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 (ZLJ115^T 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 6.0–9.5. 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 ZLJ115^T 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 phyletic 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 polyphasic evidence from this study, a novel genus and species are proposed, *Sporacetigenium mesophilum* gen. nov., sp. nov.; strain ZLJ115^T (= DSM 16796^T = AS 1.5019^T) is the type strain of *Sporacetigenium mesophilum*.

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 ZLJ115^T 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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Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Sporacetigenium mesophilum* ZLJ115^T is AY682207.

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

by microscope examination. Routine cultivation was in PYG broth in anaerobic tubes (18 × 180 mm) sealed with butyl-rubber stoppers 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 ZLJ115^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 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 ZLJ115^T 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 ZLJ115^T 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 ZLJ115^T produced acid from ribose but not lactose, while strain L4-2 was the reverse. The end products of glucose fermentation by strains ZLJ115^T and L4-2 were 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 1535^{NT}, the end products of strain ZLJ115^T 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. H₂S and NH₃ were produced from PYG liquid.

The similarity of the complete 16S rRNA gene sequences between strains ZLJ115^T 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 *Caloranaerobacter azorensis* MV1087^T and *Clostridium acidurici* ATCC 7906^T (Fig. 1). Phylogenetic analysis showed that the strains were affiliated to the phylum of low-G+C-content

Fig. 1. Phylogenetic dendrogram showing the position of *Sporacetigenium mesophilum* ZLJ115^T 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.

Gram-positive bacteria, and belonged to cluster XI of the genus *Clostridium* and related genera (Collins *et al.*, 1994). Strain ZLJ115^T 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^T and other related species in *Clostridium* cluster XI were all lower than 92.0% and the similarity between strain ZLJ115^T and the type species of the genus *Clostridium* (*Clostridium butyricum*, type strain ATCC 19398^T) 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^T 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^T 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 xylose, arabinose and ribose while the four related species did not.

Cell-wall hydrolysates of strains ZLJ115^T 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 A4β (L-Orn–D-Asp) type. The predominant cellular fatty acids of strains ZLJ115^T and L4-2 were C_{14:0} (16.5 and 7.1%, respectively), C_{16:1ω7c} (14.1 and 9.4%) and C_{16:0} (14.0 and 17.0%), together with 3-OH C_{16:0} (13.6%) and C_{17:1 iso I} (8.4%) in strain ZLJ115^T and C_{18:1ω9c} (32.8%) and C_{18:1ω7c} (8.4%) in strain L4-2. This differs from *C. paradoxum* (60% iso-C_{15:0} at pH 7.5) and *C. thermoalcaliphilum* (iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{13:0}, C_{16:0}, iso-C_{17:0}, C_{14:0} and C_{18:0}).

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^T 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 [Spor.a.ce.ti.ge'ni.um. Gr. n. *spora* seed; L. n. *acetum* vinegar; Gr. v. *gemmao* 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 aesculin are hydrolysed, whereas gelatin is not. The major fermentation products from glucose include acetate, ethanol, hydrogen and carbon dioxide.

Table 1. Characteristics that differentiate strains ZLJ115^T and L4-2 from their phylogenetic relatives

Strains: 1, strain ZLJ115^T; 2, strain L4-2; 3, *C. paradoxum* DSM 7308^T (data from Li *et al.*, 1993); 4, *C. thermoalcaliphilum* DSM 7309^T (Li *et al.*, 1994); 5, *T. thalassicus* DSM 15285^T (Slobodkin *et al.*, 2003); 6, *T. formicigenes* DV1184^T (Urios *et al.*, 2004). +, Positive; –, negative; V, variable; NR, not reported; +W, weakly positive.

Characteristic	1	2	3	4	5	6
DNA G+C content (mol%)	53.9	54.3	30	32	24	29
Products from PYG*	A2ivibp	A2ivibp	Aivsb	Aivlsm	A2	A2F
Temperature for growth (°C):						
Range	20–42	20–42	30–63	27–57.5	33–60	35–55
Optimum	37	37	56	48–51	50	45
pH for growth:						
Range	6.0–9.5	6.0–9.5	7.3–11.0	7.0–11.0	4.8–8.5	5.0–8.0
Optimum	7.5	7.5	9.8–10.3	9.5–10.0	6.5–6.8	6.0
Spore formation	+	+	+	–	+	+
Acid produced from:						
Xylose	+	+	–	–	–	–
Arabinose	+	+	–	NR	–	–
Ribose	+	–	–	–	NR	–
Galactose	+W	+W	–	–	NR	–
Rhamnose	+W	+W	–	–	NR	–
Cellobiose	+W	+W	V	+	–	–
Fructose	+	+	+	+	–	+
Lactose	–	+	V	–	NR	–
Cell-wall type	meso-DAP	meso-DAP	meso-DAP	L-Orn–D-Asp	NR	NR

*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 × 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, melibiose, 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, lecithinase 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 C_{14:0} (7.1–16.5%), C_{16:1ω7c} (9.4–14.1%) and C_{16:0} (14.0–17.0%), together with 3-OH C_{16:0} (13.6%) and C_{17:1} iso I (8.4%) detected in the type strain.

The type strain is strain ZLJ115^T (=DSM 16796^T = AS 1.5019^T), isolated from sludge of an anaerobic digester treating municipal solid waste and sewage.

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References

- Alain, K., Pignet, P., Zbinden, M. & 8 other authors (2002). *Caminiella sporogenes* gen. nov., sp. nov., a novel thermophilic spore-forming bacterium isolated from an East-Pacific Rise hydrothermal vent. *Int J Syst Evol Microbiol* **52**, 1621–1628.

- Cato, E. P., George, W. L. & Finegold, S. M. (1986). Genus *Clostridium* Prazmowski 1880, 23^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1141–1200. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Chen, S. & Dong, X. (2004). *Acetanaerobacterium elongatum* gen. nov., sp. nov., from paper mill wastewater. *Int J Syst Evol Microbiol* **54**, 2257–2262.
- Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Felsenstein, J. (1993). PHYLIP – Phylogenetic Inference Package, version 3.5c. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.
- Fendrich, C., Hippe, H. & Gottschalk, G. (1990). *Clostridium halophilum* sp. nov. and *C. litorale* sp. nov., an obligate halophilic and a marine species degrading betaine in the Stickland reaction. *Arch Microbiol* **154**, 127–132.
- Holdeman, L. V., Cato, E. P. & Moore, W. E. C. (1977). *Anaerobe Laboratory Manual*, 4th edn. Blacksburg, VA: Virginia Polytechnic Institute and State University.
- Hungate, R. E. (1969). A roll-tube method for cultivation of strict anaerobes. *Methods Microbiol* **3B**, 117–132.
- Kumar, S., Tamura, K., Jakobsen, I.-B. & Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.
- Lechevalier, M. P. & Lechevalier, H. A. (1980). The chemotaxonomy of actinomycetes. In *Actinomycete Taxonomy*, Special Publication 6, pp. 227–291. Arlington, VA: Society for Industrial Microbiology.
- Li, Y., Mandelco, L. & Wiegel, J. (1993). Isolation and characterization of a moderately thermophilic anaerobic alkaliphile, *Clostridium paradoxum* sp. nov. *Int J Syst Bacteriol* **43**, 450–460.
- Li, Y., Engle, M., Weiss, N., Mandelco, L. & Wiegel, J. (1994). *Clostridium thermoalcaliphilum* sp. nov., an anaerobic and thermo-tolerant facultative alkaliphile. *Int J Syst Bacteriol* **44**, 111–118.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.
- Miller, L. T. (1982). Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. *J Clin Microbiol* **16**, 584–586.
- Nandi, R. & Sengupta, S. (1998). Microbial production of hydrogen: an overview. *Crit Rev Microbiol* **24**, 61–84.
- Sasser, M. (1990). *Identification of bacteria by gas chromatography of cellular fatty acids*. Technical Note 101. Newark, DE: MIDI, Inc.
- Slobodkin, A. L., Tourova, T. P., Kostrikina, N. A., Chernyh, N. A., Bonch-Osmolovskaya, E. A., Jeanthon, C. & Jones, B. E. (2003). *Tepidibacter thalassicus* gen. nov., sp. nov., a novel moderately thermophilic, anaerobic, fermentative bacterium from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol* **53**, 1131–1134.
- Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Woods & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Urios, L., Cueff, V., Pignet, P. & Barbier, G. (2004). *Tepidibacter formicigenes* sp. nov., a novel spore-forming bacterium isolated from a Mid-Atlantic Ridge hydrothermal vent. *Int J Syst Evol Microbiol* **54**, 439–443.