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

Shuangya Chen,1 Lili Niu2 and Yongxiang Zhang3

Correspondence
Shuangya Chen
shuangych@yahoo.com
1Laboratory of Microbiology, Xiamen Inspection & Quarantine Technology Center, Xiamen, 361026, PR China
2Shanghai Key Laboratory of Bio-energy Crops, School of Life Sciences, Shanghai University, Shanghai, 200444, PR China
3Zhangzhou Inspection & Quarantine Bureau, Zhangzhou, 363000, PR China

A spore-forming anaerobic bacterium, designated strain P6T, 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 CO2 and H2 were also produced. The DNA G+C content of strain P6T was 55.6 mol%. The major cellular fatty acids were iso-C15 : 0, anteiso-C15 : 0 and iso-C14 : 0 3-OH. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain P6T 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 P6T 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 P6T (=JCM 14006T =AS 1.5064T).

Complex organic matter is degraded completely to CO2 and CH4 by the association of several trophic microorganisms 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 P6T). 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.

Strain P6T 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 % N2 (100 kPa) at 37 °C. Acetivibrio cellulolyticus ATCC 33288T was obtained from the American Type Culture Collection. Clostridium aldrichii DSM 6159T and Clostridium thermocellum DSM 1237T were obtained from the Deutsche Sammlung von Mikroorganismen. PY medium plus 1 % cellulose was used for cultivation of these reference strains.

Cell morphology was examined by light microscopy (Olympus BH-2) as well as electron microscopy (Hitachi...
The 16S rRNA gene of strain P6T was amplified by PCR spectrophotometer (Beckman) with denaturation (Marmur & Doty, 1962) by using a DU800 diameter after cultivation at 37°C for 48 h. Growth was determined by measuring the OD<sub>600</sub> 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 P6T was amplified by PCR and sequenced as described by Chen & Dong (2004). The 16S rRNA gene sequence of strain P6T 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 P6T 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 P6T grew exclusively in pre-reduced media and growth was completely inhibited by air. It was unable to utilize inorganic nitrogen compounds such as NH<sub>4</sub>Cl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> and KNO<sub>3</sub> as sole nitrogen source. Yeast extract (0.2 %) was required for growth. Strain P6T 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 P6T was 6.2 h when grown in PYG broth at 37°C.

Strain P6T 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<sub>2</sub> and CO<sub>2</sub> were also observed. Strain P6T did not use sulfate as electron acceptor. No H<sub>2</sub>S was produced from peptone or thiosulfate.

The DNA G+C content of strain P6T was 55.6 mol%. The predominant cellular fatty acids were iso-C<sub>15 : 0</sub> (24.92 %) and anteiso-C<sub>15 : 0</sub> (22.22 %); iso-C<sub>14 : 0</sub> 3-OH (13.77 %), iso-C<sub>14 : 0</sub> (4.84 %) and C<sub>16 : 0</sub> (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 P6T, the complete 16S rRNA gene sequence (1510 bp) was compared with the most similar sequences retrieved from GenBank. Phylogenetic analysis showed that strain P6T 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 P6T 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<sup>T</sup> and Clostridium perfringens ATCC 13124<sup>T</sup>. Treeing analysis showed that strain P6T and Fastidiosipila sanguinis CCUG 47711<sup>T</sup> formed a deep branch but were related only distantly (87.8 % 16S rRNA gene sequence similarity). Strain P6T showed highest levels of 16S rRNA gene sequence similarity with Clostridium straminisolvens DSM 16021<sup>T</sup> (90.0 %), C. thermocellum DSM 1237<sup>T</sup> (89.8 %), A. cellulolyticus ATCC 33288<sup>T</sup> (89.7 %) and C. aldrichii DSM 6159<sup>T</sup> (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 P6T could represent a novel species of a new genus in this cluster.

Strain P6T 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 P6T was a sugar-fermenting bacterium, produced acid from glucose and did not degrade cellulose (Table 1). The DNA G+C content of strain P6T (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 P6T differed from C. straminisolvens...
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.**

Cells are Gram-positive, non-motile and oval. Obligately anaerobic. No microaerophilic or aerobic growth occurs. The predominant cellular fatty acids are C₁₅ components. Mesophilic (20–42 °C) and grow at neutral pH. Chemoorganotrophic. Oxidase and catalase are not produced. Inorganic nitrogen compounds such as NH₄Cl, (NH₄)₂SO₄, (NH₄)₂HPO₄ and KNO₃ 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 aceti*genes.*

**Description of *Saccharofermentans aceti*genes sp. nov.**

*Saccharofermentans aceti*genes [a. ce. ti’ ge. nes. L. n. acetum vinegar; N.L. suff. -genes (from Gr. v. genien) to produce] producing; N.L. adj. aceti*genes* 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, sucrrose, 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-xylene, 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, lecitinhase, lipase and indole are not produced. Methyl red test is positive and Voges–Proskauer test is negative. Nitrate is not reduced. No H₂S is produced from peptone or thiosulfate. The major cellular fatty acids are iso-C₁₅ : 0, anteiso-C₁₅ : 0 and iso-C₁₄ : 0 3-OH. The G+C content of the genomic DNA of the type strain is 55.6 mol%.

The type strain, P6ᵀ (=JCM 14006ᵀ =AS 1.5064ᵀ), was isolated from an up-flow anaerobic sludge blanket reactor treating brewery wastewater.

**Acknowledgements**

We are grateful to Professor Wenyong Zhuang, Chinese Academy of Sciences, for suggesting the genus and species name and Dr Yajun Song, Chinese Academy of Military Medical Sciences, for analysis of cellular fatty acids. This work was supported by the Xiamen Inspection-Quarantine Technology Center.

**References**


