Sporacetigenium mesophilum gen. nov., sp. nov., isolated from an anaerobic digester treating municipal solid waste and sewage

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Two mesophilic, anaerobic bacterial strains (ZLJ115T and L4-2) were isolated from the sludge of an anaerobic digester treating municipal solid waste and sewage in Fujian province, China. The strains were Gram-positive, spore-forming, motile rods (0.9–1.0 × 3.6–7.3 μm). Growth of the strains was observed at 20–42 °C and pH 6–9.5. Both strains fermented several mono- and disaccharides. The main fermentation products from glucose were acetate, ethanol, hydrogen, and carbon dioxide. Optimal hydrogen production by the new isolates was observed at pH 8.8 and 39 °C, and 1.4 mol H₂ was detected from fermentation of 1 mol glucose. The DNA G+C contents of strains ZLJ115T and L4-2 were 53.9 and 54.3 mol%, respectively. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the isolates represented a novel phylogenetic sublineage within cluster XI of the clostridia, clustering with four thermophilic species, with <93.8% 16S rRNA gene sequence similarity to previously described species. Phenotypically, the new isolates were distinguished from their phylogenetic relatives by growing mesophilically and by fermenting a variety of pentoses, as well as their higher genome DNA G+C content. On the basis of the polyphasic evidence from this study, a novel genus and species are proposed, Sporacetigenium mesophilum gen. nov., sp. nov.; strain ZLJ115T ( = DSM 16796T = AS 1.5019T) is the type strain of Sporacetigenium mesophilum.

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Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of Sporacetigenium mesophilum ZLJ115T is AY682207.

Fatty acid profiles of the novel strains are available as supplementary material in IJSEM Online.

Hydrogen is a clean energy source which has potential applications for the future. Although hydrogen is traditionally produced by hydrocarbon reformation or electrolysis of water, it might also be produced by micro-organisms (Nandi & Sengupta, 1998) via either fermentation or photosynthesis. Among the fermentative bio-hydrogen-producing bacteria, the clostridia are the most common group. Basically, there are two types of hydrogen-producing metabolism represented in the clostridia: most of them perform butyrate fermentation, by producing large amounts of butyrate and acetate as concomitant main products during hydrogen-producing fermentation, while only a few produce ethanol and acetate as concomitant products (Cato et al., 1986). According to phylogenetic analysis based on 16S rRNA gene sequences, 19 clusters were proposed within the genus Clostridium (Collins et al., 1994). Collins et al. (1994) also demonstrated that Clostridium was not a monophyletic group and that many species needed to be reclassified. Members of cluster XI exhibit a wide range of physiological and metabolic characteristics, and the cluster includes alkaliphiles (Li et al., 1993, 1994), halophiles (Fendrich et al., 1990) and thermophiles (Alain et al., 2002).

During the survey and isolation of hydrogen-producing bacteria from a variety of polysaccharides and proteinaceous compounds, we isolated two obligately anaerobic, spore-forming bacterial strains from the sludge of an anaerobic digester treating municipal solid waste and sewage in Zhangzhou city, Fujian province, PR China. The strains produced H₂, acetic acid and ethanol from glucose fermentation. Phylogenetically, the strains were affiliated to cluster XI of the clostridia; however, they were distantly related to any described members in this cluster. Based on their distinctive phenotypic, genomic and phylogenetic characteristics, a novel genus and species are proposed.

Strains ZLJ115T and L4-2 were isolated in pre-reduced peptone/yeast extract/glucose (PYG) medium (Holdeman et al., 1977) by serial dilution and the 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. Culture purity was also checked.

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by microscope examination. Routine cultivation was in PYG broth in anaerobic tubes (18 × 180 mm) sealed with butyl-rubber stops under a gaseous atmosphere of 100 % N₂ (100 kPa) at 37 °C.

Cell morphology was examined under a light microscope (Olympus BH-2) as well as an electron microscope (Hitachi H-600A) after negative staining with uranyl acetate. Generation time of the strains was determined by monitoring the OD₆₀₀ of a PYG broth culture at 37 °C at 1 h intervals up to 48 h. Temperature profiles were determined in PYG broth using a water bath (Guangming medical instrument plant, Beijing) at temperatures of 15–55 °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 M). Growth was determined by measuring the OD₆₀₀ of the 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 Beckman DU800 spectrophotometer with Escherichia coli K-12 as the reference. The 16S rRNA gene was amplified by PCR and sequenced as described previously (Chen & Dong, 2004). DNA–DNA relatedness was determined on the basis of the DNA–DNA liquid reassociation rate (De Ley et al., 1970) at 65 °C using a Beckman DU 800 spectrophotometer.

The 16S rRNA gene sequence of strain ZLJ115ᵀ 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 the 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.

Short-chain fatty acids and gases produced from fermentation in PYG medium were measured using a gas chromatograph (GC-14B; Shimadzu) according to Chen & Dong (2004). Diagnostic isomers of diaminopimelic acid (DAP) in the cell wall were determined with 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 ZLJ115ᵀ and L4-2 were Gram-positive rods (0.9–1.0 × 3.6–7.3 μm), occurring singly or in short chains and motile by peritrichous flagella. The Gram-positive cell wall structure was also confirmed by the KOH lysis test (Smibert & Krieg, 1994). In the late stationary phase of growth, ovoid endospores were formed in the ends of cells, resulting in swollen cells. Colonies on PYG agar were milk-white, smooth, circular, entire and translucent, slightly convex and reached 1 mm in diameter after cultivation at 37 °C for 48 h.

Strains ZLJ115ᵀ and L4-2 grew exclusively in pre-reduced media and growth was inhibited completely by air. Both strains used peptone as the sole nitrogen source, but not inorganic nitrogen compounds such as NH₄Cl, (NH₄)₂SO₄, (NH₄)₂HPO₄ or KNO₃. Very weak growth was observed on yeast extract, peptone, tryptone or amino acids as the sole energy and carbon source. Both strains grew at 20–42 °C and pH 6.0–9.5, with optimum growth at 37–39 °C and approximately pH 7.5. The strains could grow in the presence of 0–4 % (w/v) NaCl. The mean generation time of the two strains was 8 h when grown in PYG at 37 °C.

The two strains exhibited almost identical physiological and biochemical profiles determined with conventional methods as well as with the API 50CH system. Both isolates hydrolysed aesculin and starch, but not gelatin. Milk was not curdled. Indole was not produced. Nitrate was not reduced. The strains produced acid from a few sugars such as glucose, arabinose, fructose, maltose and xylose (details in the species description below). The differences between the two isolates were that strain ZLJ115ᵀ produced acid from ribose but not lactose, while strain L4-2 was the reverse. The end products of glucose fermentation by strains ZLJ115ᵀ and L4-2 was acetate, ethanol, H₂ and CO₂, additional trace products including propionic acid, isobutyric acid and isovaleric acid were also observed from PY liquid. When co-cultured with a methanogen, Methanobacterium formicicum DSM 1535⁷ᵀ, the end products of strain ZLJ115ᵀ from glucose were shifted to acetate, CH₄ and CO₂, and no ethanol or hydrogen were produced at all (data not shown). Hydrogen production by the new isolates was at a maximum at pH 8.8 and 39 °C, and 1·4 mol H₂ was detected from fermentation of 1 mol glucose. Sulfur (150 mM) and sulfate (20 mM) were not reduced. H₂ and NH₃ were produced from PYG liquid.

The similarity of the complete 16S rRNA gene sequences between strains ZLJ115ᵀ and L4-2 was 98·9 % and their DNA G+C contents were 53·9 and 54·3 mol%, respectively. The two strains exhibited about 98 % DNA–DNA relatedness; however, they gave slightly different cellular fatty acid profiles (Supplementary Table S1 available in IJSEM Online). The above results indicated single species status for the two isolates.

To ascertain the phylogenetic position of the isolates, complete 16S rRNA gene sequences (1495 bp) were compared with the most similar sequences retrieved from GenBank. On the basis of a consensus 1362 bp 16S rRNA gene sequence, a phylogenetic tree was constructed, rooted with Caloramabacter azorensis MV1087ᵀ and Clostridium acidurici ATCC 7906ᵀ (Fig. 1). Phylogenetic analysis showed that the strains were affiliated to the phylum of low-G+C-content
Strain ZLJ115<sup>T</sup> showed the highest 16S rRNA gene sequence similarity to *Clostridium thermoalcaliphilum* (93·8 %) and *Tepidibacter formicigenes* (93·7 %), and 98 % bootstrap support confirmed that the new isolates were clustered with *C. thermoalcaliphilum*, *Clostridium paradoxum*, *T. formicigenes* and *Tepidibacter thalassicus*. The similarities between strain ZLJ115<sup>T</sup> and other related species in *Clostridium* cluster XI were all lower than 92·0 % and the similarity between strain ZLJ115<sup>T</sup> and the type species of the genus *Clostridium* (*Clostridium butyricum*) was only 81·8 %. The great sequence divergence indicated that the novel strains could represent a new genus in this cluster.

Phenotypic features of strains ZLJ115<sup>T</sup> and L4-2 also distinguished them from related bacteria (Table 1). Firstly, the phylogenetically closest related species *C. thermoalcaliphilum*, *T. formicigenes*, *T. thalassicus* and *C. paradoxum* grow moderately thermophilically, at temperatures up to 55–60 °C, while strains ZLJ115<sup>T</sup> and L4-2 are mesophilic, growing at temperatures below 45 °C. Secondly, the DNA G+C contents of the new isolates (53.9–54.3 mol%) are far higher than those of the related species (24–32 mol%), proving that they belonged to a different genus. Furthermore, the PYG fermentation products of the new isolates included ethanol, propionic acid, isobutyric acid and isovaleric acid, while *C. paradoxum* and *C. thermoalcaliphilum* did not produce ethanol from glucose and *T. formicigenes* and *T. thalassicus* did not produce propionic acid, isobutyric acid or isovaleric acid. In addition, different profiles of fermentable sugars among the species were detected (Table 1); the new isolates fermented xylene, arabinose and ribose while the four related species did not.

Cell-wall hydrolysates of strains ZLJ115<sup>T</sup> and L4-2 were rich in meso-DAP, similar to *C. paradoxum* but different from *C. thermoalcaliphilum*, the cell wall of which is of the A4<sup>c</sup> (L-Orn–D-Asp) type. The predominant cellular fatty acids of strains ZLJ115<sup>T</sup> and L4-2 were C<sub>14:0</sub> (16·5 and 7·1 %, respectively), C<sub>16:0</sub> (14·1 and 9·4 %) and C<sub>16:1ω7c</sub> (14·0 and 17·0 %), together with 3-OH C<sub>16:0</sub> (13·6 %) and C<sub>17:1iso I</sub> (8·4 %) in strain ZLJ115<sup>T</sup> and C<sub>18:1ω9c</sub> (32·8 %) and C<sub>18:1ω7c</sub> (8·4 %) in strain L4-2. This differs from *C. paradoxum* (60 % iso-C<sub>15:0</sub> at pH 7·5) and *C. thermoalcaliphilum* (iso-C<sub>15:0</sub> anteiso-C<sub>15:0</sub> iso-C<sub>13:0</sub> C<sub>16:0</sub> iso-C<sub>17:0</sub> C<sub>14:0</sub> and C<sub>18:0</sub>). The strains had the characteristics of *Clostridium* species (anaerobic, spores formed), while they were phylogenetically more closely related to other genera than to the type species of the genus *Clostridium* (*C. butyricum*). On the basis of the distant phylogenetic relationship with related taxa, unique chemotaxonomic characteristics, divergent DNA G+C contents and distinct physiological and biochemical traits, it is evident that isolates ZLJ115<sup>T</sup> and L4-2 represent a distinct genus within the cluster XI subgroup; therefore the name *Sporacetigenium mesophilum* gen. nov., sp. nov. is proposed.

### Description of *Sporacetigenium* gen. nov.

*Sporacetigenium* [Spo.ra.ce.ti.ge’ni.um. Gr. n. spora seed; L. n. *aceta*m vinegar; Gr. v. *genao* to produce; N.L. neut. n. *Sporacetigenium* spored vinegar (acetate) producer].

Gram-positive, motile, spore-forming rods. Obligately anaerobic. No microaerophilic or aerobic growth occurs. The peptidoglycan of the cell wall contains meso-DAP. Strains are mesophilic (temperature range ≥20 to ≤42 °C) and grow in neutral to alkaline pH. Chemo-organotrophy. Oxidase and catalase are not produced. Peptone may serve as nitrogen source. A few mono- and disaccharides are fermented. Starch and ascesulin are hydrolysed, whereas gelatin is not. The major fermentation products from glucose include acetate, ethanol, hydrogen and carbon dioxide.

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![Fig. 1. Phylogenetic dendrogram showing the position of *Sporacetigenium mesophilum* ZLJ115<sup>T</sup> and related species based on 16S rRNA gene sequences. The tree was constructed using the neighbour-joining method and was rooted with *Caloranaerobacter azorensis* and *Clostridium acidurici*. Solid circles indicated that the corresponding nodes (groups) were also recovered in the maximum-likelihood and maximum-parsimony methods. Numbers at nodes represented percentage levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets. GenBank accession numbers are given in parentheses. Bar, 2 % sequence divergence.](image-url)
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Table 1. Characteristics that differentiate strains ZLJ115T and L4-2 from their phylogenetic relatives

Strains: 1, strain ZLJ115T; 2, strain L4-2; 3, C. paradoxum DSM 7308T (data from Li et al., 1993); 4, C. thermoalcaliphilum DSM 7309T (Li et al., 1994); 5, T. thalassicus DSM 15285T (Slobodkin et al., 2003); 6, T. formigenes DV1184T (Urios et al., 2004). +, Positive; −, negative; V, variable; NR, not reported; +w, weakly positive.

<table>
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<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<th>4</th>
<th>5</th>
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<td>30</td>
<td>32</td>
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<td>A2ivibp</td>
<td>Avibs</td>
<td>Avibsm</td>
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<td>27–57.5</td>
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<td>pH for growth:</td>
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<td>Range</td>
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<td>9.5–10.0</td>
<td>6.5–6.8</td>
<td>6–0</td>
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<td>−</td>
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<td>Acid produced from:</td>
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<td>−</td>
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<td>NR</td>
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<td>meso-DAP</td>
<td>meso-DAP</td>
<td>L-Orn–D-Asp</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

*a, Acetic acid; b, butyric acid; f, formic acid; ib, isobutyric acid; l, lactic acid; m, malic acid; p, propionic acid; s, succinic acid; iv, isovaleric acid; 2, ethanol. Capitals indicate major products.

Sulfate is not reduced. The G+C contents of the genomic DNA of the known strains are 53.9–54.3 mol%. Only one species, Sporacetigenium mesophilum, is described so far, and it is designated the type species.

Description of Sporacetigenium mesophilum sp. nov.

Sporacetigenium mesophilum (Gr. neut. n. meson the middle; N.L. adj. philus from Gr. adj. philos loving; N.L. neut. adj. mesophilum friendly to the middle, mesophilic, referring to its preference for moderate temperatures).

Morphology and general characters are as described for the genus. Cells are 0.9–1.0 µm x 3.6–7.3 µm in size. Optimal growth occurs at 37 °C. The pH range for growth is 5.0–9.5 with an optimum at pH 6.5–7.0. Acid is produced from a few mono- and disaccharides such as D-glucose, D-fructose, L-arabinose, D-xylose, and D-maltose. D-Galactose, D-mannose, cellobiose, sucrose, rhamnose, trehalose, melibiase, melezitose and raffinose are fermented weakly. Acid is not produced from sorbose, starch, inulin, glycogen, salicin, amygdalin, glycerol, adonitol, dulcitol, erythritol, inositol, mannitol or sorbitol. Fermentation of D-lactose and ribose is variable. The following compounds are not utilized: methanol, ethanol, 1-propanol, citrate, fumarate, malate, succinate, malonic acid, hippurate, sodium gluconate, butanedioic acid, β-hydroxybutyric acid, phenylacetic acid, cellulose and xylan. Milk is not curdled. Urease, lecinthinase and lipase are not produced. Methyl red test is positive while Voges–Proskauer test is negative. Nitrate is not reduced. H2S and NH3 are produced from PYG. The predominant cellular fatty acids are C14:0 (7.1–16.5 %), C16:0 (9.4–14.1 %) and C16:1o7c (14.0–17.0 %), together with 3-OH C16:0 (13.6 %) and C17:1 iso I (8.4 %) detected in the type strain.

The type strain is strain ZLJ115T (=DSM 16796T=AS 1.5019T), isolated from sludge of an anaerobic digester treating municipal solid waste and sewage.

Acknowledgements

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References


