Clostridium aciditolerans sp. nov., an acid-tolerant spore-forming anaerobic bacterium from constructed wetland sediment

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An obligately anaerobic, spore-forming, moderately acid-tolerant bacterium, strain JW/YJL-B3^T, was isolated from a sediment sample from a constructed wetland system receiving acid sulfate water. Based on 16S rRNA gene sequence analysis, the isolate belonged to the Firmicutes branch with *Clostridium drakei* SL1^T (96.2 % gene sequence similarity) as its closest relative. The G+C content of the genomic DNA was 30.8 mol% (HPLC). Cells were straight to curved rods, 0.5–1.0 µm in diameter and 3.0–9.0 µm in length. The temperature range for growth was 20–45 °C, with an optimum around 35 °C. Growth was not detected below 18 °C or above 47 °C. The pH range for growth was broad, pH^{25 °C} 3.8–8.9, with an optimum at 7.0–7.5. However at pH 4.5, the strain grew at 52 % of the optimal growth rate. The salinity range was 0–1.5 % NaCl (w/v). Strain JW/YJL-B3^T utilized beef extract, Casamino acids, peptone, tryptone, arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, ribose, sucrose, xylose, pyruvate, glutamate and inulin as a carbon and energy source. There were no indications of growth under aerobic or autotrophic conditions. The isolate produced acetate, butyrate and ethanol as fermentation end products from glucose. Based on these characteristics and other physiological properties, the isolate is placed into the novel taxon, *Clostridium aciditolerans* sp. nov., with strain JW/YJL-B3^T (=DSM 17425^T=ATCC BAA-1220^T) as the type strain.

Micro-organisms are the most dominant form of life in acidic environments. However under this condition, many micro-organisms are killed, distorted or become cytoplasm deficient (Wortman *et al.*, 1986). A few anaerobic micro-organisms have been isolated and characterized from low pH environments, including methanogens (Williams & Crawford, 1985; Horn *et al.*, 2003) and clostridia (Hamman & Ottow, 1976; Kuhner *et al.*, 2000). Since members of the genus *Clostridium* are metabolically versatile and capable of forming endospores, they are ubiquitous and can be found in various extreme environments such as the deep-sea, hot springs and the intestinal tracts of animals.

In this paper, we report the characterization of an anaerobic, acid-tolerant, spore-forming bacterium which falls into the radius of the genus *Clostridium sensu stricto* (Wiegel *et al.*, 2005), formerly known as Collins' cluster I and II of the genus *Clostridium* (Collins *et al.*, 1994).

A constructed wetland system was built to treat acid sulfate water at the US Department of Energy's Savannah River Site

near Aiken, SC, USA. The acid sulfate water (pH ~2.0) was introduced at the top of each cylindrical cell in the system, containing sand mixed with horse manure, wood chips and limestone. Within a few months of operation, the sediment developed a series of different reaction zones that were distinguishable based on a variety of physico-chemical attributes (Thomas, 2003; Lee, 2005). Sediment samples for the enrichments were taken from the top layer of the core where conditions were relatively oxidized, the pH was around 3.5 and the sediment was dominated by iron oxyhydroxide.

Enrichments under anaerobic and acidic conditions (pH 3.5, 4.5 and 5.5) were prepared using a medium designed for sulfate-reducing bacteria (Widdel & Bak, 1992) using a modified Hungate technique (Ljungdahl & Wiegel, 1986). The medium contained either acetate (15 mM) or lactate (15 mM) as the sole carbon source and energy source supplemented with 0.1 % yeast extract. The enrichments were incubated at 37 °C for up to one month.

A pure isolate, designated strain JW/YJL-B3^T, was obtained from the enrichments by three subsequent rounds of single colony isolation using the agar (1.5 % w/v)-shake-roll-tube

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JW/YJL-B3^T is DQ114945.

technique (Ljungdahl & Wiegel, 1986). Colonies of the isolate appeared after 1–2 days and were irregular, mostly translucent, filamentous and less than 1.5 mm in diameter.

For the phylogenetic analysis, DNA was extracted from the isolate as described previously (Lee *et al.*, 2005) and amplified with a bacterial domain-specific primer set for the 16S rRNA gene, 27 forward and 1492 reverse (Lane, 1991). The PCR amplification was carried out with initial denaturation at 94 °C for 2 min and followed by 10 cycles of denaturation (94 °C, 30 s), annealing (58 °C, 30 s) and extension (72 °C, 1 min), 10 cycles of denaturation (94 °C, 30 s), annealing (58 °C, 45 s) and extension (72 °C, 1 min 15 s) and 10 cycles of denaturation (94 °C, 30 s), annealing (58 °C, 1 min) and extension (72 °C, 1 min 30 s). Final extension was for 7 min at 72 °C. PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced by Macrogen Inc. (Seoul, Korea). Retrieved 16S rRNA gene sequences were analysed using BLAST and aligned manually against sequences obtained from the GenBank database using CLUSTAL_X v1.81 (Thompson *et al.*, 1997) and GeneDoc v2.6.02 (www.psc.edu/biomed/genedoc). Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) and FITCH (Fitch & Margoliash, 1967) using the Jukes and Cantor model (Jukes & Cantor, 1969) with the PHYLIP v3.6a2.1 phylogenetic analysis package (Felsenstein, 2001).

A nearly complete 16S rRNA gene sequence was obtained for strain JW/YJL-B3^T, comprising of 1391 bp [approximate positions 47–1467 according to the *Escherichia coli* (GenBank accession number X80725) numbering scheme]. Based on 16S rRNA gene sequence similarity, strain JW/YJL-B3^T fell into the radius of *Clostridium sensu stricto* (Wiegel *et al.*, 2005). The closest relative was *Clostridium drakei* (96.2 % gene sequence similarity) (Fig. 1).

Based on the phylogenetic analysis, further culturing of the isolate was performed in phosphate-buffered basal media containing (l⁻¹): 0.69 g NaH₂PO₄·H₂O, 2.13 g Na₂HPO₄, 0.5 g NH₄Cl, 0.01 g MgSO₄·7H₂O, 0.01 g CaCl₂, 2 g glucose, 0.5 g yeast extract, 1 mg resazurin and 0.05 g cysteine.HCl. Growth of pure cultures was determined by measuring the optical density at 600 nm using a spectrophotometer (Spectronic 21; Bausch & Lomb).

Morphology was studied using light microscopy (VANOX phase-contrast microscope; Olympus) and transmission electron microscopy (100CX; JEOL). Vegetative cells grown in phosphate-buffered medium were straight rods, occurring singly or in pairs. The cells were 0.5–1.0 µm in diameter and 3.0–9.0 µm in length. Endospores detected in the late exponential growth phase were subterminal and oval in shape without swelling of the cells. Cells of strain JW/YJL-B3^T had peritrichous flagella (Fig. 2b), but motility was not observed during light microscopy. Cells stained Gram-negative at all growth phases (Doetsch, 1981), while electron microscopy and phylogenetic position indicated a Gram-type positive cell-wall structure (Fig. 2c) as expected from the 16S rRNA gene sequence-based phylogeny. Thus, the novel strain is Gram-stain negative but is Gram-type positive (Wiegel, 1981).

The temperature range for growth of the isolate was measured at pH 5.0 using a temperature gradient incubator (Scientific Industries). Strain JW/YJL-B3^T grew at between 20 and 45 °C, with an optimum around 35 °C. Growth was not detected below 18 °C or above 47 °C. The pH^{25 °C} (Wiegel, 1998) range for growth was determined using media buffered with 10 mM each of MES, HEPES and TAPS in combination with 2 mM phosphate. The pH^{25 °C} range for growth was pH 3.8–8.9, with an optimum at pH 7.0–7.5. Although the optimum pH was in the neutral range, the

Fig. 1. A phylogenetic dendrogram based on 16S rRNA gene sequences showing the position of strain JW/YJL-B3^T (bold type) among members of the genus *Clostridium sensu stricto*. The 16S rRNA gene sequence data used represents *E. coli* DSM 30083^T nucleotide positions 47–1467. The tree was constructed using the neighbour-joining method with Jukes and Cantor distance corrections. Numbers at the nodes represent the bootstrap values (1000 replicates); values above 80 % are considered significant. Bar, 1 nucleotide substitution per 100 nucleotides.

Fig. 2. Light micrograph (a) and electron micrographs of a negatively stained cell depicting retarded peritrichous flagellation (b) and an ultrathin section depicting the Gram-type type positive cell wall (c) of strain JW/YJL-B3^T. Bars, 10 µm (a), 1 µm (b) and 0.5 µm (c).

strain grew well under mildly acidic conditions, i.e., growth at pH 4.5–5.0 occurred at 52 % of the optimal growth rate. Growth was not detected at or below pH 3.5 or at or above pH 9.2. The salinity range for growth was obtained using phosphate-buffered medium supplemented with various concentrations of NaCl and KCl (9:1). The isolate grew optimally in the absence of NaCl and KCl, but showed growth up to 1.5 % (w/v) salinity. No growth was detected at 2 % salinity. The doubling time at 37 °C and pH²⁵ °C 6.5 with 0.3 % glucose was 1.7 h. Detailed results of other morphological and physiological characteristics are

summarized and compared with closely related species in Table 1.

Biochemical features of the novel isolate were tested using the API ZYM system (bioMérieux) and positive reactions were recorded for alkaline phosphatase, esterase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Strain JW/YJL-B3^T exhibited positive reactions for the methyl red test, indole production and lecithinase, but was negative for the Voges–Proskauer reaction (Smibert & Krieg, 1994) and lipase. Gelatin was hydrolysed, but not casein. Haemolysis

Table 1. Morphological and physiological characteristics of strain JW/YJL-B3^T and its closest relatives

Strains: 1, JW/YJL-B3^T (data from this study); 2, *Clostridium drakei* SL1^T (Küsel *et al.*, 2000; Liou *et al.*, 2005); 3, *Clostridium scatologenes* ATCC 25775^T (Küsel *et al.*, 2000; Liou *et al.*, 2005); 4, *Clostridium carboxidivorans* P7^T (Liou *et al.*, 2005). NR, Not reported.

Characteristic	1	2	3	4
Source	Constructed wetland sediment	Acidic coal mine pond	Soil	Agricultural settling lagoon
Cell size (µm)	0.5–1 × 3–9	0.6 × 3–4	3–21 (length)	0.5 × 3
Temperature range (°C)	20–45	18–42	18–42	24–42
Optimum temperature (°C)	35.5	30–37	37–40	37–40
pH range	3.8–8.9	4.6–7.8	4.6–8.0	4.4–7.6
Optimum pH	7.0–7.5	5.4–7.5	5.4–7.0	5.0–7.0
Salinity range (% NaCl)	0–1.5	NR	NR	NR
Optimum salinity (% NaCl)	0	NR	NR	NR
DNA G+C content (mol%)*	30.8	32	31	31
Gram stain	–	–	NR	+
Spores observed	– (subterminal, oval)	– (terminal)	+	Rare (subterminal to terminal)
Growth with H ₂ /CO ₂	–	+	+	+
Utilization of:				
Cellulose	–	+	+	+
Inositol	–	+	+	+
Lactose	+	–	–	–
Maltose	+	–	+	–
Mannitol	–	+	–	+
Raffinose	+	–	–	–
Sorbitol	–	–	+	–
Trehalose	–	–	–	+

*DNA G+C content was determined by nuclease digest.

was detected on blood agar. The utilization of various carbon sources was studied using phosphate-buffered basal medium supplemented with 0.02 % yeast extract and the test substrate. Strain JW/YJL-B3^T used beef extract, Casamino acids, peptone, tryptone, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, ribose, sucrose, xylose, pyruvate, glutamate and inulin as carbon and energy sources. Acetate, lactate, arabinose, trehalose, inositol, mannitol, sorbitol, xylitol and cellulose did not support growth of the strain. There was no indication of autotrophic growth under an atmosphere of 15 p.s.i. of CO₂ and H₂ (80:20). The strain grew in classical peptone–sugar media including peptone–yeast extract (PY), peptone–yeast extract–glucose (PYG), reinforced clostridial medium (RCM, Difco) and thioglycolate broth (Difco). The fermentation end products from glucose (20 mM) were acetate, butyrate and ethanol. The potential use of electron acceptors was determined in cultures incubated for at least 2 weeks using phosphate-buffered basal medium containing 0.2 % Casamino acids as an electron donor and an inoculum of 3 % (v/v) of an exponentially growing culture. The use of electron acceptors was determined by measuring growth (OD₆₀₀), sulfide, ammonium or nitrite production or colour-change. However, none of the following electron acceptors tested at 20 mM was utilized: fumarate, nitrate, nitrite, sulfate, thiosulfate, elemental sulfur, Fe(III), 9,10-anthraquinone 2,6-disulfonate (AQDS), Mn(IV) or sulfite at 2 mM. Oxygen was not used as a terminal electron acceptor when tested in a 150 ml serum bottle laid horizontally containing 5 ml culture (basal medium) and air as head gas.

Strain JW/YJL-B3^T was resistant only to tetracycline at 10 µM and sensitive to 10 µM ampicillin, chloramphenicol, erythromycin, rifampicin and streptomycin and 100 µM tetracycline. The G+C content of genomic DNA, determined by the HPLC method as described previously (Mesbah *et al.*, 1989; Lee *et al.*, 2005), was 30.8 mol% (mean of 4 replicates of the nuclease digest and HPLC runs).

Based on morphological, physiological and phylogenetic characteristics, we propose to place strain JW/YJL-B3^T as the type strain of a novel taxon, *Clostridium aciditolerans*, sp. nov., belonging to the genus *Clostridium sensu stricto* within the family *Clostridiaceae* (Garrity *et al.*, 2004; Wiegel *et al.*, 2005).

Description of *Clostridium aciditolerans* sp. nov.

Clostridium aciditolerans (a.ci.di.to'le.rans. N.L. n. *acidum* an acid; L. part. adj. *tolerans* tolerating; N.L. part. adj. *aciditolerans* acid-tolerating).

Cells are straight to slightly curved rods, 0.5–1 µm in diameter and 3.0–9.0 µm in length. Spores are subterminal and oval in shape and do not swell the cell. Retarded peritrichous flagellation is observed. Although the type strain stains Gram-negative at all growth phases, the strain is Gram-type positive. The temperature range for growth is

20–45 °C, with an optimum around 35 °C. No growth is observed at or below 18 °C or at or above 47 °C. The pH^{25 °C} range for growth is from pH 3.8 to 8.9, with an optimum at pH 7.0–7.5. Growth at pH 4.5–5.0 takes place at 52 % of the optimal growth rate; no growth is observed at or below pH 3.5 or at or above pH 9.2. The salinity range for growth is from 0 to 1.5 % NaCl (w/v). In the presence of 0.02 % yeast extract, the following substrates serve as carbon and energy source: beef extract, Casamino acids, peptone, tryptone, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, ribose, sucrose, xylose, pyruvate, glutamate and inulin. Fe(III), nitrate, thiosulfate, elemental sulfur, sulfate, sulfite, MnO₄ and fumarate are not used as electron acceptors. The main organic fermentation end-products from glucose are acetate, butyrate and ethanol. The strain is resistant to tetracycline (10 µM) and sensitive to ampicillin (10 µM) chloramphenicol (10 µM), erythromycin (10 µM), rifampicin (10 µM) and streptomycin (10 µM). The G+C content of the genomic DNA is 30.8 mol% (HPLC).

The type strain, JW/YJL-B3^T (=DSM 17425^T=ATCC BAA-1220^T), was isolated from a sediment sample from a constructed wetland system receiving acid sulfate water.

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