Proteiniborus ethanoligenes gen. nov., sp. nov., an anaerobic protein-utilizing bacterium

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A novel anaerobic, mesophilic, protein-utilizing bacterial strain, GW^T, was isolated from the mesophilic hydrogen-producing granular sludge used to treat food industry wastewater. The strain was a Gram-positive, non-spore-forming and non-motile rod. Growth of the strain was observed at 20–48 °C and at pH 6.4–10.0. The strain used yeast extract and peptone as carbon and energy sources. Weak growth was also observed with tryptone and Casamino acids as carbon and energy sources. The strain used none of the tested carbohydrates, alcohols or fatty acids. The fermentation products in peptone-yeast broth included ethanol, acetic acid, hydrogen and carbon dioxide. Gelatin was not hydrolysed. Nitrate was reduced. Indole was produced. NH_3 and H_2S were not produced. The DNA G+C content of strain GW^T was 38.0 mol%. The predominant cellular fatty acids were the saturated fatty acids $C_{14:0}$ (15.58 %), $C_{16:0}$ (25.40 %) and $C_{18:0}$ (12.03 %). Phylogenetic analysis based on 16S rRNA gene sequence similarity revealed that strain GW^T represented a new branch within cluster XII of the *Clostridium* subphylum, with <89.6 % 16S rRNA gene sequence similarities to all described species. On the basis of polyphasic evidence from this study, strain GW^T represents a new genus and novel species, for which the name *Proteiniborus ethanoligenes* gen. nov., sp. nov. is proposed. The type strain is GW^T (=CGMCC 1.5055^T=JCM 14574^T).

Hydrogen is a clean and renewable energy source, and bioproduction of hydrogen by micro-organisms falls into two main categories: photosynthetic biohydrogenesis and fermentative biohydrogenesis by anaerobic bacteria. One of the advantages of the fermentative biohydrogenesis process is the possibility of using organic wastes for bio-energy production through anaerobic degradation. Anaerobic degradation is a widely applied and economically attractive technology used in the treatment of various organic wastes (Lettinga, 1995). Currently most investigations concerning anaerobic degradation of organic materials have focused on the metabolism of carbohydrates, especially in the fermentative biohydrogenesis process. However, many industrial and agricultural wastes also contain a considerable amount of proteinaceous materials and fat. Although part of this can be digested by some saccharide users, the protein-specific degraders are needed to remove proteinaceous materials efficiently from the wastewater.

In recent years, a few protein-specific anaerobic degraders, such as *Clostridium thiosulfatireducens* (Hernández-Eugenio *et al.*, 2002), '*Clostridium tunisiense*' (Thabet *et al.*, 2004) and *Proteiniphilum acetatigenes* (Chen & Dong, 2005), have been isolated from upflow anaerobic sludge blanket (UASB) reactor sludge. They were all proteolytic, chemo-organotrophic anaerobic bacteria characterized by using only proteinaceous compounds, but not any tested carbohydrates, as a carbon and energy source. During a study on the microbial composition of a fermentative hydrogen-producing reactor, several hydrogen-producing anaerobic bacterial strains were isolated with a rich medium (glucose-peptone-yeast extract), among which was a proteinaceous compound-specific strain. Phylogenetically the strain was affiliated to the phylum of low G+C Gram-positive bacteria but was distantly related to all the existing species. Hence a novel mesophilic, protein-utilizing, hydrogen-producing anaerobe is described in this paper.

A pre-reduced PY medium (Holdeman *et al.*, 1977) was used for isolation and routine cultivation. Granular sludge from a laboratory-scale UASB reactor for treating food industry wastewater and producing hydrogen was used as inoculum. Granules were crushed with a mortar in an anaerobic glove box (Forma scientific 1209) and then inoculated into the pre-reduced PY broth under 100 % N_2. After the enrichments were incubated at 37 °C for 24 h, the Hungate roll-tube technique (Hungate, 1969) was

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Proteiniborus ethanoligenes* GW^T is EF116488.

An electron micrograph of an ultrathin section of strain GW^T is available with the online version of this paper.

performed. Single colonies in the roll-tube 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 of strain GW^T was obtained. The purity of the culture was examined under a light microscope.

Substrate utilization studies were performed in a basal medium containing different substrates as follows: Casamino acids, peptone, yeast extract and tryptone (0.2 %, final concentration); sugars, fatty acids (20 mM, final concentration) and amino acids (0.2 %, final concentration). The basal medium contained (l^{-1}): 1 g NH_4Cl, 0.3 g KH_2PO_4, 0.3 g K_2HPO_4, 0.6 g NaCl, 0.1 g $CaCl_2.2H_2O$, 0.1 g $MgCl_2.6H_2O$ and 1 mg resazurin. The pH was adjusted to 7.5 with 1 M NaOH.

Cell morphology was examined under a light (Olympus BH-2) and electron (Hitachi H-600A) microscope. For electron microscopy studies, bacterial cells grown in PY at 37 °C for 24 h 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 strain GW^T was determined by monitoring the OD_{600} of the PY culture at 37 °C at 1 h intervals up to 48 h. The temperature profile was determined in PY using a water bath with temperature controller between temperatures of 15 to 55 °C at 1 °C intervals. The pH range for growth was determined for the culture 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 conventional methods. All tests were performed in duplicate. The fermentation products, short-chain fatty acids, alcohols and gases were measured using a gas chromatograph (GC-14B Shimadzu) as described previously (Chen & Dong, 2004). The diagnostic isomers of diaminopimelic acid and amino acids in the cell wall were determined with established TLC procedures (Lechevalier & Lechevalier, 1980). Cellular fatty acids were extracted, methylated and analysed using the standard MIDI (Microbial Identification) system (Miller, 1982; Sasser, 1990).

Genomic DNA was extracted and purified 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). The 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 GW^T was submitted to GenBank and EMBL to search for similar sequences using the BLAST algorithm (Altschul *et al.*, 1990). Sequences with higher similarities were retrieved from the database and aligned and similarity analysis was performed using the CLUSTAL_X program (Thompson *et al.*, 1997). Phylogenetic trees were constructed using the neighbour-joining, maximum-likelihood and maximum-parsimony methods implemented in the program MEGA3 (Kumar *et al.*, 2004). The resultant tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings.

Cells of strain GW^T were non-motile short rods (supplementary Fig. S1 in IJSEM Online), 0.5–0.6 μm × 1.4–3.8 μm, and arranged singly or in clumps. The cells stained Gram-negative in all the growth phases; however, a Gram-positive bacterial cell wall ultrastructure was revealed by electron microscopy (Fig. 1). Spores were never observed. Colonies on PY agar were white, smooth, circular and entire and slightly convex and reached 0.5 mm after cultivation at 37 °C for 72 h.

Strain GW^T grew strictly anaerobically and growth occurred from 20 to 48 °C and at pH 6.4–10.0, with optimum growth at 37 °C and at approximately pH 8.5–8.8. The strain could grow in the presence of 0–2 % (w/v) NaCl. The doubling time of strain GW^T was 3.3 h when growing on PY at 37 °C. The strain used proteinaceous compounds exclusively, and did not use any of the tested carbohydrates, alcohols or fatty acids. Yeast extract and peptone could be used as carbon and energy sources. Weak growth was also observed with tryptone and Casamino acids, but the strain did not use any of the single amino acids. When growing in 0.4 % yeast extract and 0.2 % peptone medium, 15 mM ethanol, 10 mM hydrogen, 6 mM acetate and a trace amount of propionate were produced. Strain GW^T hydrolysed casein, but not gelatin or DNA, and did not curdle milk. Indole was produced from yeast extract and peptone; however, NH_3 was not produced. Nitrate could be utilized as electron acceptor. No H_2S was produced from peptone or thiosulfate.

The cell wall hydrolysate of strain GW^T was rich in L-lysine, but no diaminopimelic acids were detected. The cellular fatty acids of strain GW^T were characterized mainly by saturated fatty acids, predominantly $C_{14:0}$ (15.58 %), $C_{16:0}$ (25.40 %) and $C_{18:0}$ (12.03 %); $C_{18:1}\omega 9c$ (11.20 %), $C_{16:1}\omega 7c$ (6.18 %), iso$C_{17:1}$ I (9.49 %) and iso$C_{15:0}$ (4.30 %) were also relatively abundant.

The genomic DNA G+C content of strain GW^T was determined as 38.0 mol%.

To ascertain the phylogenetic position of the novel strain, the complete 16S rRNA gene sequence (1523 bp) of strain GW^T was compared with the most similar sequences and those of the representatives of the '*Clostridia*' retrieved from GenBank. On the basis of a consensus 1367 bp of the 16S rRNA gene sequence, a phylogenetic tree rooted with *Peptostreptococcus hydrogenalis* GIFU 7662^T was constructed. Phylogenetic analysis showed that strain GW^T was affiliated to the low G+C Gram-positive bacteria phylum, and belonged to cluster XII of the '*Clostridia*'

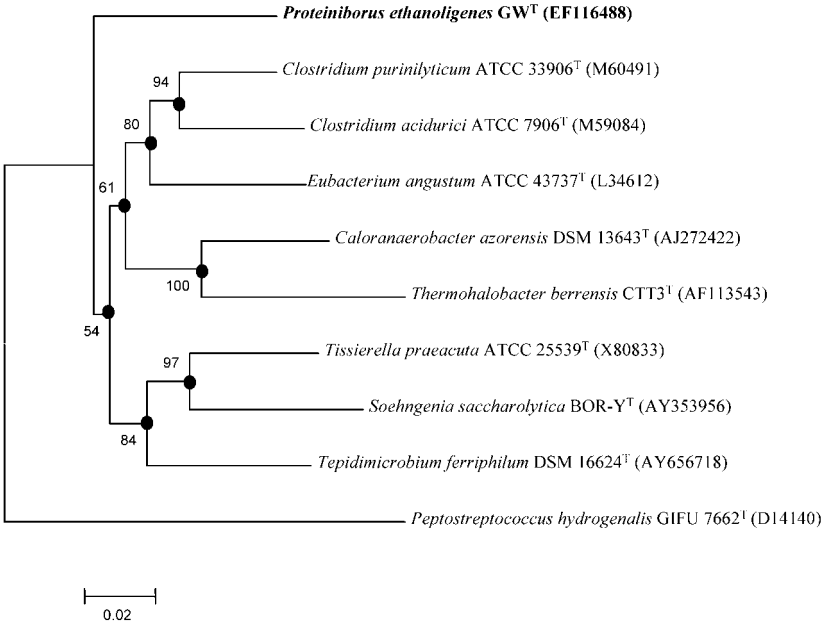

Fig. 1. Phylogenetic dendrogram of 16S rRNA gene sequences indicating the position of strain GW^T among the bacterial members in cluster XII of the '*Clostridia*'. The tree rooted with *Peptostreptococcus hydrogenalis* was constructed using the neighbour-joining method. Solid circles indicate that the corresponding nodes (groups) are also recovered in UPGMA and minimum evolution methods. Numbers at nodes represent levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets. GenBank accession numbers of 16S rRNA sequences are given in parentheses. Bar, 2 % sequence divergence.

(Collins *et al.*, 1994). The closest relatives were *Clostridium purinilyticum*, *Clostridium acidurici* and *Eubacterium angustum*, with sequence similarity levels of 89.7, 88.9 and 88.8 %, respectively. The similarity levels of the 16S rRNA gene sequence with that of other members in phylogenetic cluster XII ranged between 86 and 88 %. Trees constructed by neighbour-joining, UPGMA and minimum evolution showed the same topology. The 16S rRNA sequence of strain GW^T is not closely related to that of previously described taxa and represents a novel genus.

Strain GW^T also showed distinct phenotypic features distinguishing it from representative members in the same cluster (Table 1). *Clostridium purinilyticum*, *Clostridium acidurici* and *Eubacterium angustum* grew exclusively on purines, such as uric acid and adenine (Dürre *et al.*, 1981; Cato *et al.*, 1986; Beuscher & Andreesen, 1984), however strain GW^T could not use these substances at all. Also, the three species were not able to use proteinaceous materials as sole carbon and energy sources. Moreover, strain GW^T was also different from the species *Caloranaerobacter*

Table 1. Characteristics differentiating strain GW^T from its phylogenetic relatives

Species: 1, *Proteiniborus ethanoligenes* JCM 14574^T; 2, *Clostridium purinilyticum* ATCC 33906^T (Dürre *et al.*, 1981); 3, *Eubacterium angustum* ATCC 43737^T (Beuscher & Andreesen, 1984); 4, *Caloranaerobacter azorensis* DSM 13643^T (Wery *et al.*, 2001); 5, *Thermohalobacter berrensis* CTT3^T (Cayol *et al.*, 2000). Symbols: +, positive; −, negative; NR, not reported.

Characteristic	1	2	3	4	5
Inhabiting niche	UASB sludge	Farm soil	Sewage sludge	Deep-sea hydrothermal vent	Solar saltern
Gram type	+	+	+	−	−
Spore formation	−	+	−	−	−
Motility	−	+	−	+	+
Optimum temp. (°C)	37	36	37	65	65
Optimum pH	8.5–8.8	7.3–7.8	8.0–8.2	7.0	7.0
Glucose fermentation	−	−	−	+	+
Major products from PYG*	A2p	−	−	Aiv	A2
Peptone used as carbon source	+	−	−	−	−
Purine utilized	−	+	+	−	−
Hydrogen produced	+	+	+	−	+
Hydrolysis of gelatin	−	−	+	NR	−
Nitrate reduced	+	−	−	−	−
G+C content (mol%)	38.0	29	40.3	27	33

*Products: a, acetic acid; p, propionic acid; iv, isovaleric acid; 2, ethanol. Upper-case and lower-case letters indicate major and minor fermentation products, respectively.

azorensis and *Thermohalobacter berrensis* in the same phylogenetic branch in the optimal growth temperature and substrate range: while strain GW^T is mesophilic and a protein-consumer, the other two species grow optimally at 65 °C and are sugar-consumers (Wery *et al.*, 2001; Cayol *et al.*, 2000).

On the basis of the physiological properties and the phylogenetic analyses, strain GW^T is proposed as a novel genus and novel species, *Proteiniborus ethanoligenes* gen. nov., sp. nov., affiliated with the low G+C Gram-positive bacteria phylum.

Description of *Proteiniborus* gen. nov.

Proteiniborus (Pro.tei.ni′bo.rus. N.L. n. *proteinum* protein; Gr. adj. *boros* gluttonous; N.L. masc. n. *Proteiniborus* protein-consumer).

Gram-positive, non-motile, non-spore-forming rod. Anaerobic and mesophilic. Cell wall peptidoglycan contains abundant L-lysine but not diaminopimelic acids. Cellular fatty acids consist mainly of saturated fatty acids, predominantly $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$. Chemo-organotrophic. Yeast extract and peptone can be used as energy sources. The fermentation products from PY include ethanol, acetic acid, hydrogen and carbon dioxide. Nitrate but not thiosulfate is reduced. The G+C content of the genomic DNA of the known strain is 38.0 mol%. The type species of the genus is *Proteiniborus ethanoligenes*, a member of cluster XII of the '*Clostridia*'.

Description of *Proteiniborus ethanoligenes* sp. nov.

Proteiniborus ethanoligenes (e.tha.no.li′ge.nes. N.L. n. *ethanol-is* ethanol; Gr. v. *gennao* produce; N.L. part. adj. *ethanoligenes* ethanol-producing).

Morphology and general characteristics are as described for the genus. Cells are 0.5–0.6 μm wide and 1.4–3.8 μm long. Colonies on PY agar are white, smooth, circular and entire and slightly convex and reach 0.5 mm after cultivation at 37 °C for 72 h. Growth occurs between 20 and 48 °C (optimum 37 °C) and at pH 6.4–10.0 (optimum 8.5–8.8). Besides yeast extract and peptone, weak growth is also observed on tryptone and Casamino acids. Ethanol, acetic acid and hydrogen are the main products from yeast extract and peptone, and a trace amount of propionic acid is also produced. The following substrates are not used: L-serine, L-threonine, L-alanine, L-histidine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-valine, L-glutamine, L-arginine, L-tyrosine, tryptophan, L-isoleucine, L-proline, aspartate, L-cysteine, L-arabinose, cellobiose, aesculin, D-fructose, D-galactose, D-glucose, glycogen, inulin, D-lactose, maltose, mannose, melibiose, raffinose, rhamnose, ribose, sucrose, salicin, sorbose, starch, trehalose, D-xylose, adonitol, amygdalin, dulcitol, erythritol, inositol, mannitol, sorbitol, ribitol, methanol, ethanol, l-propanol, citrate, fumarate, malate, succinate, malonate, hippurate, sodium gluconate, butane diacid, β-hydroxybutyric acid, phenylacetic acid, cellulose and xylan. Milk is not curdled. Indole is produced. No NH_3 is produced from yeast extract or peptone. Methyl red and Voges–Proskauer tests are negative. Casein is degraded and nitrate is reduced. Gelatin and DNA are not hydrolysed.

The type strain is GW^T (=CGMCC 1.5055^T=JCM 14574^T), isolated from granular sludge from a laboratory-scale UASB hydrogen-producing reactor used to treat food industry wastewater.

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