Hydrogenoanaerobacterium saccharovorans gen. nov., sp. nov., isolated from H₂-producing UASB granules

Lei Song^{1,2} and Xiuzhu Dong¹

Correspondence

Xiuzhu Dong

dongxz@sun.im.ac.cn

¹State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, PR China

²Graduate School of Chinese Academy of Sciences, Beijing, PR China

Two strictly anaerobic bacterial strains, designated SW512^T and W72, were isolated from a laboratory-scale H₂-producing up-flow anaerobic sludge blanket (UASB) reactor. Cells were Gram-stain-negative, non-motile and 0.3–0.4×2.0–14.5 µm; they did not form spores. Both strains grew at 24–45 °C (no growth at ≤22 °C or ≥46 °C), with optimum growth at 37 °C. The pH range for growth was 4.5–9.0 (no growth at pH ≤4.2 or pH ≥9.3), with optimum growth at pH 6.5–7.0. Several kinds of mono-, di- and oligosaccharides supported growth. The main end products of glucose fermentation were ethanol, acetate, hydrogen and carbon dioxide (according to the equation 1 mol glucose→1.1 mol acetate+0.6 mol ethanol+2.6 mol H₂+1.6 mol CO₂). The DNA G+C contents of strains SW512^T and W72 were 41.9±0.5 and 42.8±0.4 mol% (T_m method), respectively. Phylogenetic analysis based on 16S rRNA gene sequence similarities indicated that the two strains represent a new phyletic sublineage within the family 'Ruminococcaceae', with <91.3% 16S rRNA gene sequence similarity to recognized species. On the basis of the polyphasic evidence in this study, it is proposed that the two strains represent a novel species in a new genus, for which the name *Hydrogenoanaerobacterium saccharovorans* gen. nov., sp. nov. is proposed; the type strain of the type species is SW512^T (=AS 1.5070^T=JCM 14861^T).

Hydrogen is considered to be a promising green alternative to fossil fuels since it is an efficient energy carrier and does not produce any greenhouse gases during combustion. The up-flow anaerobic sludge blanket (UASB) reactor has been widely used for biohydrogen production in recent years. One of its outstanding characteristics is that the anaerobic micro-organisms can be self-immobilized to form active sludge granules. During the development of granulation, large amounts of biomass and diverse microbes can be retained in the reactor, which greatly promotes treatment capacity and efficiency.

During a survey on the diversity of strict anaerobic bacteria in a laboratory-scale H₂-producing UASB reactor, two bacterial strains that produced large amounts of hydrogen from carbohydrates were isolated. In this study, a taxonomic investigation of the two strains is presented

and it is proposed that these two strains can be classified in a novel species in a new genus.

Anaerotruncus colihominis DSM 17241^T was purchased from the DSMZ, Braunschweig, Germany. Strains SW512^T and W72 were isolated in the pre-reduced basal medium described by McNerney *et al.* (1979) supplemented with 1% glucose as carbon source by serial dilution and the roll-tube technique (Hungate, 1969). Routine cultivation was in the same medium in anaerobic tubes sealed with butyl rubber stoppers under a gaseous atmosphere of 100% N₂ (100 kPa) at 37 °C. A single colony was picked and transferred to the same broth and incubated at 37 °C for 24 h. Culture purity was checked by microscopic examination. Colonies of strain SW512^T were smooth, circular, white and translucent, measuring 1.0–1.5 mm in diameter after cultivation at 37 °C for 48 h.

Gram staining was carried out according to Johnson *et al.* (1995) and cell morphology was examined by light microscopy (BH-2; Olympus) and by electron microscopy (H-600A; Hitachi) of cells that had been negatively stained with uranyl acetate. Cells of strain SW512^T and W72 were non-motile, thin rods (0.3–0.4×2.0–14.5 µm), occurring singly or in pairs (Fig. 1a). The cells stained Gram-negative

Abbreviation: UASB, up-flow anaerobic sludge blanket.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Hydrogenoanaerobacterium saccharovorans* sp. nov. SW512^T and W72 are EU158190 and EU170433, respectively.

A dendrogram constructed using the UPGMA method based on 16S rRNA gene sequence similarities between strain SW512^T and related strains is available with the online version of this paper.

Fig. 1. Electron micrographs of cells of strain SW512^T. (a) Negatively stained cells (bar, 1.0 μm). (b) Ultrathin cell section (bar, 0.1 μm).

throughout the whole exponential growth phase; however, the ultrathin cell section electron micrograph (Fig. 1b) showed an atypical Gram-positive structure cell wall. Strain SW512^T grew exclusively in pre-reduced media and growth was completely inhibited under a low concentration of air (2.0–3.0 %). No spores were observed by microscopy either after cultivation on low nutrient agar at 37 °C for 1, 3 and 7 days or by heat-treatment at 80 °C for 10 min.

The growth temperature range for strain SW512^T, determined using a water bath, was 24–45 °C, with optimum growth at 37 °C. No growth was detected at ≤ 22 °C or ≥ 46 °C after incubation for 72 h. The pH range for growth of strain SW512^T was 4.5–9.0 and it grew optimally at pH 6.5–7.0 in medium adjusted to various pH values with HCl or NaOH (1 mol l⁻¹). No growth was detected at pH ≤ 4.2 or pH ≥ 9.3 after incubation for 72 h. The biochemical traits of the two strains were determined using conventional methods (Kämpfer *et al.*, 1991). Experiments showed that the two strains utilized several kinds of mono-, di- and oligosaccharides and exhibited almost identical physiological and biochemical profiles except that strain

SW512^T fermented arabinose, but strain W72 did not. A detailed profile of fermentable sugars is given in the species description below. The strains required yeast extract and peptone for growth but inorganic nitrogen compounds, such as NH₄Cl, (NH₄)₂SO₄, (NH₄)₂HPO₄ and KNO₃, could not serve as nitrogen sources.

The growth yield of strain SW512^T in peptone-yeast extract-glucose (PYG) medium was 1.420 g cell dry mass (l culture)⁻¹ after cultivation under optimum growth conditions for 48 h. The main end products from glucose were ethanol, acetate, hydrogen and carbon dioxide, determined by GC (GC-14B; Shimadzu) according to Chen & Dong (2004). Strain SW512^T produced 2.6 mol H₂ per mol glucose fermented under optimal hydrogen-producing conditions (pH 7.8 and 39 °C).

Cellular fatty acids were extracted, methylated and analysed using the standard MIDI (Microbial Identification) system (Miller, 1982; Sasser, 1990). The cellular fatty acids of strain SW512^T were mainly comprised of saturated fatty acids and iso-branched fatty acids, predominantly C_{16:0} (29.1 %), C_{14:0} (15.6 %), iso-C_{17:1} ω 9c (12.0 %), iso-C_{15:0} (11.6 %) and iso-C_{13:0} (5.2 %). This fatty acid profile clearly differed from those of phylogenetically related *Clostridium* species; the cellular fatty acid profiles of mesophilic members are characterized by higher percentages of unsaturated fatty acids and do not contain branched-chain fatty acids (Kaneda, 1991).

Genomic DNA extraction was performed as described previously (Marmur, 1961). The DNA G+C contents of strains SW512^T and W72 were determined as 41.9 ± 0.5 and 42.8 ± 0.4 mol%, respectively, by the thermal denaturation method (Marmur & Doty, 1962) using a DU800 spectrophotometer (Beckman) with *Escherichia coli* K-12 as reference.

To determine the phylogenetic affiliation of strains SW512^T and W72, the complete 16S rRNA genes were amplified by PCR and sequenced as described previously (Chen & Dong, 2004). Sequences were submitted to GenBank and the BLAST algorithm was used to search for similar sequences. The best matching sequences were retrieved from the database and aligned; similarity analysis was performed using the CLUSTAL_X program (Thompson *et al.*, 1997). On the basis of a consensus 16S rRNA gene sequence of 1358 bp, an integrated phylogenetic tree rooted with *Clostridium butyricum* ATCC 19398^T was constructed (Fig. 2) 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. Strains SW512^T and W72 were closely related to each other genetically and phylogenetically, displaying 99.8 % 16S rRNA gene sequence similarity; this information and the almost identical physiological and biochemical profiles indicate that the two isolates can be considered to be a

Fig. 2. Phylogenetic dendrogram of strains SW512^T and W72 and strains of related species based on 16S rRNA gene sequence similarities. The tree was rooted with *Clostridium butyricum* ATCC 19398^T and constructed using the neighbour-joining method. Solid circles indicate that the corresponding nodes (groups) are also recovered with the maximum-likelihood and maximum-parsimony methods. Numbers at nodes represent levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets. GenBank accession numbers are given in parentheses. Bar, 2% sequence divergence.

single species. Phylogenetically, the two strains formed a distinct lineage within the family ‘*Ruminococcaceae*’, which consists of phenotypically and phylogenetically diverse groups. Strain SW512^T showed the highest 16S rRNA gene sequence similarity (91.3%) to the type strain of *Acetanaerobacterium elongatum*; similarities were less than 91.0% with type strains of other related species. A phylogenetic tree constructed using the UPGMA method (see Supplementary Fig. S1 in IJSEM Online) also showed the clustering of the two strains with members of the family ‘*Ruminococcaceae*’. The relatively large sequence divergence indicated that the strains in this study are members of a new genus in this family.

In addition to the large sequence divergence, the two strains also showed distinct phenotypic features that enabled them to be distinguished from representative members in the same cluster (Table 1). They differed from the type strain of *Acetanaerobacterium elongatum* in terms of motility (the two novel strains are non-motile), as well as in biochemical traits such as the milk reaction and fermentable sugars. They differed from the type strain of *Anaerotruncus colihominis* as they were unable to produce butyric acid from glucose or produce indole.

On the basis of the distant phylogenetic relationship with related taxa, unique chemotaxonomic characteristics, DNA

G+C content and physiological and biochemical traits described above, it is evident that strains SW512^T and W72 represent a novel species in a distinct genus of the family ‘*Ruminococcaceae*’ within the order *Clostridiales*, for which the name *Hydrogenoanaerobacterium saccharovorans* gen. nov., sp. nov. is proposed.

Description of *Hydrogenoanaerobacterium* gen. nov.

Hydrogenoanaerobacterium (Hy.dro.gen.o.an.ae.ro.bac.te’ri.um. N.Gr. n. *hydrogenon* hydrogen; Gr. pref. *an-* without; Gr. n. *aer* air; Gr. neut. n. *baktron* a small rod; N.L. neut. n. *Hydrogenoanaerobacterium* an anaerobic, hydrogenogenic, rod-shaped bacterium).

Gram-stain-negative, but with an atypical Gram-positive structure cell wall. Non-spore-forming, non-motile, straight, thin rods. Obligately anaerobic. Mesophilic and neutrophilic. Cellular fatty acids consist mainly of saturated fatty acids and iso-branched fatty acids, predominantly C_{14:0}, iso-C_{15:0}, C_{16:0} and iso-C_{17:1ω9c}. Various mono-, di- and oligosaccharides, including glucose, are fermented. The major end products from glucose are ethanol, acetate, hydrogen and carbon dioxide.

The type species is *Hydrogenoanaerobacterium saccharovorans*.

Table 1. Differential characteristics of *Hydrogenoanaerobacterium saccharovorans* sp. nov. and its nearest phylogenetic relatives

Strains: 1, *H. saccharovorans* SW512^T; 2, *H. saccharovorans* W72; 3, *Acetanaerobacterium elongatum* JCM 12359^T (Chen & Dong, 2004); 4, *Anaerotruncus colihominis* DSM 17241^T (Lawson *et al.*, 2004).

Characteristic	1	2	3	4
Products from glucose*	A2	A2	A2	AB
H ₂ production†	4	4	4	+
Motility	—	—	+	—
Optimum growth temperature (°C)	37	37	37	36–40
Gelatinase	+	+	+	—
Milk reaction	—	—	C‡	—§
DNA G + C content (mol%)	41.9 ± 0.5	42.8 ± 0.4	50.5	54
Acid production from:				
Arabinose	+	—	+	—
Cellobiose	—	—	+	+
Fructose	—	—	+	+
Glycogen	+	+	—	—§
Inositol	—	—	—	+
Inulin	+	+	+	+§
Mannitol	—	—	—	—
Mannose	+	+	—	+
Raffinose	+	+	+	—
Salicin	—	—	+	—
Starch	—	—	—	—
Sucrose	+	+	+	—
Trehalose	+	+	—	+

*Fermentation products: A, acetate; B, butyrate; 2, ethanol.

†On a scale of + (very small amount) to 4 (abundant).

‡C, Curdled milk.

§Data from this study.

Description of *Hydrogenoanaerobacterium saccharovorans* sp. nov.

Hydrogenoanaerobacterium saccharovorans (sac.cha.ro.vo'rans. Gr. n. *saccharon* sugar; L. part. adj. *vorans* devouring, digesting; N.L. part. adj. *saccharovorans* sugar-digesting).

Cells are 0.3–0.4 × 2.0–14.5 µm. Surface colonies on PYG agar are smooth, circular, white, semi-opaque and 1.0–1.5 mm in diameter after cultivation at 37 °C for 48 h. Grows at 24–45 °C (no growth at ≤22 °C or ≥46 °C), with optimum growth at 37 °C. The pH range for growth is 4.5–9.0 (no growth at pH ≤4.2 or pH ≥9.3), with optimum growth at pH 6.5–7.0. Oxidase and catalase are not produced. Gelatin and aesculin are hydrolysed. Acid is produced from a number of sugars, including glycogen, raffinose, sucrose, glucose, ribose, mannose, lactose, arabinose, maltose, inulin and trehalose. Acid is not produced from glycerol, mannitol, fructose, xylose, sorbitol, cellobiose, rhamnose, inositol, salicin or starch. Complex substrates such as yeast extract and peptone are also fermented. The major end products of glucose fermentation are ethanol, acetate, hydrogen and carbon dioxide according to the equation 1 mol glucose → 1.1 mol acetate + 0.6 mol ethanol + 2.6 mol H₂ + 1.6 mol CO₂. Milk reaction is

negative. Nitrate is not reduced. H₂S, but not indole, is produced on PYG. The major cellular fatty acids are C_{16:0}, C_{14:0}, iso-C_{17:1}ω9c, iso-C_{15:0} and iso-C_{13:0}.

The type strain, SW512^T (=AS 1.5070^T=JCM 14861^T), was isolated from a laboratory-scale UASB reactor at ambient temperature. The DNA G + C content of the type strain is 41.9 ± 0.5 mol%.

Acknowledgements

We thank Professor Hanqing Yu for donating sludge granules. This study was supported by the National Basic Research Program of China (2004CB719602).

References

- Chen, S. & Dong, X. (2004). *Acetanaerobacterium elongatum* gen. nov., sp. nov., from paper mill waste water. *Int J Syst Evol Microbiol* **54**, 2257–2262.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Felsenstein, J. (1993). PHYLIP (phylogenetic inference package), version 3.5c. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.

- Hungate, R. E. (1969).** A roll tube method for cultivation of strict anaerobes. *Methods Microbiol* **3B**, 117–132.
- Johnson, M. J., Thatcher, E. & Cox, M. E. (1995).** Techniques for controlling variability in gram staining of obligate anaerobes. *J Clin Microbiol* **33**, 755–758.
- Kämpfer, P., Steiof, M. & Dott, W. (1991).** Microbiological characterization of a fuel-oil contaminated site including numerical identification of heterotrophic water and soil bacteria. *Microb Ecol* **21**, 227–251.
- Kaneda, T. (1991).** Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. *Microbiol Rev* **55**, 288–302.
- Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001).** MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.
- Lawson, P. A., Song, Y., Liu, C., Molitoris, D. R., Vaisanen, M. L., Collins, M. D. & Finegold, S. M. (2004).** *Anaerotruncus colihominis* gen. nov., sp. nov., from human faeces. *Int J Syst Evol Microbiol* **54**, 413–417.
- Marmur, J. (1961).** A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Marmur, J. & Doty, P. (1962).** Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.
- McInerney, M. J., Bryant, M. P. & Pfennig, N. (1979).** Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. *Arch Microbiol* **122**, 129–135.
- Miller, L. T. (1982).** Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxyl acids. *J Clin Microbiol* **16**, 584–586.
- Sasser, M. (1990).** *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.