Acetanaerobacterium elongatum gen. nov., sp. nov., from paper mill waste water

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Two mesophilic anaerobic bacterial strains (Z7^T and Z1) were isolated from waste water sludge of the Xinanzhang paper mill, Beijing, China. The strains were Gram-positive, non-spore-forming and motile. Cells were thin rods (0·2–0·4 × 4·0–8·0 μm). Growth of the strains was observed at 20–42 °C and pH 5·0–7·5. Both strains hydrolysed gelatin and aesculin and fermented several kinds of mono-, di- and oligosaccharides. The 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$C_{15:0}$ (42·83 %) and iso$C_{14:0}$ (32·11 %). The DNA G+C contents of strains Z7^T and Z1 were 50·4 and 48·6 mol%, respectively. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the isolates represent a new phyletic sublineage within the *Clostridium leptum* rRNA cluster, with <91 % 16S rRNA gene sequence similarity to currently described species. On the basis of polyphasic evidence from this study, *Acetanaerobacterium elongatum* gen. nov., sp. nov., a novel genus and species, is proposed, with strain Z7^T (=JCM 12359^T=AS 1.5012^T) as the type strain.

Hydrogen is considered to be an ideal, clean energy source that has potential as an alternative to fossil fuels, supplies of which are gradually being exhausted. Of the approaches to hydrogen production, microbial conversion from biomass is an energy-saving process with economic feasibility. Microbial hydrogen production falls into two categories, namely photosynthetic and fermentative procedures. So far, a variety of micro-organisms has been found to produce hydrogen from fermentation of an organic complex, including obligate anaerobes such as *Clostridium* and *Ruminococcus* species, facultative anaerobes such as *Enterobacter aerogenes* and *Escherichia coli* and aerobes such as *Alcaligenes* and *Bacillus* species (Nandi & Sengupta, 1998).

During the survey and isolation of hydrogen-producing bacteria from a variety of polysaccharides and proteinaceous compounds, two obligately anaerobic, non-spore-forming bacterial strains were isolated from waste water sludge of the Xinanzhang paper mill, Beijing, China. The strains produced H_2 gas, acetic acid and ethanol from glucose fermentation. Phylogenetically, the strains were affiliated to 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, *Acetanaerobacterium elongatum* gen. nov., sp. nov.

Strains Z7^T and Z1 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 37 °C for 2 days. The roll-tube procedure was repeated several times until a pure culture was obtained. Culture purity was also checked by microscopic examination. 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 37 °C.

Cell morphology was examined under a light microscope (Olympus BH-2) and an EM (Hitachi H-600A). For EM studies, bacterial cells grown in PYG at 37 °C for 2 days were coated with a palladium/iridium alloy with a high vacuum evaporator (Hitachi HUS-5GB). 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).

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Acetanaerobacterium elongatum* Z7^T and Z1 are AY487928 and AY518589, respectively.

Short chain fatty acids and gases were measured by GC (GC-14B; Shimadzu) using stainless steel columns packed with porapak GDX-401 (60–80 mesh). Fatty acids and alcohols were detected with a flame-ionization detector. For measurement of non-volatile fatty acids, methyl esters were derived from the samples according to Holdeman *et al.* (1977) before being analysed. Column temperatures were 220, 130 and 150 °C for the measurement of volatile fatty acids, alcohols and non-volatile fatty acids, respectively. Gaseous products were detected with a thermal conductivity detector. The column temperature was 30 °C. N_2 was used as the carrier gas in all analyses.

Generation time of the strains was determined by monitoring OD_{600} of the PYG culture at 37 °C at 1 h intervals up to 48 h. Temperature profiles were determined in PYG using a water bath (Guangming Medical Instrument Plant) at temperatures of 15 to 55 °C at 1 °C intervals. 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). All tests were performed in duplicate.

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 by PCR using a pair of universal primers: 27F (5′-AGAGTTTGATCC/ATGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′), corresponding to base positions 8–27 and 1541–1525 of the 16S rRNA gene of *Escherichia coli* (Winker & Woese, 1991), respectively. The genomic DNA extracted was used as a template and PCR amplification was performed with Thermolyne Amplitron I (Barnstead Thermolyne). PCR products were purified using a UNIQ-10 PCR product purification kit (Sangon) and ligated into vector pUCm-T (Sangon) as recommended by the manufacturer. Primers T7 (5′-GTAATACGACTC-ACTATAGG-3′) and M13R (5′-CAGGAAACAGCTATG-ACCAT-3′) were used for sequencing the 16S rRNA gene fragment. 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 $Z7^T$ 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 and similarity analysis was performed using the program CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic trees were constructed 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.

The diagnostic isomers of diaminopimelic acid in the cell wall were determined using 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).

Cells of strains $Z7^T$ and Z1 were thin rods to filaments (0·2–0·4 × 4·0–8·0 μm), occurring singly or in pairs and motile by means of two peritrichous flagella (Fig. 1a). The cell wall was Gram-positive, as confirmed by the KOH lysis test (Smibert & Krieg, 1994) and ultrathin section EM of strain $Z7^T$ (Fig. 1b). No spores were observed and resistance to treatment at 80 °C for 10 min was not observed. Colonies on PYG agar were white, smooth, circular, entire,

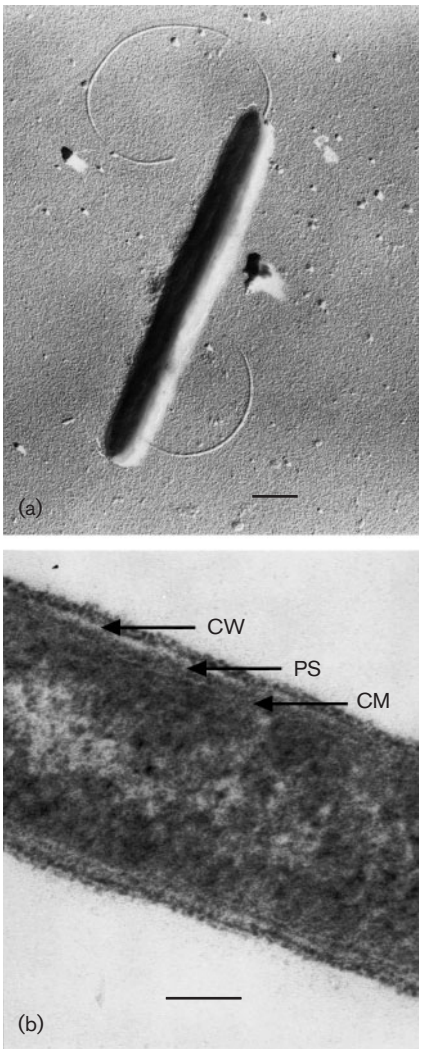

Fig. 1. EMs of cells of strain $Z7^T$. (a) TEM; bar, 0·5 μm. (b) Ultrathin section EM (CW, cell wall; PS, periplasm; CM, cytoplasmic membrane); bar, 0·05 μm.

translucent and slightly convex, reaching 1·5–3·0 mm in diameter after cultivation at 37 °C for 72 h.

Strains $Z7^T$ and Z1 grew exclusively in pre-reduced media and growth was completely inhibited by air. Both strains required amino acids and peptides as sole nitrogen sources, but not inorganic nitrogen compounds such as NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and KNO_3. Yeast extract (0·2 %) was required for growth on amino acids, but not for growth on peptides. Both strains grew at 20–42 °C and pH 5·0–7·5, with optimum growth at 37 °C and approximately pH 6·5–7·0. The strains could grow in the presence of 0–2 % (w/v) NaCl. The mean generation time of the two strains was 4·78 h when grown in PYG at 37 °C. The growth yield of strain $Z7^T$ in PYG was 1·041 g cell dry mass $(l\ culture)^{-1}$.

The two strains exhibited almost identical physiological and biochemical profiles determined using conventional methods, as well as the API 50CH system, except that strain $Z7^T$ strongly fermented melezitose, whereas strain Z1 fermented melezitose weakly. Both isolates hydrolysed gelatin, curdled milk and produced acid from a few sugars such as raffinose and sucrose (detailed data are listed in the species description). The products of glucose fermentation by strains $Z7^T$ and Z1 were acetate, ethanol, H_2 and CO_2. The strains did not use sulfate as electron acceptor. No H_2S was produced from peptone or thiosulfate.

The cell wall hydrolysates of strains $Z7^T$ and Z1 were rich in LL-diaminopimelic acid. The cellular fatty acids of strain $Z7^T$ were characterized mainly by iso-branched fatty acids, predominantly $isoC_{15:0}$ (42·83 %) and $isoC_{14:0}$ (32·11 %); $anteisoC_{15:0}$ (6·04 %) and $C_{14:0}$ 2-OH (8·03 %) 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 $Z7^T$ and Z1 was 98·7 % and their DNA G+C contents were 50·4 and 48·6 mol%, respectively. All the results above indicate the single species status of the two isolates.

To ascertain the phylogenetic position of the isolates, the complete 16S rRNA gene sequences (1533 bp) were compared with the most similar sequences and those of representatives of the *Clostridiaceae* retrieved from GenBank. On the basis of a consensus 1372 bp of the 16S rRNA gene sequence, a phylogenetic tree, rooted with *Bacillus subtilis* NCDO 1769^T, was constructed (Fig. 2). 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, including spore-forming *Clostridium* species (*Clostridium sporosphaeroides*, *C. leptum* and *Clostridium cellulosi*), *Ruminococcus* species (*Ruminococcus albus*, *Ruminococcus flavefaciens* and *Ruminococcus callidus*), *Anaerofilum* species (*Anaerofilum agile* and *Anaerofilum pentosovorans*) (Zellner *et al.*, 1996), *Eubacterium* species, *Faecalibacterium prausnitzii* (Duncan *et al.*, 2002) and *Anaerotruncus colihominis* (Lawson *et al.*, 2004). Strain $Z7^T$ showed the highest 16S rRNA gene sequence similarity (90·9 %) to *Anaerotruncus colihominis*; similarities were 87·0–90·1 % with other related species in the *C. leptum* subgroup and lower than 86·0 % with other representatives of the *Clostridiaceae*. The large

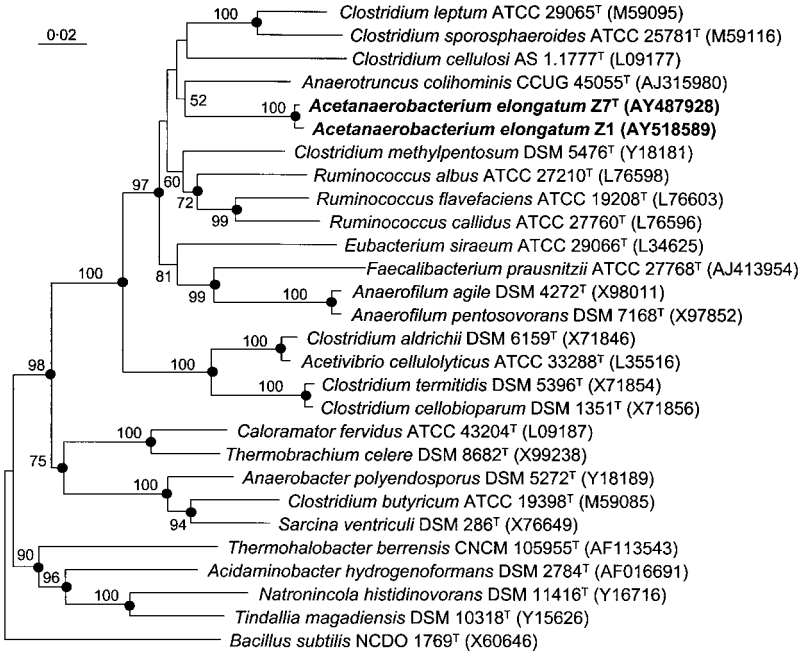

Fig. 2. Phylogenetic dendrogram of *Acetanaerobacterium elongatum* gen. nov., sp. nov. $Z7^T$ and related species based on 16S rRNA gene sequence similarity. The tree was rooted with *Bacillus subtilis* and constructed using the neighbour-joining method. Solid circles indicate that the corresponding nodes (groups) were also recovered in maximum-likelihood and maximum-parsimony methods. Numbers at nodes represent levels of bootstrap support (%) based on neighbour-joining analysis of 1000 resampled datasets. GenBank accession numbers are given in parentheses. Bar, 2 % sequence divergence.

sequence divergence indicated that the isolates represent a new genus in this cluster.

Strains Z7^T and Z1 also showed distinct phenotypic features, enabling them to be distinguished from representative members in the same cluster. They differed from *Anaerotruncus colihominis* by the latter's production of butyric acid from glucose fermentation, production of indole and the wide pH range for growth (pH 5·5–11·0). Unlike related *Clostridium* species, the isolates were non-spore-forming, hydrolysed gelatin and had different sugar fermentation profiles (see Table 1). They differed from *Ruminococcus* species in their thin rod shape, different biochemical traits and a variation in the DNA G+C content of 4–10 mol%. They produced a large amount of hydrogen, but no lactic acid from glucose fermentation, enabling them to be distinguished from *Anaerofilum* species. They differed from *Eubacterium siraeum* by the latter's inability to ferment glucose and other features shown in Table 1. *F. prausnitzii* has a Gram-negative cell wall and produced butyrate, D-lactate and formate, but no hydrogen from glucose fermentation, enabling it to be differentiated from the two strains.

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 Z7^T and Z1 represent a distinct genus within the *C. leptum* rRNA subgroup; *Acetanaerobacterium elongatum* gen. nov., sp. nov. is therefore proposed.

Table 1. Characteristics that can be used to differentiate *Acetanaerobacterium elongatum* gen. nov., sp. nov. from its phylogenetic relatives

Strains: 1, *Acetanaerobacterium elongatum* gen. nov., sp. nov. JCM 12359^T; 2, *Anaerotruncus colihominis* CCUG 45055^T (data from Lawson *et al.*, 2004); 3, *C. cellulosi* AS 1.1777^T (He *et al.*, 1991); 4, *C. sporosphaeroides* ATCC 25781^T (Cato *et al.*, 1986); 5, *C. leptum* ATCC 29065^T (Cato *et al.*, 1986); 6, *Anaerofilum agile* DSM 4272^T (Zellner *et al.*, 1996); 7, *Anaerofilum pentosovorans* DSM 7168^T (Zellner *et al.*, 1996); 8, *E. siraeum* ATCC 29066^T (Moore & Moore, 1986); 9, *F. prausnitzii* ATCC 27768^T (Moore *et al.*, 1984); 10, *Clostridium methylpentosum* DSM 5476^T (Himelbloom & Canale-Parola, 1989); 11, *R. flavefaciens* ATCC 19208^T (Bryant *et al.*, 1986). Symbols: +, >90 % of strains positive; −, <10 % of strains positive; D+, 60–89 % of strains positive; D−, 11–39 % of strains positive; d, 40–60 % of strains positive; W, weak reaction; C, curdled milk; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
DNA G+C content (mol%)	50·5	54	35	27	51–52	54·5	55	45	52–57	46	39–44
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Ring	Coccus
Spore production	−	−	+	+	+	−	−	−	−	+	−
Products from PYG*	A2	AB	A2	ABp	A(2)	LA2F	LA2F	−	FBAL	−	S2(L)
H_2 production†	4	+	4	4	4	−	−	−	−	−	4
Motility	+	−	+	−	−	+	−	−	−	−	−
Optimum growth temperature (°C)	37	36–40	55–60	37–45	37	37	25–40	37–45	37	45	37–42
Gelatinase	+	−	−	−	−	NR	NR	−	NR	NR	−
Milk reaction	C	NR	C	−	−	NR	NR	D−	NR	NR	−
Acid production from:											
Inositol	−	−	+	−	−	NR	NR	NR	NR	−	−
Glycogen	−	NR	+	−	D−	NR	NR	−	NR	−	−
Mannitol	−	−	+	−	−	NR	NR	−	NR	−	−
Mannose	−	+	+	−	−	NR	+	D+	NR	−	d
Starch	−	−	+	−	−	−	−	D−	w	−	NR
Trehalose	−	d	+	−	D+	+	+	−	w	−	−
Cellobiose	+	+	+	−	−	+	+	+	w	−	+
Fructose	+	+	+	−	D−	+	+	D−	+	−	−
Arabinose	+	−	+	−	−	NR	+	−	−	+	d
Inulin	+	NR	+	−	−	NR	NR	NR	+	−	−
Salicin	+	−	+	−	−	+	NR	−	+	−	−
Raffinose	+	−	+	−	−	−	−	−	−	−	−
Sucrose	+	NR	−	−	D+	−	−	D−	W	−	d

*Products from PYG (given in decreasing order of amounts 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.

†On a scale of − (negative) to 4 (abundant).

Description of *Acetanaerobacterium* gen. nov.

Acetanaerobacterium (A.cet.an.ae.ro.bac.te′ri.um. L. n. *acetum* vinegar; Gr. pref. *an* not; Gr. n. *aer* air; Gr. neut. n. *baktron* a small rod; N.L. neut. n. *Acetanaerobacterium* vinegar-producing anaerobic small rod).

Gram-positive, motile, non-spore-forming, straight, thin rods. Obligately anaerobic. No microaerophilic or aerobic growth occurs. Cell wall peptidoglycan contains LL-diaminopimelic acid. Cellular fatty acids consist mainly of iso-branched fatty acids, predominantly $isoC_{15:0}$ and $isoC_{14:0}$. Mesophilic: grows at 20–42 °C. Grows at neutral pH. Chemo-organotrophic. Oxidase and catalase are not produced. 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 acetate, ethanol, hydrogen and carbon dioxide; lactic acid, propionate and succinate are not produced. Sulfate is not reduced. The G+C contents of the genomic DNA of the known strains are 48·6 and 50·4 mol%. Only one species, *Acetanaerobacterium elongatum*, is described so far; this species has been designated the type species.

Description of *Acetanaerobacterium elongatum* sp. nov.

Acetanaerobacterium elongatum (e.lon.ga′tum. L. neut. part. adj. *elongatum* elongated).

Morphology and general characters are as described for the genus. Cells are 0·2–0·4 × 4·0–8·0 μm. Colonies on PYG agar are circular, slightly convex, white and translucent, reaching 1·5–3·0 mm in diameter after 3 days incubation at 37 °C. Optimal growth occurs at 37 °C. The pH range for growth is 5·0–7·5, with optimum growth at pH 6·5–7·0. Acid is produced from a few sugars, including D-glucose, D-fructose, D-galactose, L-arabinose, D-xylose, cellobiose, D-maltose, sucrose, raffinose, inulin and salicin. Acid is not produced from sorbose, ribose, D-lactose, mannose, melibiose, rhamnose, trehalose, starch, glycogen, amygdalin, adonitol, dulcitol, erythritol, inositol, mannitol, sorbitol or ribitol. No acid is produced from the following compounds: methanol, ethanol, 1-propanol, pyruvate, citrate, fumarate, malate, succinate, malonic acid, hippurate, sodium gluconate, butane diacid, β-hydroxybutyric acid, phenylacetic acid, cellulose and xylan. Curdles milk. Starch is hydrolysed and arginine dihydrolase is produced. Urease, lecithinase and lipase are not produced. Methyl red and Voges–Proskauer tests are negative. Nitrate is not reduced. No H_2S is produced from peptone or thiosulfate. The major cellular fatty acids are $isoC_{15:0}$ (42·83 %), $isoC_{14:0}$ (32·11 %), $anteisoC_{15:0}$ (6·04 %) and $C_{14:0}$ 2-OH (8·03 %).

The type strain is $Z7^T$ (=JCM 12359^T=AS 1.5012^T), isolated from the anaerobic sludge of paper mill waste water.

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