Saccharofermentans acetigenes gen. nov., sp. nov., an anaerobic bacterium isolated from sludge treating brewery wastewater

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A spore-forming anaerobic bacterium, designated strain P6^T, was isolated from the sludge of an up-flow anaerobic sludge blanket reactor treating brewery wastewater. Cells were Gram-positive, oval and 0.6–0.9 μm by 1.2–1.8 μm in size. Growth was observed at 20–42 °C and at pH 5.0–7.5. It fermented several hexoses, polysaccharides and alcohols. Sucrose and aesculin could also be fermented. The main end products of fermentation from glucose were acetate, lactate and fumarate; trace CO_2 and H_2 were also produced. The DNA G+C content of strain P6^T was 55.6 mol%. The major cellular fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{14:0}$ 3-OH. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain P6^T represented a novel phyletic sublineage in clostridial cluster III, and showed <91 % similarity to the type strains of recognized species in this cluster. Phenotypically, the new isolate was distinguished from its phylogenetic relatives (e.g. *Clostridium straminisolvens*, *Clostridium thermocellum*, *Acetivibrio cellulolyticus* and *Clostridium aldrichii*) by producing acid from glucose and its inability to degrade cellulose. On the basis of evidence from this polyphasic study, strain P6^T is considered to represent a novel species of a new genus, for which the name *Saccharofermentans acetigenes* gen. nov., sp. nov. is proposed. The type strain of *Saccharofermentans acetigenes* is P6^T (=JCM 14006^T =AS 1.5064^T).

Complex organic matter is degraded completely to CO_2 and CH_4 by the association of several trophic micro-organisms in methanogenic environments (Zehnder, 1978). The presence of members of the class *Clostridia* in brewery wastewater was revealed by 16S rRNA gene sequence analysis (Liu *et al.*, 2002). During a survey of the microbial community in sludge treating brewery wastewater in Fujian Province, China, we isolated a novel obligately anaerobic, spore-forming bacterial strain (designated P6^T). This strain fermented several sugars and produced mainly acetic acid from glucose fermentation. Phylogenetically, the strain was affiliated to clostridial cluster III of the low-G+C content Gram-positive bacteria (Collins *et al.*, 1994), but was distantly related to all recognized genera and species in this cluster. Based on its distinctive phenotypic, genotypic and phylogenetic characteristics, this strain is shown to represent a novel species of a new genus.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P6^T is AY 949857.

Strain P6^T was isolated in pre-reduced peptone-yeast extract-glucose (PYG) medium (Holdeman *et al.*, 1977) by serial dilution with 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 rolling tube procedure was repeated several times until a pure culture was obtained. The purity of the isolate was confirmed by the homogeneous morphology of colonies on the agar surface as well as cell type observed by microscopy (see below). Routine cultivation was in anaerobically prepared PYG broth in tubes (18×150 mm) sealed with butyl rubber stoppers under a gaseous atmosphere of 100 % N_2 (100 kPa) at 37 °C. *Acetivibrio cellulolyticus* ATCC 33288^T was obtained from the American Type Culture Collection. *Clostridium aldrichii* DSM 6159^T and *Clostridium thermocellum* DSM 1237^T were obtained from the Deutsche Sammlung von Mikroorganismen. PY medium plus 1 % cellobiose was used for cultivation of these reference strains.

Cell morphology was examined by light microscopy (Olympus BH-2) as well as electron microscopy (Hitachi

H-600A) after negative staining with uranyl acetate. Generation time was determined by monitoring OD_{600} of a PYG broth culture at 37 °C at 1 h intervals up to 48 h. Temperature profiles were determined in PYG broth by using a water bath (MEMMERT WB 22) at 15–55 °C (1 °C intervals). The pH range for growth of strain P6^T was determined in PYG broth adjusted to pH 4.5–9.5 with HCl or NaOH (1 M). Growth was determined by measuring the OD_{600} of the cultures at 1, 3 and 7 days. Biochemical traits were determined by using both conventional methods and the API 50CH system (bioMérieux). All tests were performed in duplicate. Short-chain fatty acids and gases produced from fermentation in PYG medium were measured by using a gas chromatograph (Shimadzu GC-14B) according to the method described by Chen & Dong (2004).

Cellular fatty acids were extracted, methylated and analysed by using the standard MIDI (Microbial Identification) system (Miller, 1982; Sasser, 1990). Genomic DNA was extracted and purified as described by Marmur (1961). The G+C content of the DNA was determined by thermal denaturation (Marmur & Doty, 1962) by using a DU800 spectrophotometer (Beckman) with *Escherichia coli* K-12 as reference.

The 16S rRNA gene of strain P6^T was amplified by PCR and sequenced as described by Chen & Dong (2004). The 16S rRNA gene sequence of strain P6^T was submitted to GenBank to search for similar sequences by using the BLAST algorithm. The most closely matching sequences were retrieved from the database and were aligned by using the CLUSTAL X program (Thompson *et al.*, 1997). Phylogenetic trees were reconstructed via the neighbour-joining, minimum-evolution, UPGMA and maximum-parsimony methods as implemented in the program MEGA2 (Kumar *et al.*, 2001), and the resultant tree topologies were evaluated by bootstrap analysis of 1000 datasets.

Cells of strain P6^T were Gram-positive and oval (0.6–0.9 by 1.2–1.8 μm). Motility was not observed. Endospores were formed, resulting in swollen cells. Colonies on PYG agar were white, round and translucent, and about 1 mm in diameter after cultivation at 37 °C for 48 h.

Strain P6^T grew exclusively in pre-reduced media and growth was completely inhibited by air. It was unable to utilize inorganic nitrogen compounds such as NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and KNO_3 as sole nitrogen source. Yeast extract (0.2 %) was required for growth. Strain P6^T grew at 20–42 °C and at pH 5.0–7.5, with optimum growth at 37 °C and approximately pH 6.5. Growth was observed in the presence of 0–2 % (w/v) NaCl. The mean generation time of strain P6^T was 6.2 h when grown in PYG broth at 37 °C.

Strain P6^T hydrolysed aesculin but not gelatin, and produced acid from several sugars, such as D-glucose, D-fructose, sucrose, starch, aesculin, adonitol, dulcitol, inositol and mannitol. No acid was produced from cellulose or xylan (detailed data are given in the species description below). Milk was not curdled. Indole was not produced. Nitrate was not reduced. The main end products of glucose fermentation were acetate, lactate and fumarate; additional trace products including H_2 and CO_2 were also observed. Strain P6^T did not use sulfate as electron acceptor. No H_2S was produced from peptone or thiosulfate.

The DNA G+C content of strain P6^T was 55.6 mol%. The predominant cellular fatty acids were iso-$C_{15:0}$ (24.92 %) and anteiso-$C_{15:0}$ (24.22 %); iso-$C_{14:0}$ 3-OH (13.77 %), iso-$C_{14:0}$ (4.84 %) and $C_{16:0}$ (4.81 %) were also relatively abundant. This profile differed from those of phylogenetically related species of the genus *Clostridium*, mesophilic members of this genus being characterized by a higher percentage of unsaturated fatty acids and the absence of branched-chain fatty acids (Kaneda, 1991). It also differed from the thermophilic bacterium *C. thermocellum*, for which 75 % of the cellular fatty acids were branched.

To ascertain the phylogenetic position of strain P6^T, the complete 16S rRNA gene sequence (1510 bp) was compared with the most similar sequences retrieved from GenBank. Phylogenetic analysis showed that strain P6^T could be accommodated in clostridial cluster III of the low-G+C content Gram-positive bacteria, and that it was related most closely to cloned 16S rRNA gene sequences of uncultured bacteria. A phylogenetic tree including strain P6^T and other representatives of clusters III, IV, XIII, II and I in the family *Clostridiaceae* was reconstructed (Fig. 1), based on a consensus length of 1365 bp of the 16S rRNA gene sequence, and was rooted with *Clostridium butyricum* ATCC 19398^T and *Clostridium perfringens* ATCC 13124^T. Treeing analysis showed that strain P6^T and *Fastidiosipila sanguinis* CCUG 47711^T formed a deep branch but were related only distantly (87.8 % 16S rRNA gene sequence similarity). Strain P6^T showed highest levels of 16S rRNA gene sequence similarity with *Clostridium straminisolvens* DSM 16021^T (90.0 %), *C. thermocellum* DSM 1237^T (89.8 %), *A. cellulolyticus* ATCC 33288^T (89.7 %) and *C. aldrichii* DSM 6159^T (89.7 %); levels of similarity ranged between 87.8 and 88.0 % with the type strains of other related species in cluster III. Bootstrap resampling showed that this relationship was statistically significant (99 % recovery in 1000 resamplings). The high level of sequence divergence indicated that strain P6^T could represent a novel species of a new genus in this cluster.

Strain P6^T also showed distinct phenotypic features that could be used to distinguish it from phylogenetically related members in the same cluster. First, all of its closest relatives, namely *C. straminisolvens* (Kato *et al.*, 2004), *C. thermocellum* (McBee, 1954), *A. cellulolyticus* (Patel *et al.*, 1980) and *C. aldrichii* (Yang *et al.*, 1990), were cellulolytic bacteria, whereas strain P6^T was a sugar-fermenting bacterium, produced acid from glucose and did not degrade cellulose (Table 1). The DNA G+C content of strain P6^T (55.6 mol%) was far higher than those of related species (38–41.3 mol%), also suggesting that it belonged to a different genus. Strain P6^T differed from *C. straminisolvens*

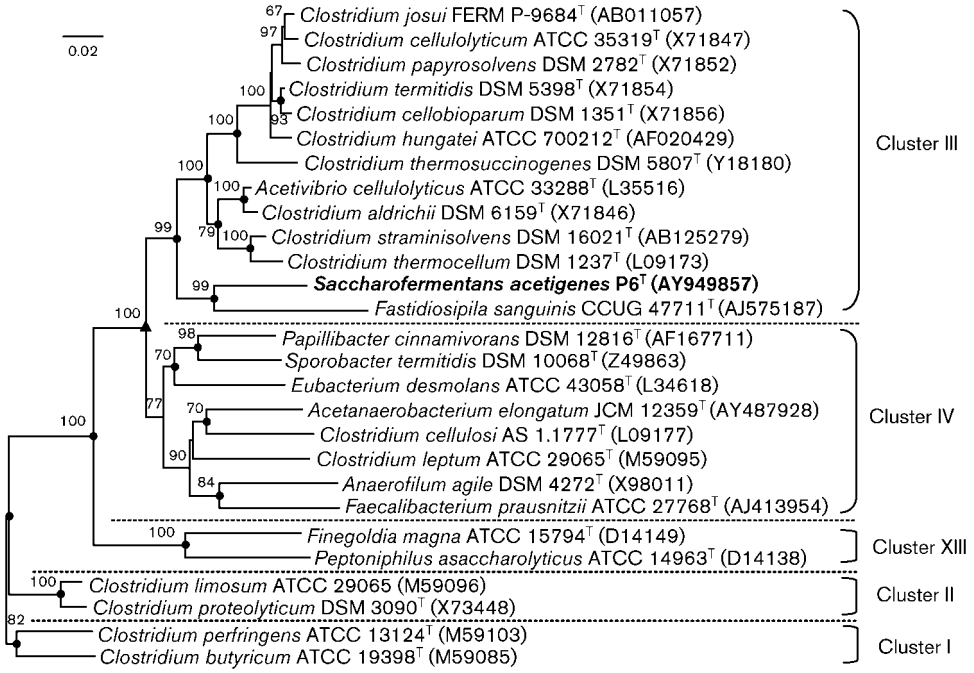

Fig. 1. Neighbour-joining phylogenetic dendrogram showing the position of strain P6^T among related species based on 16S rRNA gene sequences. The tree was rooted with *C. butyricum* ATCC 19398^T and *C. perfringens* ATCC 13124^T. Solid circles indicate that the corresponding nodes (groups) were also recovered with the minimum-evolution and maximum-parsimony methods. The solid triangle indicates that the corresponding node was also recovered with the minimum-evolution and UPGMA methods. Numbers at branch points are 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.

Table 1. Differential characteristics between strain P6^T and its phylogenetic relatives

Strains: 1, P6^T; 2, *Clostridium straminisolvens* DSM 16021^T; 3, *Clostridium thermocellum* DSM 1237^T; 4, *Acetivibrio cellulolyticus* ATCC 33288^T; 5, *Clostridium aldrichii* DSM 6159^T; 6, *Fastidiosipila sanguinis* CCUG 47711^T.

Characteristic	1	2[a]	3	4	5	6[b]
Acid production from:						
Glucose	+	−	−[c,d]	−[d,e]	−[d,f]	−
Fructose	+	−	−[c,d]	−[d,e]	−[d,f]	−
Sucrose	+	−	−[c,d]	−[d,e]	−[d,f]	−
Starch	+	−	−[c,d]	−[d,e]	−[d,f]	−
Mannitol	+	−	−[c,d]	−[d,e]	−[d,f]	−
Cellulose	−	+	+[c,d]	+[d,e]	+[d,f]	−
Spore formation	+	+	+[c,d]	−[d,e]	+[d,f]	−
Optimum temperature (°C)	37	50–55	60[c,d]	35[e]	35[f]	37
DNA G+C content (mol%)	55.6	41.3	38[d]	38[d,e]	40[f], 39[d]	32.9

Data from: *a*, Kato *et al.* (2004); *b*, Falsen *et al.* (2005); *c*, McBee (1954); *d*, this study (conventional methods used in acid production tests); *e*, Patel *et al.* (1980); *f*, Yang *et al.* (1990).

and *C. thermocellum* based on its optimum temperature for growth. The optimum temperature for growth of strain P6^T was 37 °C, compared with reported values of 50–55 and 60 °C for *C. straminisolvens* and *C. thermocellum*, respectively. Strain P6^T differed from *A. cellulolyticus* by the latter's Gram-negative cell wall, and it did not form spores. Also, the new isolate was obligately anaerobic, whereas *C. straminisolvens* was able to grow aerobically. Although strain P6^T appeared to be affiliated to *F. sanguinis* (Falsen *et al.*, 2005), *F. sanguinis* differed in that cells were non-spore-forming cocci, it had a much lower DNA G+C content of 32.9 mol% and it was unable to ferment carbohydrates.

On the basis of the distant phylogenetic relationship with related taxa and physiological and biochemical traits, it was evident that the novel strain was a member of a new genus within clostridial cluster III. Therefore, we suggest that strain P6^T represents a novel species of a new genus, for which the name *Saccharofermentans acetigenes* gen. nov., sp. nov. is proposed.

Description of *Saccharofermentans* gen. nov.

Saccharofermentans (sac.cha.ro.fer.men′tans. Gr. n. *sakchâr* sugar; L. part. adj. *fermentans* fermenting; N.L. neut. n. *Saccharofermentans* sugar-fermenting).

Cells are Gram-positive, non-motile and oval. Obligately anaerobic. No microaerophilic or aerobic growth occurs. The predominant cellular fatty acids are C_{15} components. Mesophilic (20–42 °C) and grow at neutral pH. Chemo-organotrophic. Oxidase and catalase are not produced. Inorganic nitrogen compounds such as NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and KNO_3 do not serve as sole nitrogen sources. A few sugars are fermented. Cellulose is not degraded. Aesculin is hydrolysed but gelatin is not. The major fermentation products from glucose include acetate, lactate and fumarate. Sulfate is not reduced. The type species is *Saccharofermentans acetigenes*.

Description of *Saccharofermentans acetigenes* sp. nov.

Saccharofermentans acetigenes [a.ce.ti′ge.nes. L. n. *acetum* vinegar; N.L. suff. *-genes* (from Gr. v. *gennaô* to produce) producing; N.L. adj. *acetigenes* vinegar- or acetic acid-producing].

Morphology and general characteristics are as described for the genus. Cells are 0.6–0.9 by 1.2–1.8 μm in size. Optimal growth occurs at 37 °C. The pH range for growth is 5.0–7.5 with optimum growth at pH 6.5. Acid is produced from D-glucose, D-fructose, aesculin, sucrose, starch, dulcitol, mannitol, inositol and adonitol. Cellobiose, lactose, melibiose, trehalose, amygdalin and erythritol are weakly fermented. Acid is not produced from L-arabinose, D-galactose, maltose, D-xylose, glycogen, inulin, mannose, raffinose, rhamnose, ribose, salicin, sorbose or sorbitol. No acid is produced from methanol, ethanol, 1-propanol, citrate, fumarate, malate, succinate, malonic acid, hippurate, sodium gluconate, succinic acid, β-hydroxybutyric acid, phenylacetic acid, cellulose or xylan. Milk is not curdled. Urease, lecithinase, lipase and indole are not produced. Methyl red test is positive and Voges–Proskauer test is negative. Nitrate is not reduced. No H_2S is produced from peptone or thiosulfate. The major cellular fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{14:0}$ 3-OH. The G+C content of the genomic DNA of the type strain is 55.6 mol%.

The type strain, P6^T (=JCM 14006^T =AS 1.5064^T), was isolated from an up-flow anaerobic sludge blanket reactor treating brewery wastewater.

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References

Chen, S. & Dong, X. (2004). *Acetanaerobacterium elongatum* gen. nov., sp. nov., from paper mill wastewater. *Int J Syst Evol Microbiol* **54**, 2257–2262.

Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.

Falsen, E., Collins, M. D., Welinder-Olsson, C., Song, Y., Finegold, S. M. & Lawson, P. A. (2005). *Fastidiosipila sanguinis* gen. nov., sp. nov., a new Gram-positive, coccus-shaped organism from human blood. *Int J Syst Evol Microbiol* **55**, 853–858.

Holdeman, L. V., Cato, E. P. & Moore, W. E. C. (1977). *Anaerobe Laboratory Manual*, 4th edn. Blacksburg, VA: Virginia Polytechnic Institute and State University.

Hungate, R. E. (1969). A roll-tube method for cultivation of strict anaerobes. *Methods Microbiol* **3B**, 117–132.

Kaneda, T. (1991). Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. *Microbiol Rev* **55**, 288–302.

Kato, S., Haruta, S., Cui, Z. J., Ishii, M., Yokota, A. & Igarashi, Y. (2004). *Clostridium straminisolvens* sp. nov., a moderately thermophilic, aerotolerant and cellulolytic bacterium isolated from a cellulose-degrading bacterial community. *Int J Syst Evol Microbiol* **54**, 2043–2047.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.

Liu, W. T., Chan, O. C. & Fang, H. H. (2002). Characterization of microbial community in granular sludge treating brewery wastewater. *Water Res* **36**, 1767–1775.

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.

McBee, R. H. (1954). The characteristics of *Clostridium thermocellum*. *J Bacteriol* **67**, 505–506.

Miller, L. T. (1982). Single derivatization method for routine analysis of bacterial whole-cell fatty acid-methyl esters, including hydroxy acids. *J Clin Microbiol* **16**, 584–586.

Patel, G. B., Khan, A. W., Agnew, B. J. & Colvin, J. R. (1980). Isolation and characterization of an anaerobic, cellulolytic microorganism, *Acetivibrio cellulolyticus* gen. nov., sp. nov. *Int J Syst Bacteriol* **30**, 179–185.

Sasser, M. (1990). *Identification of bacteria by gas chromatography of cellular fatty acids*, Technical Note 101. Newark, DE: MIDI Inc.

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.

Yang, J. C., Chynoweth, D. P., Williams, D. S. & Li, A. (1990). *Clostridium aldrichii* sp. nov., a cellulolytic mesophile inhabiting a wood-fermenting anaerobic digester. *Int J Syst Bacteriol* **40**, 268–272.

Zehnder, A. J. B. (1978). Ecology of methane formation. In *Water Pollution Microbiology*, pp. 349–376. Edited by R. Mitchell. New York: Wiley.