was not produced. H_2S was not produced from peptone or thiosulfate.

The similarity between the partial 16S rRNA gene sequences (500 bp) of strains TB107^T and TB6-6 was 99·6 % and their

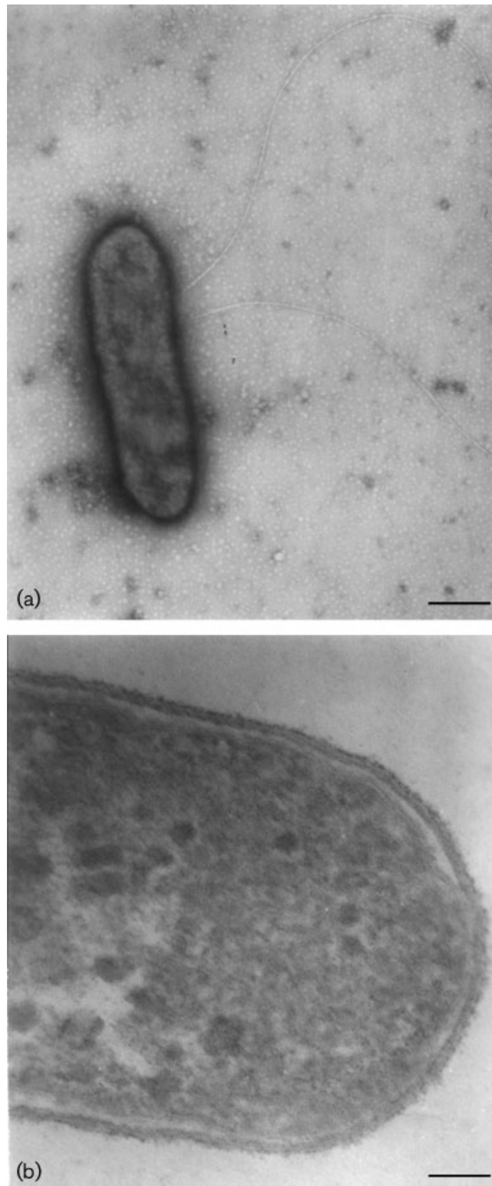

Fig. 1. Transmission electron micrographs of a cell of strain TB107^T (a) and an ultrathin section (b). Bars, 0·5 μm (a) and 0·1 μm (b).

G + C contents were 46·6 and 48·9 mol%, respectively. All of the above results indicate the single species status of the two isolates.

To ascertain the phylogenetic position of the new isolates, the complete 16S rRNA gene sequence (1523 bp) of strain TB107^T was compared with the most similar sequences retrieved from GenBank. On the basis of a consensus 1424 bp of the 16S rRNA gene sequence, a phylogenetic tree rooted with *Capnocytophaga gingivalis* ATCC 33624^T and *Capnocytophaga haemolytica* JCM 8565^T was constructed (Fig. 2). Phylogenetic analysis showed that strain TB107^T belonged to the CFB group (Paster *et al.*, 1994) and clustered with *Dysgonomonas* species (89·6–90·6 % sequence similarity). Bootstrap resampling showed that this relationship was statistically significant (97 % recovery in 1000 resamplings). Other remotely related taxa included the genera *Bacteroides* (85–87 % sequence similarity), *Porphyromonas* (84–88 % sequence similarity), *Prevotella* (79–85 % sequence similarity) and *Tannerella forsythensis* (89·3 % sequence similarity). Based on the sequence divergence, it was evident that the new isolates could not be assigned to any of the described genera in this cluster.

Strains TB107^T and TB6-6 also showed distinct phenotypic features that distinguished them from representative members in the same cluster (Table 1). They differed from *Dysgonomonas* species by the latter's cell shape of coccobacilli, facultative anaerobic growth, lower genomic DNA G + C content (38 mol%) and the ability to ferment glucose. Unlike the non-motile fusiform species *T. forsythensis*, the new isolates did not produce phenylacetic acid and butyric acid from glucose. The novel species differed from *Bacteroides distasonis* and *Bacteroides merdae* by the latter being non-motile and able to produce a large amount of succinic acid during glucose fermentation.

The cellular fatty acids of strain TB107^T were characterized mainly by iso-branched fatty acids, predominantly anteiso-$C_{15:0}$ (46·21 %). $C_{15:0}$ (8·90 %), iso-$C_{17:0}$ 3-OH (5·93 %) and anteiso-$C_{17:0}$ (5·15 %) fatty acids were also relatively abundant, and were proportionally different to those of phylogenetically related genera and species (see the supplementary table in IJSEM Online). Previous studies revealed that iso-$C_{15:0}$ accounted for 33–58 % of the fatty acids of *Porphyromonas* strains, and that anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{17:0}$ and $C_{16:0}$ were the major cellular fatty acids of *Bacteroides* and *Prevotella* (Moore *et al.*, 1994).

An obligate syntrophic propionate-degrading bacterium, *Syntrophobacter sulfatireducens* DSM 16706^T, was also isolated from the same upflow anaerobic sludge blanket (UASB) reactor as strain TB107^T (Chen *et al.*, 2005). This syntrophic strain converted 20 mM propionate to acetate and methane in co-culture with *M. formicicum* DSM 1535^T in 50–60 days, with a propionate-degradation rate of 0·75 mM per day during the exponential phase. When in the triculture with strain TB107^T, 20 mM propionate could be degraded in less than 30 days and the degradation rate was accelerated to about 1·25 mM per day. It appeared that acceleration of the propionate-degradation rate of the syntrophic propionate co-culture by strain TB107^T was due to the provision of unknown nutrient factors or the depletion of pyruvate.

Description of *Proteiniphilum* gen. nov.

Proteiniphilum (Pro.tei′ni.phi.lum. N.L. neut. n. *proteinum* protein; Gr. adj. *philos* loving; N.L. neut. n. *Proteiniphilum* protein loving).

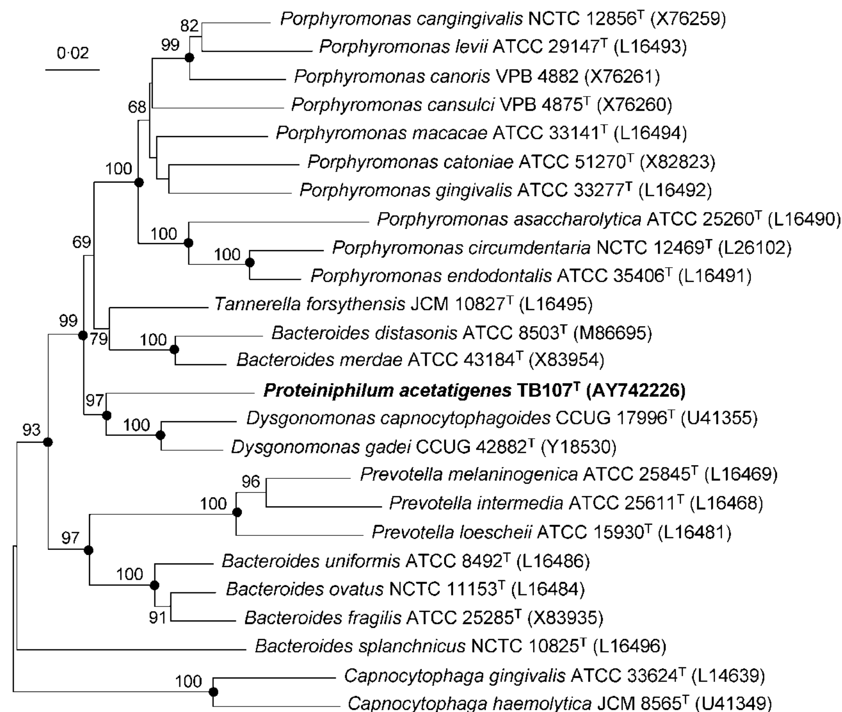

Fig. 2. Phylogenetic dendrogram of *Proteiniphilum acetatigenes* TB107^T and related species based on 16S rRNA gene sequence similarity. The tree was rooted with *C. gingivalis* ATCC 33624^T and *C. haemolytica* JCM 8565^T, and was constructed using the neighbour-joining method. Solid circles indicate that the corresponding nodes (groups) were also recovered using maximum-likelihood and maximum-parsimony methods. Numbers at nodes represent percentage levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets. GenBank accession numbers of 16S rRNA gene sequences are given in parentheses. Bar, 2 % sequence divergence.

Gram-negative, motile, non-spore-forming rods. Obligately anaerobic. Microaerophilic or aerobic growth does not occur. Cellular fatty acids mainly consist of iso-branched fatty acids, predominantly anteiso-$C_{15:0}$. Mesophilic. Grow at 20–45 °C. Chemoorganotrophic. Cytochrome oxidase and catalase are not produced. Proteolytic. Yeast extract and peptone can be used as energy sources. Carbohydrates and alcohols are not used. Gelatin is not hydrolysed. Not resistant to 20 % bile. The major fermentation products from PY are acetic acid and propionic acid. Nitrate is not reduced. The G+C contents of the genomic DNA of the known strains of the type species are 46·6 and 48·9 mol%. Only one species, *Proteiniphilum acetatigenes*, is described so far; this species has been designated the type species.

Table 1. Characteristics that can be used to differentiate *Proteiniphilum acetatigenes* sp. nov. TB107^T from its phylogenetic relatives

Species: 1, *Proteiniphilum acetatigenes* TB107^T; 2, *Dysgonomonas capnocytophagoides* CCUG 17996^T (Hofstad *et al.*, 2000); 3, *Dysgonomonas gadei* CCUG 42882^T (Hofstad *et al.*, 2000); 4, *T. forsythensis* JCM 10827^T (Tanner *et al.*, 1986; Sakamoto *et al.*, 2002); 5, *B. distasonis* ATCC 8503^T (Holdeman *et al.*, 1984); 6, *B. merdae* ATCC 43184^T (Johnson *et al.*, 1986). Symbols: +, positive; −, negative.

Characteristic	1	2	3	4	5	6
Inhabiting niche	UASB sludge	Clinical specimen	Infected gall bladder	Periodontal pockets	Faeces	Faeces
Cell shape	Rod	Coccobacilli to short rod	Coccobacilli	Fusiform	Rod	Rod
Motile	+	−	−	−	−	−
Relation to O_2	Obligate anaerobe	Facultative anaerobe	Facultative anaerobe	Obligate anaerobe	Obligate anaerobe	Obligate anaerobe
DNA G+C content (mol%)	46·6	38	38	46	43–45	44
Glucose fermentation	−	+	+	−	+	+
Major products from PY or PYG*	AP(s)	PLS	[+]	ABiVPPa	SAp(ivibl)	SApibiv
Resistant to ox bile	−	+	+	−	+	+

*Products from PY or peptone-yeast extract-glucose (PYG) medium: upper-case letters, >1 meq of acid per 100 ml broth; lower-case letters, <1 meq of acid per 100 ml; A, acetic acid; B, butyric acid; L, lactic acid; P, propionic acid; S, succinic acid; iB, isobutyric acid; iV, isovaleric acid; Pa, phenylacetic acid; [+], acid is produced. Products in parentheses may or may not be detected.

Description of *Proteiniphilum acetatigenes* sp. nov.

Proteiniphilum acetatigenes (a.ce′ta.ti.gen.es. N.L. *acetas -atis* acetate; Gr. v. *gennao* produce; N.L. part. adj. *acetatigenes* acetate-producing).

Morphology and general characteristics are as described for the genus. Cells are 0·6–0·9 × 1·9–2·2 μm. Colonies on PY agar are circular, slightly convex, white, translucent, and reach 1·5 mm in diameter after 3 days incubation at 37 °C. Optimum growth occurs at 37 °C. The pH range for growth is 6·0–9·7, with optimum growth occurring at pH 7·5–8·0. In addition to yeast extract and peptone, pyruvate, glycine and L-arginine can be used as carbon and energy sources. Weak growth is observed with tryptone, L-serine, L-threonine and L-alanine. Acetic acid is the main product from fermentation of yeast extract, peptone, pyruvate and L-arginine. Propionic acid is also produced from yeast extract. The following substrates are not used: L-histidine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-valine, L-glutamine, tryptophan, L-tyrosine, L-isoleucine, L-proline, L-aspartate, L-cysteine, L-arabinose, cellobiose, aesculin, D-fructose, D-galactose, D-glucose, glycogen, inulin, D-lactose, D-maltose, mannose, melibiose, raffinose, rhamnose, ribose, sucrose, salicin, sorbose, starch, trehalose, D-xylose, adonitol, amygdalin, dulcitol, erythritol, inositol, mannitol, sorbitol, ribitol, methanol, ethanol, 1-propanol, citrate, fumarate, malate, succinate, malonate, hippurate, sodium gluconate, butane diacid, β-hydroxybutyric acid, phenylacetic acid, cellulose and xylan. Milk is not curdled. Starch and aesculin are hydrolysed. Indole is not produced. Urease, lecithinase and lipase are not produced. Methyl red and Voges–Proskauer tests are negative. H_2S is not produced from peptone or thiosulfate. NH_3 is produced from yeast extract, peptone and L-arginine. The major cellular fatty acids are anteiso-$C_{15:0}$ (46·21 %), $C_{15:0}$ (8·90 %), iso-$C_{17:0}$ 3-OH (5·93 %) and anteiso-$C_{17:0}$ (5·15 %).

The type strain is TB107^T (=JCM 12891^T=AS 1.5024^T), which was isolated from the granule sludge of a UASB reactor treating brewery wastewater.

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