Ethanoligenens harbinense gen. nov., sp. nov., isolated from molasses wastewater

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Two strictly anaerobic bacterial strains (YUAN-3^T and X-29) were isolated from anaerobic activated sludge of molasses wastewater in a continuous stirred-tank reactor. The strains were Gram-positive, non-spore-forming, mesophilic and motile. Cells were regular rods (0·4–0·8 × 1·5–8·0 μm) and occurred singly, in pairs and sometimes in chains of up to eight. Autoaggregative and autofluorescent growth of strain YUAN-3^T and non-aggregative growth of strain X-29 were observed at 20–44 °C and pH 3·5–9·0. Both strains hydrolysed gelatin and aesculin and fermented several kinds of mono-, di- and oligosaccharides. Fermentation end products formed from glucose were acetate, ethanol, hydrogen and carbon dioxide. The predominant cellular fatty acids were the branched-chain fatty acids iso-$C_{16:0}$ (44·18 %) and iso-$C_{12:0}$ (26·67 %). The DNA G+C contents of strains YUAN-3^T and X-29 were 47·8 and 49·0 mol%, respectively. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the isolates represent a novel phyletic sublineage within the *Clostridium cellulosi* rRNA cluster, with <92 % 16S rRNA gene sequence similarity to currently known species. On the basis of polyphasic evidence from this study, it is proposed that the unknown bacterium should be classified in a new genus as a novel species, *Ethanoligenens harbinense* gen. nov., sp. nov. The type strain of *Ethanoligenens harbinense* is YUAN-3^T (=JCM 12961^T=CGMCC 1.5033^T).

Hydrogen-producing micro-organisms can be divided into two main categories: photosynthetic organisms that produce hydrogen in the light and anaerobic bacteria that produce hydrogen via fermentation metabolism. At present, the majority of fermentative hydrogen-producing micro-organisms are obligate anaerobes belonging to the genera *Clostridium* and *Ruminococcus*, facultative anaerobes such as *Enterobacter aerogenes* and *Escherichia coli* and aerobes belonging to the genera *Alcaligenes* and *Bacillus* (Nandi & Sengupta, 1998). During a survey of the microbial community in a hydrogen-producing bioreactor, two obligately anaerobic, autoaggregative, non-spore-forming bacterial strains were isolated from anaerobic sludge of molasses wastewater in a continuous stirred-tank reactor (CSTR). The strains produced H_2, CO_2, ethanol and acetic acid from glucose fermentation. Phylogenetically, the strains were affiliated with the *Clostridium leptum* rRNA subgroup (Collins *et al.*, 1994); however, they were only distantly related to any existing genera in this cluster. Based on the distinctive phenotypic, genomic and phylogenetic characteristics of these two strains, it is proposed that they represent a novel species in a new genus.

Strains YUAN-3^T and X-29 were isolated in pre-reduced peptone/yeast extract/glucose (PYG) medium (Holdeman *et al.*, 1977) by serial dilution and the Hungate roll-tube technique (Hungate, 1969). Single colonies were picked and transferred to the same broth and incubated at 35 °C for 2 days. The roll-tube procedure was repeated several times until a pure culture was obtained. Routine cultivation was in PYG broth in anaerobic tubes (18 × 180 mm) sealed with butyl rubber stoppers under a gaseous atmosphere of 100 % N_2 (200 kPa) at 35 °C.

Morphology and life cycle were examined in cultures grown on PYG medium using a light microscope (Olympus BX-51) and an EM (FEI TECNI G^2). For EM studies, bacterial cells grown in PYG at 35 °C for 2 days were stained with phosphotungstic acid (3 %). For ultrathin section examination of the cell wall, bacterial cells were fixed with osmic acid and embedded in araldite; samples were then sliced and stained with lead citrate (Reynolds, 1963).

Gases were measured by GC (GC-SC2) equipped with a thermal conductivity detector and a 2·0 m stainless steel

Published online ahead of print on 25 November 2005 as DOI 10.1099/ijs.0.63926-0.

Abbreviations: CSTR, continuous stirred-tank reactor; DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains YUAN-3^T and X-29 are AY295777 and AY833421, respectively.

Micrographs of colonies and cells of strain YUAN-3^T are available as supplementary material in IJSEM Online.

column packed with TDS-01 (60/80 mesh). N_2 was used as carrier gas at a flow rate of 70 ml min^{-1}. Fatty acids and alcohols were detected by GC (GC-122) equipped with a hydrogen flame-ionization detector and a 2·0 m stainless steel column packed with GDX103 (60/80 mesh). For measurement of non-volatile fatty acids, methyl esters were derived from samples according to Holdeman *et al.* (1977) before being analysed. Column temperatures were 220, 190 and 190 °C for the measurement of volatile fatty acids, alcohols and non-volatile fatty acids, respectively. N_2 was used as carrier gas at a flow rate of 60 ml min^{-1}.

Generation time of the strains was determined by monitoring OD_{600} of the PYG culture at 35 °C at 2 h intervals up to 48 h. Temperature profiles were determined in PYG broth using a water bath at temperatures of 15–55 °C at intervals of 1 °C. The pH range for growth was determined for the culture in PYG 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 and the API 50CH system (bioMérieux). Enzyme activities were analysed using both conventional methods and the API ZYM system (bioMérieux). All tests were performed in duplicate.

For extraction of cell walls, crude cells were disrupted by sonication, separated from unbroken cells by fractional centrifugation and purified using trypsin and 2 % SDS as reported previously (Evtushenko *et al.*, 2000). The diagnostic isomers of diaminopimelic acid (DAP) were detected by the TLC method (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 bacterial DNA mini kit (Watson Biotechnologies) according to the manufacturer's instructions. The G + C content of the DNA was determined by the thermal denaturation method (Marmur & Doty, 1962) using a DU800 spectrophotometer with *Escherichia coli* K-12 as the reference. The 16S rRNA gene was amplified by PCR using a pair of universal primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′), corresponding to base positions 8–27 and 1525–1541, respectively, of the 16S rRNA gene of *Escherichia coli* (Winker & Woese, 1991). The genomic DNA extracted was used as a template and PCR amplification was performed with the GenAmp PCR System 9700. PCR products were detected by agarose gel electrophoresis and visualized by UV fluorescence after ethidium bromide staining. PCR products were purified using a UNIQ-10 PCR product purification kit (Sangon) and cloned in *Escherichia coli* JM109 using the pGEM-T plasmid vector system (Promega) as recommended by the manufacturer. Primers T7 (5′-GTAATACGACTCACTATAG-G-3′) and M13R (5′-CAGGAAACAGCTATGACCAT-3′) were used for sequencing the 16S rRNA gene fragment. Sequencing was performed by Bioasia Biological 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 YUAN-3^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; similarity analysis was performed using the program CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic trees were constructed from the evolutionary distance matrix calculated using the neighbour-joining method (Saitou & Nei, 1987) with Kimura's two-parameter method (Kimura, 1980). Neighbour-joining analysis was performed with the program MEGA3 (Kumar *et al.*, 2004). Robustness of the resultant tree topology was evaluated by bootstrap resampling analysis (Felsenstein, 1985) with 1000 replicates.

Colonies of strains YUAN-3^T and X-29 on PYG agar were milk-white, circular, opaque and slightly concave with an obvious ring margin, reaching 1·5–3·0 mm in diameter after cultivation at 35 °C for 48 h. Cells were Gram-positive, non-spore-forming rods (0·4–0·8 × 1·5–8·0 μm), motile by means of peritrichous flagella. Strain YUAN-3^T was autoaggregative and autofluorescent, whereas strain X-29 showed non-aggregative and non-fluorescent growth. The young culture (12–24 h) consisted of rods or filaments (0·4–0·8 × 4·0–8·0 μm), occurring singly or in pairs; strain YUAN-3^T began to form autoaggregative granules in shake cultures. In 3- to 4-day-old cultures, the rods and filaments fragmented into shorter elements (about 1·5–4·0 μm). In older cultures, regular rods predominated and occurred singly or in pairs; strain YUAN-3^T formed large autoaggregative granules (generally 0·5–5·0 mm, but sometimes up to 1·5 cm) in shake cultures and the culture medium was clear. However, cultures of strain X-29 were non-aggregative and homogeneous. Polysaccharide capsules (0·1–0·4 μm) were usually observed on agar media and in liquid culture; those of strain YUAN-3^T were usually thicker than those of strain X-29. A Gram-positive cell wall and cell inclusion bodies (polyphosphate and poly-β-hydroxybutyrate) were confirmed by ultrathin section EM of strain YUAN-3^T (available as supplementary material in IJSEM Online).

Strains YUAN-3^T and X-29 grew in pre-reduced media and growth was completely inhibited by air. Any one of yeast extract, tryptone or peptone could be used as a nitrogen source by both strains but, apart from $(NH_4)_2HPO_4$, inorganic nitrogen compounds such as NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$ and KNO_3 could not be used. Both strains grew at 20–44 °C and pH 3·5–9·0, with optimum growth at 35 °C and approximately pH 4·5–5·0. The strains could grow in the presence of 0–2 % (w/v) NaCl. The mean generation time of the two strains was 4·2 h when grown in PYG broth at 35 °C. The growth yield of strain YUAN-3^T in PYG broth was 1·205 g cell dry mass (l culture)$^{-1}$.

Apart from the fact that strain YUAN-3^T strongly autoaggregated, whereas strain X-29 did not, the two strains

exhibited almost identical physiological and biochemical characteristics as determined using routine methods and the API 50CH and API ZYM systems. Both isolates hydrolysed gelatin, curdled milk and produced acid from a few sugars such as raffinose and sucrose (detailed results are listed in the species description). YUAN-3^T did not produce acid from mannitol, but X-29 did. YUAN-3^T weakly hydrolysed starch and cellulose, but X-29 did not. Strain YUAN-3^T was positive for leucine arylamidase, whereas strain X-29 was negative. The products of glucose fermentation by strains YUAN-3^T and X-29 were acetate, ethanol, H_2 and CO_2.

The cell wall hydrolysates of the two strains were rich in LL-DAP. The cellular fatty acids of strain YUAN-3^T were characterized mainly by iso-branched fatty acids, predominantly iso-$C_{16:0}$ (44·18 %) and iso-$C_{12:0}$ (26·67 %); anteiso-$C_{17:0}$ (5·25 %) and $C_{16:0}$ (4·98 %) fatty acids were also relatively abundant. The cellular fatty acid compositions were obviously different from those of phylogenetically related *Clostridium* species; most of the mesophilic members are characterized by a higher percentage of unsaturated fatty acids and the absence of branched-chain fatty acids (Kaneda, 1991).

Similarity between the complete 16S rRNA gene sequences of strains YUAN-3^T and X-29 was 100 % and their DNA G+C contents were 47·8 and 49·0 mol%, respectively. All the results above indicate the single species status of the two isolates.

To determine the phylogenetic position of the isolates, the complete 16S rRNA gene sequences (1506 bp) were compared with the most similar sequences and those of representatives of the *Clostridiaceae* retrieved from GenBank. On the basis of a consensus 1334 bp sequence of the 16S rRNA gene, a phylogenetic tree, rooted with *Acidaminobacter hydrogenoformans* DSM 2784^T, was constructed (Fig. 1). Phylogenetic analysis showed that the strains belonged to the *C. leptum* rRNA subgroup (Collins *et al.*, 1994). This group consists of phenotypically and phylogenetically diverse groups (Chen & Dong, 2004), including spore-forming *Clostridium* species (*Clostridium sporosphaeroides*, *C. leptum* and *C. cellulosi*), *Ruminococcus* species (*Ruminococcus albus*, *R. flavefaciens*, *R. bromii* and *R. callidus*), *Anaerofilum* species (*Anaerofilum agile* and *A. pentosovorans*) (Zellner *et al.*, 1996), *Eubacterium* species, *Faecalibacterium prausnitzii* (Duncan *et al.*, 2002) and *Papillibacter cinnamivorans* (Defnoun *et al.*, 2000). Strain YUAN-3^T showed the highest 16S rRNA gene sequence similarity (91·8 %) to *C. cellulosi*; similarities were 84·0–88·8 % with other related species in the *C. leptum* subgroup and lower than 86·0 % with other representatives of the *Clostridiaceae*. The large sequence divergence indicated that the isolates represent a new genus in this cluster.

Strains YUAN-3^T and X-29 also showed distinct phenotypic, physiological and biochemical features, enabling them to be distinguished from representative members in the same cluster. The two isolates were non-spore-forming and hydrolysed gelatin, characteristics that, along with their different sugar fermentation profiles (see Table 1), enabled them to be distinguished from related *Clostridium* species. They differed from *Ruminococcus* species in their rod shape, different biochemical traits and DNA G+C content. They differed from *Eubacterium siraeum* in glucose fermentation and other features shown in Table 1. They produced a large amount of hydrogen, enabling them to be distinguished from *Anaerofilum* species. *F. prausnitzii* has a Gram-negative cell wall and produces butyrate, D-lactate and formate but no hydrogen from glucose fermentation, enabling it to be

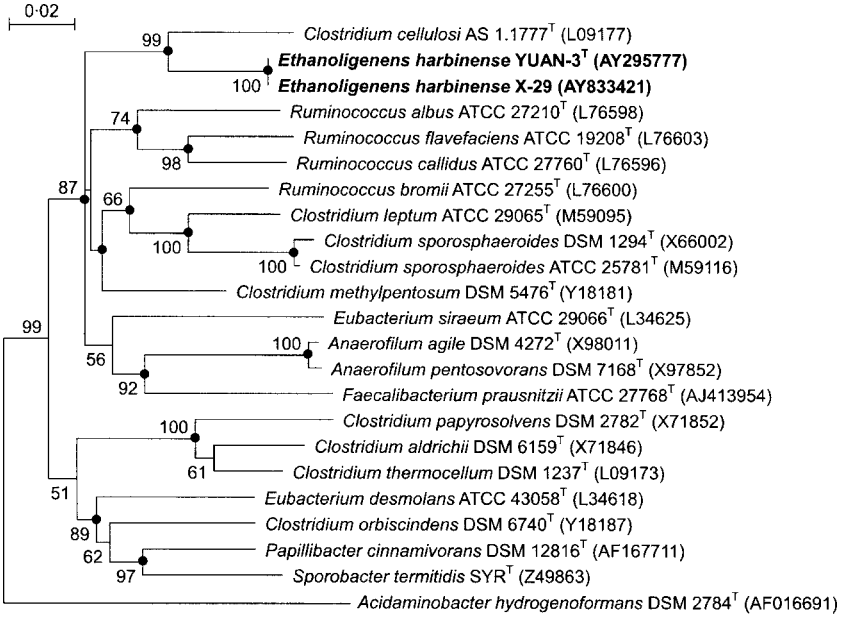

Fig. 1. Phylogenetic tree showing the relationships between strains YUAN-3^T and X-29 and related species based on 16S rRNA gene sequences. The sequence of *Acidaminobacter hydrogenoformans* DSM 2784^T served as an outgroup sequence. The tree was constructed using the neighbour-joining method. Solid circles indicate that the corresponding nodes (groups) were also recovered using the maximum-parsimony method. Numbers within the dendrogram indicate the percentages of occurrence of the branching order in 1000 bootstrapped trees. Accession numbers of nucleotide sequences are given in parentheses. Bar, 2 % sequence divergence.

Table 1. Characteristics that differentiate *Ethanoligenens harbinense* gen. nov., sp. nov. from its phylogenetic relatives

Strains: 1, *Ethanoligenens harbinense* gen. nov., sp. nov. YUAN-3^T; 2, *C. cellulosi* AS 1.1777^T (He *et al.*, 1991); 3, *C. sporosphaeroides* ATCC 25781^T (Cato *et al.*, 1986); 4, *C. leptum* ATCC 29065^T (Cato *et al.*, 1986); 5, *A. agile* DSM 4272^T (Zellner *et al.*, 1996); 6, *A. pentosovorans* DSM 7168^T (Zellner *et al.*, 1996); 7, *Eubacterium siraeum* ATCC 29066^T (Moore & Holdeman Moore, 1986); 8, *F. prausnitzii* ATCC 27768^T (Moore *et al.*, 1984); 9, *C. methylpentosum* DSM 5476^T (Himelbloom & Canale-Parola, 1989); 10, *R. flavefaciens* ATCC 19208^T (Bryant, 1986). +, Positive; −, negative; W, weak reaction; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10
DNA G+C content (mol%)	48	35	27	51–52	54·5	55	45	52–57	46	39–44
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Ring	Coccus
Spore production	−	+	+	+	−	−	−	−	+	−
Products from PYG*	A2†	A2	ABp	A(2)	LA2F	LA2F	−	FBAL	−	S2(L)
H_2 production	+‡	+	+	+	−	−	−	−	−	+
Motility	+	+	−	−	+	−	−	−	−	−
Autoaggregation	+	−	−	−	−	−	−	−	−	−
Optimum growth temperature (°C)	35	55–60	37–45	37	37	25–40	37–45	37	45	37–42
Gelatinase	+	−	−	−	ND	ND	−	ND	ND	−
Milk reaction§	C	C	−	−	ND	ND	ND	ND	ND	−
Acid production from:										
Inositol	−	+	−	−	ND	ND	ND	ND	−	−
Glycogen	−	+	−	ND	ND	ND	−	ND	−	−
Mannitol	−	+	−	−	ND	ND	−	ND	−	−
Mannose	+	+	−	−	ND	+	ND	ND	−	ND
Starch	W	+	−	−	−	−	ND	W	−	ND
Trehalose	+	+	−	ND	+	+	−	W	−	−
Cellobiose	+	+	−	−	+	+	+	W	−	+
Fructose	+	+	−	ND	+	+	ND	+	−	−
Arabinose	−	+	−	−	ND	+	−	−	+	ND
Inulin	−	+	−	−	ND	ND	ND	+	−	−
Salicin	+	+	−	−	+	ND	−	+	−	−
Raffinose	+	+	−	−	−	−	−	−	−	−
Sucrose	+	−	−	ND	−	−	ND	W	−	ND

*Products from PYG (in decreasing order of amount usually detected): A, acetate; B, butyrate; F, formate; L, lactate; P, propionate; S, succinate; 2, ethanol. Upper-case letters indicate at least 1 mg (ml culture)$^{-1}$ and lower-case letters indicate less than 1 mg (ml culture)$^{-1}$. In all cases, ethanol is a major product. Products in parentheses are not detected uniformly.

†1·13 mol ethanol (mol glucose)$^{-1}$ and 0·69 mol acetate (mol glucose)$^{-1}$ are produced after 32 h at 35 °C.

‡Maximal H_2 yield is 2·81 mol H_2 (mol glucose)$^{-1}$ for YUAN-3^T cultured on PYG at 35 °C.

§C, Curdles milk; −, does not curdle milk.

differentiated from the two isolates. Unlike *P. cinnamivorans*, they were motile by peritrichous flagella.

On the basis of the distant phylogenetic relationship with related taxa, unique chemotaxonomic characteristics, DNA G+C content and physiological and biochemical traits, it is evident that isolates YUAN-3^T and X-29 represent a distinct genus within the *C. leptum* rRNA subgroup; *Ethanoligenens harbinense* gen. nov., sp. nov. is therefore proposed to accommodate these two strains.

Description of *Ethanoligenens* gen. nov.

Ethanoligenens [E.tha.no.li.gen′ens. N.L. n. *ethanol-is* ethanol; L. part. adj. *genens* (from L. v. *genere* to produce) producing; N.L. neut. n. *Ethanoligenens* ethanol-producing (bacterium)]. Gram-positive, motile, non-spore-forming, straight rods. Obligately anaerobic. Oxidase- and catalase-negative. Cell wall peptidoglycan contains LL-DAP. Cellular fatty acids consist mainly of iso-branched fatty acids, predominantly iso-$C_{16:0}$ and iso-$C_{12:0}$. Mesophilic. Grow at 20–44 °C and pH 3·5–9·0. Chemo-organotrophic. Amino acids and peptides may serve as nitrogen sources. Various mono-, di- and oligosaccharides are fermented. Gelatin and aesculin are hydrolysed. The major fermentation products from glucose include ethanol, acetate, hydrogen and carbon dioxide; propionate and succinate are not produced. Sulfate is not reduced. The G+C contents of the genomic DNA of the known strains are 47·8 and 49·0 mol%. Belongs to the *C. leptum* rRNA subgroup. Only one species, *Ethanoligenens harbinense*, is described so far; this species has been designated the type species.

Description of *Ethanoligenens harbinense* sp. nov.

Ethanoligenens harbinense (har.bin′en.se. N.L. neut. adj. *harbinense* from Harbin, where the type strain was isolated).

General morphological, chemotaxonomic and growth characteristics are as described for the genus. Cells are 0·4–0·8 × 1·5–8·0 μm. Colonies on PYG agar are milk-white, circular, opaque, slightly concave with an obvious ring margin, reaching 1·5–3·0 mm in diameter after cultivation at 35 °C for 48 h. Optimal growth occurs at 35 °C. The pH range for growth is 3·5–9·0, with optimum growth at pH 4·5–5·0. Acid is produced from a few sugars, including D-glucose, D-fructose, D-galactose, cellobiose, D-maltose, sucrose, raffinose, ribose, trehalose, D-lactose, D-turanose, mannose, melibiose and salicin. Acid is not produced from arabinose, inulin, sorbose, rhamnose, glycogen, adonitol, dulcitol, erythritol, inositol, sorbitol, melezitose, xylitol, D-lyxose, D-tagatose, arabitol or fucose. No acid is produced from the following compounds: methanol, ethanol, 1-propanol, pyruvate, fumarate, malate, succinate, malonic acid, hippurate, β-hydroxybutyric acid, phenylacetic acid or xylan. Curdles milk. Urease is produced. Arginine dihydrolase, lecithinase and lipase are not produced. Positive for esterase (C_4), esterase lipase (C_8), lipase (C_{14}), valine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Negative for alkaline phosphatase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase and α-glucosidase. Positive for indole, methyl red and Voges–Proskauer tests. Does not use sulfate or nitrate as electron acceptor. No H_2S is produced from peptone or thiosulfate. The major cellular fatty acids are iso-$C_{16:0}$ (44·18 %), iso-$C_{12:0}$ (26·67 %), anteiso-$C_{17:0}$ (5·25 %) and $C_{16:0}$ (4·98 %).

The type strain is YUAN-3^T (=JCM 12961^T=CGMCC 1.5033^T), isolated from the anaerobic sludge of molasses wastewater in a CSTR.

Acknowledgements

This research was supported by the Chinese National Natural Science Foundation (no. 30470054), the Key Project of Chinese National Programs for Fundamental Research and Development (973 Program, no. G2000026402) and the National Science Foundation for Distinguished Young Scholars (no. 50125823).

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