Gracilibacter thermotolerans gen. nov., sp. nov., an anaerobic, thermotolerant bacterium from a constructed wetland receiving acid sulfate water

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An obligatorily anaerobic, thermotolerant, asporogenic bacterium, strain JW/YJL-S1T, was isolated from a sediment sample of a constructed wetland system receiving acid sulfate water (pH 1.6–3.0). Cells of strain JW/YJL-S1T were straight to curved rods 0.2–0.4 μm in diameter and 2.0–7.0 μm in length, and stained Gram-negative. Growth of strain JW/YJL-S1T was observed at 25–54°C (no growth at or below 20 or at or above 58°C), with an optimum temperature range for growth of 42.5–46.5°C. The pH range for growth was 6.0–8.25 (no growth at or below pH 5.7 or at or above pH 8.5), with optimum growth at pH 6.8–7.75. The salinity range for growth was 0–1.5% (w/v) NaCl, with an optimum at 0–0.5%. During growth on glucose the isolate produced acetate, lactate and ethanol as main fermentation end products. The fatty acid composition was dominated by branched-chain compounds: i15:0, a15:0, i16:0 and i17:0. The G+C content of the genomic DNA was 42.8 mol% (HPLC). Strain JW/YJL-S1T showed polymorphism of the 16S rRNA gene. Its closest relative was the thermophilic Clostridium thermosuccinogenes DSM 5807T (a member of Clostridium cluster III) (a BLASTN search revealed Clostridium pascui DSM 10365T to have 92.7% gene sequence similarity, the highest value). The inferred phylogenetic trees placed strain JW/YJL-S1T between Clostridium clusters I/II and III. Based on the morphological and phylogenetic data presented, JW/YJL-S1T (= DSM 17427T = ATCC BAA-1219T) is proposed as the type strain of a novel species in a new genus, Gracilibacter thermotolerans gen. nov., sp. nov.

Abbreviation: PLFA, phospholipid fatty acid.

The GenBank/EMBL/DDBJ accession numbers for five clones of the 16S rRNA gene sequences of strain JW/YJL-S1T are DQ117465–DQ117469.

It is well known that various microbial communities are involved not only in the generation but also in the remediation of acid mine drainage. Most of the microorganisms isolated from mining environments are iron- and sulfur-oxidizing bacteria and sulfate-reducing bacteria. Although heterotrophic fermentative bacteria are closely associated with other microbial communities in acid mine drainage, little is known about their diversity and functions in such environments. For instance, heterotrophic fermentative bacteria can remove organic acids that can inhibit chemolithotrophic bacteria such as Leptospirillum ferrooxidans and Acidithiobacillus ferrooxidans (Johnson, 1998). Therefore, the generation of acid mine drainage can be facilitated by indigenous heterotrophic bacteria. Conversely, they also support sulfate- or metal-reducing bacteria by degrading biopolymers into monomers and fermentation products, which then serve as substrates, thus contributing to the bioremediation of acid mine drainage.

Here we report on a new isolate recovered from a constructed treatment wetland system receiving acid sulfate water. On the basis of the physiological and phylogenetic evidence presented, we propose a new genus, Gracilibacter, to accommodate this organism.

Strain JW/YJL-S1T was isolated from an MPN (most probable number) tube inoculated with sediment from the upper layer of a constructed treatment wetland system. This wetland was receiving water from an acid sulfate runoff pond from a coal pile located at the Department of Energy’s Savannah River Site near Aiken, SC, USA (Lee, 2005). The acid runoff pond water has a pH of 1.6–3.0 and relatively high sulfate and ferric iron concentrations. The uppermost sediment in the constructed wetland was dominated by iron oxyhydroxide precipitates coating and replacing an organic substrate amended with limestone. The organic substrate
used for the constructed treatment wetland was composed primarily of composted stable wastes and spent brewing grains mixed with local farmland soil from South Carolina (Thomas, 2003; Lee, 2005). Thus, no defined habitat can be given for the novel taxon described herein.

The isolate was routinely cultured in a carbonate-buffered basal medium (Widdel & Bak, 1992) supplemented with 20 mM acetate and 0.1 mM ferric citrate at pH 25°C 6.8 (Wiegel, 1998) and 37°C under anaerobic conditions (100% N₂) using a modified Hungate technique (Ljungdahl & Wiegel, 1986). Single colonies were obtained from dilution rows in agar- (1.5% w/v) shake roll-tubes. To ensure that a culture was derived from a single cell, the isolate was purified by an additional five rounds of single colony isolation using the agar-shake roll-tube method (Ljungdahl & Wiegel, 1986). Surface colonies of strain JW/YJL-S1T appeared after 2–3 days and were less than 1 mm in diameter, circular to irregular, mostly translucent and filamentous. Cell morphology was observed via light microscopy (Olympus VANOVX phase-contrast microscope) and electron microscopy (JEOL100CX transmission electron microscope). Vegetative cells in liquid culture were straight to curved, 0.2–0.4 μm in diameter and 2.0–7.0 μm in length (Fig. 1). Cells were either single or formed chains. Infrequently, cells up to 45 μm in length were detected. No active motility was observed under phase-contrast microscopy when cells were grown in carbonate-buffered basal medium. However, retarded peritrichous flagella were observed under the electron microscope (Fig. 1a; negative staining). Motility was subsequently observed during growth in SIM agar medium (Cappuccino & Sherman, 1987). No spores were detected either by microscopy or by heat treatment (10 min at 80°C). Gram staining was performed according to standard procedures (Doetsch, 1981) and showed that cells from both the early exponential and the stationary growth phases in both media stained Gram-negative. Detailed results of morphological and physiological characterization are given in the genus and species descriptions below.

Using a temperature-gradient incubator (Scientific Industries, Inc.), the temperature range for growth was determined to be 25–54°C with an optimum at 42.5–46.5°C. No growth was detected at or below 20°C or at or above 58°C. The pH range for growth was determined at 37°C in basal medium supplemented with 10 mM each of MES, HEPES and TAPS. The pH range for growth was 6.0–8.25 with an optimum at pH 7.5 6.8–7.75. No growth was detected at or below pH 5.7 or at or above pH 8.5, suggesting that strain JW/YJL-S1T originated not from the acid runoff but from the organic substrate of the constructed wetland. The salinity range for growth was 0–1.5% (w/v) with an optimum at 0.5% NaCl plus KCl at a ratio of 9:1; no growth was detected above 2% salts. The doubling time for strain JW/YJL-S1T was 3–1 h at 42°C and pH 7.5 with 0.3% yeast extract as the substrate. The isolate required yeast extract for growth. For characterization tests, cultures were incubated for up to 20 days, with growth judged positive if the optical density (at 600 nm) of the culture was twice that of a control culture containing only yeast extract (0.02%). Utilization of possible substrates (0.2%, w/v) was tested in the presence of 0.02% yeast extract. Strain JW/YJL-S1T used Casamino acids, tryptone, peptone, maltose, sucrose, arabinose, fructose, galactose, glucose, mannose, xylose, mannitol and sorbitol as carbon and energy sources. No growth was observed with cellobiose, lactose, raffinose, ribose, trehalose, inositol, xylitol, acetate, lactate, pyruvate, methanol or carboxymethylcellulose (1.0% w/v, CMC 7LT or 7M; Hercules) as carbon and energy sources. None of the following electron acceptors, tested in media containing 20 mM lactate or 0.1% yeast extracts, was utilized: fumarate, nitrate, sulfate, sulfite, thiosulfate, elemental sulfur, iron(III), anthraquinone 2,6-disulfonate or manganese(IV) at concentrations of 20 mM (sulfite was tested at 2 mM). Strain JW/YJL-S1T showed positive growth on classical peptone-sugar media including

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**Fig. 1.** Electron micrographs of cells of strain JW/YJL-S1T showing retarded flagella (a) and the prototypical image of the cell line (b). The inset in (b) shows a thin section that matches a conventional thin section of a Gram-positive bacterium that has an S layer. Bars, 2 μm (a), 4 μm (b) and 50 nm [inset in (b)].
peptone-yeast extract (PY), peptone-yeast extract-glucose (PYG), reinforced clostridial medium (RCM; Difco) and thioglycolate broth (Difco). Fermentation end products from 20 mM glucose were analysed by HPLC with an Aminex-H87 column (Bio-Rad) and Beckman detector. The main organic fermentation end products were acetate, lactate and ethanol. Bacterial cell-membrane phospholipid fatty acids (PLFAs) were extracted and isolated from lyophilized cells. Total lipids were extracted, fractionated and saponified and then methylation to obtain fatty acid methyl esters of the phospholipids as described by Guckert et al. (1985). These were then analysed by using a capillary column (30 m DB-5, 0.25 mm inner diameter) gas chromatograph (Agilent 6890) equipped with a flame-ionization detector and by using a gas chromatograph–mass spectrometer (Agilent 7972 MSD). Compounds were identified by comparison of retention times with a bacterial acid methyl ester standard (Supelco) and by analysis of the mass spectra data. The PLFA composition of strain JW/YJL-S1T was dominated by and by analysis of the mass spectra data. The PLFA composition of strain JW/YJL-S1T was dominated by branched-chain fatty acids (b15:0, a15:0, i16:0 and i17:0), which accounted for 44–7% of the total PLFAs. Significant amounts of 16:0 (29–0%) and small amounts of the unsaturated 16:1, 17:1 and 18:1 fatty acids (5–4, 5–4 and 2–5%, respectively) were also present (Table 1). This profile is similar to that reported by O’Leary & Wilkinson (1988) for closely related Gram-type positive bacteria. Antibiotic susceptibility was tested with ampicillin, chloramphenicol, erythromycin, rifampicin, streptomycin and tetracycline at concentrations of 10 and 100 μM. Strain JW/YJL-S1T was resistant only to 10 μM streptomycin. Biochemical properties of strain JW/YJL-S1T were determined using the API ZYM system (bioMérieux). Enzyme assays of strain JW/YJL-S1T were positive for esterase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase and β-glucosidase. Strain JW/YJL-S1T produced indole but not H2S in SIM medium.

For phylogenetic and G + C content analyses, DNA was extracted by using a DNeasy genomic DNA purification kit (Qiagen). The DNA G + C content was measured by HPLC as described by Mesbah et al. (1989) with the modification of Lee et al. (2005), using S1 nuclease (Invitrogen) and 0–3 M sodium acetate (pH 5–0). The G + C content of the genomic DNA was 42–8 mol% (HPLC), the mean of four replicate analyses. 16S rRNA gene sequence analysis of strain JW/YJL-S1T was carried out three times (yielding the same results) with a bacterial domain-specific primer set, 27 forward and 1492 reverse (Lane, 1991). PCR amplification was performed using the Easy-A high-fidelity PCR cloning enzyme (Stratagene) with 30 cycles of denaturation (94°C, 30 s), annealing (58°C, 30 s) and extension (72°C, 1 min) after initial denaturation at 94°C for 3 min. Final extension was for 7 min at 72°C. To check for possible heterogeneity of 16S rRNA genes, the PCR product of the 16S rRNA gene of strain JW/YJL-S1T was cloned and transformed using the TOPO TA cloning kit (Invitrogen). Plasmid DNA was extracted using an Eppendorf FastPlasmid Mini kit (Brinkmann), amplified as described above, purified using the QIAquick PCR purification kit (Qiagen) and sequenced by Macrogen Inc. (Seoul, Korea). Similarities among partial sequences were determined using SEQUENCER version 4.1.4 (Gene Codes). Retrieved 16S rRNA gene sequences were analysed using BLAST and then aligned manually using CLUSTAL X version 1.81 (Thompson et al., 1997) to create a multiple sequence alignment. A phylogenetic tree (Fig. 2) was inferred by the neighbour-joining method (Saitou & Nei, 1987) using Jukes–Cantor distance corrections (Jukes & Cantor, 1969), with the phylogenetic analysis package PHYLIP version 3.6a2.1 (Felsenstein, 2001). Five clones of the 16S rRNA gene (derived from a single cell colony) were sequenced and revealed the presence of polymorphism of the 16S rRNA gene in strain JW/YJL-S1T. Four of the five sequenced clones grouped together with more than 99% similarity. The other sequenced clone showed about 2% divergence from the rest. When these 16S rRNA gene sequences, containing 1550 bp [approximately positions –107 to 1450 according to the Escherichia coli numbering scheme (GenBank accession no. X80725)], were compared using a BLASTN search against sequences in GenBank, they yielded the same correlations, i.e. that strain JW/YJL-S1T was closely related to uncultured clones mostly obtained from methanogenic environments and to consortia including those from rice paddy-field microcosms (Chin et al., 1999; Hengstmann et al., 1999; Erkel et al., 2005), an oil reservoir (Grabowski et al., 2005), a uranium reduction enrichment plant (GenBank accession numbers DQ125504 and DQ125852) and methanogenic fermenter cultures degrading acetate (Shigematsu et al., 2003), propane or butyrate (GenBank accession numbers AB221361, AB232817, AB248637, AB232818, AB248624 and AB248638). Using BLAST search, Clostridium pasteur DSM 10365T (Clostridium cluster I based on the classification of Collins et al., 1994) had the most similar 16S rRNA gene sequence among bacteria with validly published names, with 93% similarity over the first 160 bp, 92% for 1012 bp and 96% for 54 bp. In an inferred phylogenetic tree, strain JW/YJL-S1T was placed distantly between Collins’

Table 1. PLFA composition of strain JW/YJL-S1T

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<thead>
<tr>
<th>Identified component</th>
<th>Percentage of total</th>
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<tr>
<td>14:0</td>
<td>2.3</td>
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<tr>
<td>i15:0</td>
<td>23.8</td>
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<td>a15:0</td>
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<tr>
<td>15:0</td>
<td>0.5</td>
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<tr>
<td>i16:0</td>
<td>0.6</td>
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<tr>
<td>16:1</td>
<td>5.4</td>
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<tr>
<td>16:0</td>
<td>29.0</td>
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<tr>
<td>17:1</td>
<td>5.4</td>
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<tr>
<td>i17:0</td>
<td>15.4</td>
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<td>18:0</td>
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<td>18:1</td>
<td>1.6</td>
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<td>18:1</td>
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Clostridium cluster I/II and III (Fig. 2) with Clostridium thermosuccinogenes as its closest neighbour. Note that in the recent emended description of the genus Clostridium (Wiegel et al., 2005), cluster II species such as Clostridium proteolyticum fall within cluster I and were classified as members of cluster I (the genus Clostridium sensu stricto), whereas cluster III members represent a new family. In addition to this phylogenetic evidence, strain JW/YJL-S1 T showed no evidence of endospore formation. In addition, strain JW/YJL-S1 T was not cellulytic, distinguishing it from Cl stomidium thermosuccinogenes and related cellulytic species in cluster III; nor did it produce succinic acid or grow at elevated temperatures. Strain JW/YJL-S1 T was also metabolically more versatile than O. pfnegii. Furthermore, the DNA G+C content of strain JW/YJL-S1 T (42.8 mol%) is significantly higher than that of related clostridial species. Based on the polyphasic evidence provided here, JW/YJL-S1T is proposed as the type strain of a novel species in a new genus, Gracilibacter thermotolerans gen. nov., sp. nov., belonging to the order Clostridales (Garrity et al., 2004) without assignment to a family.

Description of Gracilibacter gen. nov.

Gracilibacter (Gra.ci.li.bac’ter. L. adj. gracilis slender; N.L. masc. n. bact’er equivalent of Gr. neut. n. baktron rod or staff; N.L. masc. n. Gracilibacter slender rod, referring to its cell shape).

A member of the low-G+C (about 43 mol%) Gram-positive subphylum Bacillus–Clostridium. Specific habitat unknown. Anaerobic chemo-organotrophs. No spores observed. The type species is Gracilibacter thermotolerans.

Description of Gracilibacter thermotolerans sp. nov.

Gracilibacter thermotolerans (ther.mo.to’le.rans. Gr. n. thermé heat; L. pres. part. tolerans tolerating; N.L. part. adj. thermotolerans heat-tolerating).

Cells are straight to curved rods, 0.2–0.4 µm in diameter and 2.0–7.0 µm in length. Gram-type positive (Wiegel, 1981) but Gram staining negative at all growth phases. Autoplasts (L-shaped cells) occur infrequently during the late-stationary growth phase. Non-motile although retarded flagella (1–5 per cell) are present. PLFA profile is dominated by branched-chain fatty acids: i15:0, a15:0, i16:0 and i17:0. Temperature range for growth is 25–54 °C (no growth at and below 20 °C or at or above 58 °C), with an optimum at 42.5–46.5 °C. The pH 25 °C range for growth is 6.0–8.25 (no growth at and below pH 25 °C 5 or at or above pH 25 °C 8.5), with an optimum at 6.8–7.75. The salinity range for growth is 0–1.5 % (w/v), with an optimum at 0.5 %. In the presence of 0.02 % yeast extract, Casamino acids, tryptone, peptone, maltose, sucrose, arabinose, fructose, galactose, glucose, mannose, xylose, mannitol and sorbitol serve as carbon and energy sources. The main organic fermentation end products from glucose are acetate, lactate and ethanol. No indication of growth on H2/CO2 (80:20, v/v) or the use of iron(III), nitrate, thiosulfate, elemental sulfur, sulfate, sulfite, MnO2 or fumarate as electron acceptors. Positive for esterase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase and β-glucosidase (API ZYM). Indole is produced in SIM medium. Resistant to streptomycin (10 µM). The G+C content of the genomic DNA is 42.8 mol% (HPLC).

The type strain, JW/YJL-S1T (=DSM 17427T = ATCC BAA-1219 T), was isolated from a constructed treatment wetland system at the Department of Energy’s Savannah River Site,
Aiken, SC, USA. The 16S rRNA gene of the type and so far only strain exhibits the presence of polymorphism.

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**References**


