Clostridium huakuii sp. nov., an anaerobic, acetogenic bacterium isolated from methanogenic consortia

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A Gram-staining-positive, spore-forming, obligately anaerobic, acetogenic bacterium, designated LAM1030T, was isolated from methanogenic consortia enriched from biogas slurry collected from the large-scale anaerobic digester of Modern Farming Corporation in Hebei Province, China. Cells of strain LAM1030T were motile, straight or spiral-rod-shaped. Strain LAM1030T could utilize glucose, fructose, maltose, galactose, lactose, sucrose, mannitol, pyruvate, succinic acid and tryptophan as the sole carbon source. Acetic acid, isovaleric acid and butanoic acid were the main products of glucose fermentation. Sodium sulfite was used as an electron acceptor. Growth of strain LAM1030T was completely inhibited by the addition of ampicillin, tetracycline, gentamicin or erythromycin at a concentration of 20 μg ml⁻¹. The main polar lipids of strain LAM1030T were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, 11 unknown glycolipids and two unknown phospholipids. No respiratory quinone was detected. The major fatty acids of strain LAM1030T were C₁₆ : ₀ (21.1 %), C₁₄ : ₀ (10.3 %), summed feature 9 (including C₁₆:₀ 10-methyl and/or iso-C₁₇:₁ ω₉c) (11.3 %), summed feature 3 (including C₁₆:₁ ω₇c and/or C₁₆:₁ ω₆c) (10.6 %) and iso-C₁₅ : ₀ (6.6 %). Analysis of the 16S rRNA gene sequence indicated that strain LAM1030T belonged to the genus Clostridium and was most closely related to Clostridium subterminale DSM 6970T, Clostridium thiosulfatireducens DSM 13105T and Clostridium sulfidigenes DSM 18982T, with 97.0, 96.9 and 96.8 % similarity, respectively. The G + C content of the genomic DNA of strain LAM1030T was 31.2 ± 0.3 mol%. On the basis of its phenotypic, phylogenetic and chemotaxonomic characterization, strain LAM1030T is suggested to represent a novel species of the genus Clostridium, for which the name Clostridium huakuii sp. nov. is proposed. The type strain is LAM1030T (=ACCC 00698T =JCM 19186T).

Methanogenesis is the formation of methane by microbes, which is a key biochemical reaction during the carbon cycle on Earth, and a lot of micro-organisms are involved in the complex biochemical reaction system (Mah et al., 1977; Thauer et al., 2008; Battin et al., 2009; Bastviken et al., 2011). Acetogenic bacteria contribute significantly to the turnover of organic matter in methanogenic bioreactors and landfills (Mclnerney & Bryant, 1981; Ibba & Fynn, 1991; Wiegel, 1994; Barlaz, 1997). Many investigations have been carried out into the isolation and characterization of acetogens (Drake et al., 2008). Fontaine et al. (1942) isolated a novel organism, Clostridium thermoaceticum, which was the first reported acetogen, involved in the conversion of glucose to acetate. At the time of writing, more than 100 acetogenic species have been isolated from various habitats, and the genera Acetobacterium and Clostridium harbour most of the known species. While
studying the microbial community of methanogenic consortia enriched from biogas slurry, a Clostridium-like acetogen, designated LAM1030\textsuperscript{T}, was isolated. Based on its phenotypic and genotypic characteristics, the new isolate was considered to represent a novel species of the genus Clostridium.

Strain LAM1030\textsuperscript{T} was isolated using a modified Hungate technique (Hungate, 1969). MB medium [10.0 g peptone, 10.0 g yeast extract, 5.0 g glucose, 0.5 g cysteine, 1.0 mg resazurin and 40.0 ml salt solution (DSMZ medium 104), distilled water added to 1 l final volume, pH 7.0] was used for the methanogenic consortia enrichment. MB medium was transferred into serum bottles (100 ml) under O\textsubscript{2}-free N\textsubscript{2} and autoclaved at 121 °C for 20 min. Biogas slurry (10 ml) was added into the bottles and incubated anaerobically at 30 °C for 10 days. The Hungate technique (Hungate, 1969; Bryant, 1972) was modified to isolate anaerobes from the enrichment samples using brain heart medium (BD/Difco 241830). Strains were purified by subculturing onto fresh medium at least twice before being preserved in 25% (v/v) glycerol at −80 °C for further study.

The strain grew well in brain heart infusion medium (BD/BBL 211059) at 35 °C. Morphological characteristics of cells from an exponentially growing culture were analysed by using a light microscope (Nikon 80i) and transmission electron microscope (Hitachi 7500) (Ruan, et al., 2014). Standard physiological identifications were carried out according to the methods modified by Cowan & Steel (1965) and Lánya (1987). The optimal physiological conditions for the growth of strain LAM1030\textsuperscript{T} were investigated in brain heart infusion medium. All tests were conducted independently in duplicate. The strain was incubated in the Hungate tube at temperatures of 10, 15, 20, 25, 30, 35, 40, 45 and 50 °C, pH 5–10 (in 0.5 pH unit increments) and NaCl concentrations of 0–7% (w/v, in 0.5% increments) and also 8% and 10%. The pH was adjusted to the desired value by using sterile solutions of citric acid/Na\textsubscript{2}HPO\textsubscript{4} (pH 4.0 to 5.0), MES (pH 5.5 to 6.0), PIPES (pH 6.5 to 7.0), Tricine (pH 7.5 to 8.5), CAPSO (pH 9.0 to 9.5) or CAPS (pH 10.0 to 11.5) added at a final concentration of 30 mM (Ruan, et al., 2014). The AN MicroPlate (Biolog) and API 20A kit (bioMérieux) were used to test the utilization of various substrates as sole carbon and energy sources according to the manufacturers’ instructions. API ZYM (bioMérieux) was used to test enzyme activities. To test the reduction of electron acceptors, filter-sterilized solutions of sodium nitrite (5 mM), sodium nitrate (20 mM), sodium thiosulfate (20 mM), sodium sulfite (5 mM) or sodium sulfate (20 mM) were added to the basal medium (4 g peptone, 2 g yeast extract, 0.6 g CaCl\textsubscript{2}, 0.1 g NH\textsubscript{4}Cl, 0.2 g MgCl\textsubscript{2}, 0.1 g KCl, 2 g NaCl, 2.5 g NaHCO\textsubscript{3}, 7.2 g HEPES, distilled water added to 1 l, pH 7.0). Elemental sulfur (1%) and amorphous Fe(III) oxyhydroxide (0.2%) were added to the basal medium directly before autoclaving (Ramamoorthy, et al., 2006). The main products from glucose fermentation in the basal medium were analysed and quantified as described by Steer, et al. (2001). Antibiotic sensitivity was tested under different concentrations of ampicillin, tetracycline, gentamycin and erythromycin. Strain LAM1030\textsuperscript{T} was incubated in the presence of 0, 10, 20, 50, 100, 200 and 300 µg each antibiotic ml\textsuperscript{−1}.

Genomic DNA was extracted and purified by using a TIANamp Bacter DNA kit (Tiangen Biotech) according to the manufacturer’s instruction. The 16S rRNA gene of strain LAM1030\textsuperscript{T} was amplified by PCR with the bacterial universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). Purified PCR products of approximately 1.4 kb were sequenced by Life Technologies (Shanghai, China). Sequence similarity analysis and multiple sequence alignment were conducted by using the EzTaxon-e service (Kim, et al., 2012) and CLUSTAL X (Thompson, et al., 1994) respectively. Phylogenetic trees were reconstructed via the neighbour-joining and maximum-parsimony methods with the MEGA4 program package (Tamura, et al., 2007). Evolutionary distances were calculated according to the algorithm of Kimura’s two-parameter model (Kimura, 1980) for the neighbour-joining method. The DNA G + C content was determined by the thermal denaturation method by using a Beckman DU 800 spectrophotometer (Beckman Coulter); Escherichia coli K12 was used as the reference strain.

Chemotaxonomic analyses were performed on strain LAM1030\textsuperscript{T} and Clostridium thiosulfatiucedens DSM 13105\textsuperscript{T}. Cells were harvested in the late exponential phase of growth in brain heart infusion medium at 35 °C. Polar lipids were extracted and separated on silica gel plates (10 × 10 cm, Merck 5554) and further analysed with the method described by Minnikin, et al. (1984) and Xu, et al. (2011). Sulfuric acid was used to reveal total lipids, ninhydrin for aminolipids, α-naphthol for glycolipids and Zinzadze reagent for phospholipids; the results were analysed as described by Fang, et al. (2012). Isoprenoid quinones were extracted from lyophilized cells, which had been cultivated in brain heart infusion medium for 36 h at 35 °C, using chloroform/methanol (2:1, v/v), evaporated under vacuum conditions and re-extracted in n-hexane/water (1:1, v/v). The crude solution was purified using Sep-Pak Vac silica cartridges (Waters) and subsequently analysed by HPLC (Dionex UltiMate 3000) (Zhu, et al., 2012).

Analysis of major fatty acids was performed on strain LAM1030\textsuperscript{T} and C. thiosulfaticucedens DSM 13105\textsuperscript{T}. Both strains were incubated in trypticase soy broth (TSB; BD/Difco 211825) medium at 35 °C for 36 h. Fatty acid methyl esters were obtained from fresh cells collected from the serum bottles. The identification and quantification of the fatty acid methyl esters as well as the numerical analysis of the fatty acid profiles were carried out by using the Sherlock Microbial Identification System with the standard MIS Library Generation Software (Microbial ID) according to the manufacturer’s instruction.

The cell morphology of strain LAM1030\textsuperscript{T} was similar to that of members of the genus Clostridium, endospore-forming,
motile, straight or spiral-rod-shaped with a cell size of 0.3 μm to 0.4 μm in width and 1.5 μm to 3.0 μm in length (Fig. S1, available in the online Supplementary Material). Electron micrographs revealed a typical Gram-positive cell wall structure (Fig. S1). The pH and temperature ranges for growth were pH 6.0 to 9.0 (optimum pH 7.0–8.0) and 25 to 45 °C (optimum 35 °C), respectively. The strain grew well in medium with 10 g NaCl l⁻¹ and tolerated up to 30 g NaCl l⁻¹. Strain LAM1030T was able to utilize the following substrates as carbon and energy sources: N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, amygdalin, D-arabitol, arbutin, cellobiose, dextrin, i-erythritol, D-fructose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-glucosaminic acid, α-D-glucose, α-D-glucose 1-phosphate, D-glucose 6-phosphate, glycerol, L-xylulose, myo-inositol, α-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, melezitose, melibiose, methyl α-D-galactoside, methyl β-D-galactoside, methyl β-D-glucoside, palatinose, D-raffinose, L-rhamnose, salicin, sucrose, D-trehalose, turanose, pyruvic acid, pyruvic acid methyl ester, succinic acid, inosine, uridine, thymidine-5’-monophosphate and uridine-5’-monophosphate. Compared with the reference strains, cells of strain LAM 1030T were positive for the utilization of lactose, D-glucose, D-galactose, D-mannose, cellobiose, maltose, sucrose, D-fructose, succinic acid and D-mannitol, while negative for the utilization of L-alanine, L-glutamate, L-histidine, L-methionine, L-phenylalanine and L-tryptophan. In API ZYM tests, strain LAM1030T gave positive reactions for leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and α-mannosidase, while negative reactions were obtained for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), α-chymotrypsin, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase and α-fucosidase. A comparison of the physiological characteristics between strain LAM1030T and relative reference strains is shown in Tables 1 and S1. Acetic acid (86.1 %), isovaleric acid (12.2 %) and butanoic acid (1.7 %) were the main end-products of glucose fermentation. Sodium sulfite was used as an electron acceptor. Growth of strain LAM1030T was completely inhibited by the addition of ampicillin, tetracycline, gentamicin or erythromycin at a concentration of 20 μg ml⁻¹.

Table 1. Differential phenotypic, physiological and genotypic characteristics of strain LAM1030T and its relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Biogas slurry</td>
<td>Human infections</td>
<td>Cheese factory wastewater</td>
<td>Pond sediment</td>
</tr>
<tr>
<td>Cell width (μm)</td>
<td>0.3–0.4</td>
<td>0.5–1.9</td>
<td>0.5–0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Cell length (μm)</td>
<td>1.5–3.0</td>
<td>1.6–11.0</td>
<td>2.0–4.0</td>
<td>3–6</td>
</tr>
<tr>
<td>pH for growth</td>
<td>6.0–9.0</td>
<td>5.4–9.3</td>
<td>6.0–10.0</td>
<td>5.5–9.0</td>
</tr>
<tr>
<td>Optimum</td>
<td>7.0–8.0</td>
<td>7.5</td>
<td>7.5</td>
<td>6.6</td>
</tr>
<tr>
<td>Temperature for growth</td>
<td>25–45</td>
<td>25–45</td>
<td>20–45</td>
<td>18–48</td>
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<tr>
<td>Optimum</td>
<td>35</td>
<td>37</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>NaCl concentration for growth</td>
<td>30</td>
<td>65</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>Optimum (g l⁻¹)</td>
<td>10</td>
<td>0–10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>31.2 ± 0.3*</td>
<td>28†</td>
<td>33.3 ± 0.3*</td>
<td>32.3†</td>
</tr>
<tr>
<td>Thiosulfate reduction</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sulfite reduction</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>L-Alanine</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DL-Tryptophan</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*DNA G+C content was determined by the Tm method. †DNA G+C content was determined by HPLC.
An almost complete 16S rRNA gene sequence (1394 nt) was obtained from strain LAM1030\textsuperscript{T}. The results of 16S rRNA gene sequence analysis indicated that strain LAM1030\textsuperscript{T} belonged to the genus *Clostridium* and was most closely related to *Clostridium subterminale* DSM 6970\textsuperscript{T}, *C. thiosulfatireducens* DSM 13105\textsuperscript{T} and *C. sulfidigenes* DSM 18982\textsuperscript{T}, with 97.0, 96.9 and 96.8 % similarity, respectively (Fig. 1). The topologies of the phylogenetic tree built by using the maximum-parsimony method supported the result that strain LAM1030\textsuperscript{T} formed a stable clade with these three species (Fig. S2). The G+C content of the genomic DNA of strain LAM1030\textsuperscript{T} was 31.2 ± 0.3 mol% as determined by the Tm method, which was lower than those of *C. thiosulfatireducens* DSM 13105\textsuperscript{T} and *C. sulfidigenes* DSM 18982\textsuperscript{T} (33.3 and 32.3 mol%, respectively), but higher than that of *C. subterminale* DSM 6970\textsuperscript{T} (28 mol%). The low DNA G+C content was in the range of DNA G+C content of the genus *Clostridium*.

The main polar lipids of strain LAM1030\textsuperscript{T} were diphasphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), 11 unknown glycolipids and two unknown phospholipids. Although the main polar lipids of strain LAM1030\textsuperscript{T} and *C. thiosulfatireducens* DSM 13105\textsuperscript{T} were similar, differences existed in their content and other minor components (Figs S3 and S4). No respiratory quinone was detected in strain LAM1030\textsuperscript{T}. The fatty acid profile of strain LAM1030\textsuperscript{T} was similar to that of *C. thiosulfatireducens* DSM 13105\textsuperscript{T}. The major fatty acids of strain LAM1030\textsuperscript{T} were C\textsubscript{16:0} (21.1 %), C\textsubscript{14:0} (10.3 %), summed feature 9 (including C16:0 10-methyl and/or iso-C17:1 \textit{o9c}) (11.3 %), summed feature 3 (including C16:1 \textit{m7c} and/or C16:1 \textit{w6c}) (10.6 %) and iso-C\textsubscript{15:0} (6.6 %); detailed fatty acid compositions of strain LAM1030\textsuperscript{T} and *C. thiosulfatireducens* DSM 13105\textsuperscript{T} are shown in Table S2.

On the basis of phenotypic, phylogenetic and chemotaxonomic characteristics, strain LAM1030\textsuperscript{T} is considered to represent a novel species of the genus *Clostridium*, for which the name *Clostridium huakuii* sp. nov. is proposed.

**Description of *Clostridium huakuii* sp. nov.**

*Clostridium huakuii* (huak.u’i.i. N.L. gen. n. huakuii of Hua-Kui, named in honour of the Chinese microbiologist Hua-Kui Chen for his contributions to soil microbiology in China).

Cells of strain LAM1030\textsuperscript{T} are anaerobic, motile, Gram-staining-positive, spore-forming, straight or spiral-shaped with a cell size of 0.3 to 0.4 \textmu m in length. The pH and temperature ranges for growth are pH 6.0 to 9.0 (optimum pH 7.0–8.0) and 25 to 45 °C (optimum 35 °C), respectively. Grows well in medium with 10 g NaCl l\textsuperscript{−1} and tolerates up to 30 g NaCl l\textsuperscript{−1}. Able to utilize N-acetyl-d-glucosamine, N-acetyl-β-d-mannosamine, amygdalin, D-arabitol, arbutin, cellobiose, dextrin, L-erythritol, D-fructose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-glucosaminic acid, α-D-glucose, β-D-glucose 1-phosphate, D-glucose 6-phosphate, glycerol, L-α-glycerol phosphate, myo-inositol, α-D-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, melezitose, melibiace, methyl α-D-galactoside, methyl β-D-galactoside, methyl β-D-glucoside, palatinose, L-rhamnose, salicin, sucrose, D-trehalose, turanose, pyruvic acid, pyruvic acid methyl ester, succinic acid, inosine, uridine, thymidine-5′-monophosphate and uridine-5′-monophosphate,
but unable to utilize L-alanine, L-glutamate, L-histidine, L-methionine, L-phenylalanine or L-threonine as carbon and energy sources. Positive reactions for leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-Bl-phosphohydrolase, α-glucosidase and α-mannosidase in the API ZYM system. The main products of glucose fermentation are acetic acid, isovaleric acid and butanoic acid. Sodium sulfite can be used as an electron acceptor. Growth is completely inhibited by the addition of ampicillin, tetracycline, gentamicin or erythromycin at 20 μg ml⁻¹. C₁₆:0 3C 1₄:₀ C₁₄:₀ summed feature 9 (including C₁₆:0 10-methyl and/or iso-C₁₇:1ω9c(11.3%), summed feature 3 (including C₁₆:1ω7c and/or C₁₆:1ω6c) and iso-C₁₅:₀ are the major fatty acids. The main polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, 11 unknown glycolipids and two unknown phospholipids. No respiratory quinone is detected.

The type strain is LAM1030 T (=ACCC 00698T=JCM 19186T), which is isolated from methanogenic consortia enriched from biogas slurry collected from the large-scale anaerobic digester of Modern Farming Corporation in Hebei Province, China. The genomic DNA G+C content of the type strain is 31.2 ± 0.3 mol% as determined by the Tm method.

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