Romboutsia sedimentorum sp. nov., isolated from an alkaline-saline lake sediment and emended description of the genus *Romboutsia*

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A Gram-stain-positive, spore-forming, obligately anaerobic bacterium, designated LAM201T, was isolated from sediment samples from an alkaline-saline lake located in Daqing oilfield, Daqing City, PR China. Cells of strain LAM201T were non-motile and straight or spiral rod-shapes. Strain LAM201T was able to utilize glucose, fructose, maltose, trehalose and sorbitol as the sole carbon source. Acetic acid, ethanol, iso-butanoic acid and iso-valeric acid were the main products of glucose fermentation. The major fatty acids of LAM201T were C16:0 (26.7 %) and C18:0 (11.2 %). The main polar lipids were four unknown glycolipids and five unknown phospholipids. The predominant cell-wall sugars were ribose and galactose. The cell-wall peptidoglycan of strain LAM201T contained alanine, glycine, glutamic acid and aspartic acid. Sodium sulfite was used as the electron acceptor. The G+C content of the genomic DNA was 32 ± 0.8 mol%, as determined by the Tm method. Analysis of the 16S rRNA gene sequence indicated that the isolate belonged to the genus *Romboutsia* and was most closely related to *Romboutsia lituseburensis* DSM 797T and *Romboutsia ilealis* CRIB1 with 97.3 % and 97.2 % similarities, respectively. The DNA–DNA hybridization values between strain LAM201T and the two reference strains were 37 % and 31 %, respectively. On the basis of its phenotypic, phylogenetic and chemotaxonomic characteristics, strain LAM201T is suggested to represent a novel species within the genus *Romboutsia*, for which the name *Romboutsia sedimentorum* sp. nov. is proposed. The type strain is LAM201T (=ACCC 00717T=JCM 19607T).

The genus *Clostridium* belongs to the family *Clostridiaceae*, which is one of the largest genera of the prokaryotes, and was first described by Prazmanowski (1880) and has been adopted on the Approved Lists of Bacterial Names (Skerman et al., 1980). At the time of writing, the genus *Clostridium* comprises more than 200 species with validly published names (http://www.bacterio.net/clostridium.html). Recently, there was a proposal to reclassify a subset of species within the genus *Clostridium* to four new genera within the family *Peptostreptococcaceae* based on phenotypic and genetic considerations (Gerritsen et al., 2014). This reclassification included the proposal of the novel genus *Romboutsia* to accommodate *Romboutsia lituseburensis*, previously named *Clostridium lituseburense*, and the type species of the genus, *Romboutsia ilealis*. While studying the anaerobic alkane-degrading...
bacterial diversity of sediment samples from an alkaline-saline lake located in Daqing oilfield, Daqing City, PR China, a *Romboutsia*-like strain, designated LAM201T, was isolated. On the basis of its phenotypic, phylogenetic and chemotaxonomic characteristics, the new isolate is considered to represent a novel species of the genus *Romboutsia*.

Liquid culture medium (consisting of 5.0 g peptone, 1.0 g yeast extract, 0.5 g l-cysteine, 0.4 g K₂HPO₄, 3H₂O, 0.5 g NaCl, 0.1 g MgCl₂, 1 mg resazurin and 40 ml salt solution as DSMZ medium 104, in water, added to a volume of 1 l, pH 7.0) was used as enrichment medium. Serum bottles (100 ml) containing 20 ml of liquid enrichment culture medium were autoclaved for 30 min at 121 °C. Sediment samples (10 %, w/v) were inoculated into serum bottles under an O₂-free N₂ atmosphere and incubated at 30 °C for 10 days. The Hungate technique (Hungate, 1969; Bryant, 1972) was adopted to isolate single strains from the enrichment samples using brain heart infusion (BHI) agar (BD/Difco 241830). Strain LAM201T was purified at least twice before being preserved in 25 % (v/v) glycerol at −80 °C for further studies.

The strain grew well in BHI broth (BD/BBL 211059) at 35 °C; the OD₆₀₀ increased to 0.5 after 24 h of growth. The morphological characteristics of cells in the exponential growth phase were examined after 48 h of growth at 35 °C using a light microscope (Nikon 80i) and transmission electron microscope (Hitachi 7500) (Ruan, et al., 2014). The Gram-staining reaction was conducted according to the method described by Smibert & Krieg (1994). Spore-forming ability was observed after heat treatment at 80 °C for 10 min by light microscopy (Nikon 80i). The motility of strain LAM201T was examined from the detection of turbidity through Hungate anaerobic tubes containing semi-solid tryptic soy broth (TSB) (BD/Difco 211825) after 48 h of growth at 35 °C. The optimal growth conditions for strain LAM201T were determined using BHI broth. All tests were conducted independently and in duplicate. The strain was incubated in Hungate tubes at: temperatures of 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C and 50 °C; pH values of 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 and 10, and NaCl concentrations of 0 %, 1 %, 2 %, 2.5 %, 3 %, 3.5 %, 4 %, 4.5 %, 5 %, 5.5 %, 6 %, 6.5 %, 7 %, 8 % and 10 % (w/v). The pH was adjusted to the desired value by using sterile solutions of citric acid/Na₂HPO₄ (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 to a final concentration of 30 mM (Ruan, et al., 2014). Standard physiological identifications and hydrolysis of casein were carried out according to methods modified by Cowan & Steel (1965) and Lánya (1987). The ability to grow under aerobic, trace-O₂ and anaerobic conditions at 35 °C for up to 7 days was examined. The basal medium used for testing physiological characteristics consisted of the following components in water (l⁻¹): 2 g peptone, 1 g yeast extract, 0.6 g CaCl₂, 0.1 g NH₄Cl, 0.2 g MgCl₂, 0.1 g KCl, 2 g NaCl, 2.5 g NaHCO₃, 0.5 g Na₂S, 7.2 g HEPES, 0.5 g l-cysteine, 1 ml trace element solution as DSMZ medium 141 and 1 mg resazurin. The medium was adjusted to pH 7.0 and sterilized by autoclaving at 121 °C for 30 min. By adding 20 mM of the respective sugar to the basal medium growth on the following carbon sources was examined: glucose, fructose, xylose, maltose, arabinose, lactose, rhamnose, sucrose, mannose, ribose, galactose, cellobiose, trehalose, sorbitol and mannitol. An increase in the OD₆₀₀ of medium containing an additional carbon source, as compared to the basal medium lacking an additional carbon source (control medium), was considered to represent positive growth. Tests for the utilization of various energy sources were performed using the API 20A system (bioMérieux) according to the manufacturer’s instructions. Enzyme activity was examined using the API ZYM system (bioMérieux). To analyse the reduction of electron acceptors, sodium nitrite (5 mM), sodium nitrate (20 mM), sodium thiosulfate (20 mM), sodium sulfate (5 mM) or sodium sulfate (20 mM) were added from filter-sterilized solutions to basal medium (lacking the reductant l-cysteine, Na₂S and casamino acids), respectively. Elemental sulfur (1 %, w/v) and amorphous Fe (III) oxhydroxide (0.2 %, w/v) were added to the basal medium immediately before autoclaving (Rammamoorthy *et al.*, 2006). Ethanol, acetic acid, iso-butanolic acid, butyanoic acid, iso-valeric acid and iso-butyric acid from glucose fermentation in the basal medium were analysed and quantified as described by Steer *et al.* (2001).

The genomic DNA of strain LAM201T was extracted and purified using a TIANamp Bacter DNA kit (Tiangen Biotech), according to the manufacturer’s instructions. The 16S rRNA gene was amplified by PCR with the bacterial universal primers, 27F (S’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (S’-GTTACCTTGTGACTAC-3’). Purified PCR products of approximately 1.4 kb were sequenced by the Majorbio Company (Beijing, China). The EzTaxon-e service (Kim *et al.*, 2012) and CLUSTAL W (Thompson *et al.*, 1994) were used to determine sequence similarities and for multiple sequence alignments. Phylogenetic trees were reconstructed using the neighbour-joining and maximum-parsimony methods with the MEGA4 program package (Tamura *et al.*, 2007). For the neighbour-joining method evolutionary distances were calculated according to the algorithm of Kimura’s two-parameter model (Kimura, 1980). The DNA G+C content was determined by the thermal denaturation method using a Beckman DU 800 spectrophotometer (Beckman Coulter). *Escherichia coli* K12 was used as a reference strain. DNA–DNA reassociation analysis was performed as described by Chang *et al.* (2008).

Chemotaxonomic analyses were executed on strain LAM201T and *R. litusubeurensis* DSM 797T. The strains were grown in TSB medium (BD/Difco 211825). Cells were harvested in the late exponential phase after growth at 35 °C for 48 h. The 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). Molybdatosphoric acid was used to reveal total polar lipids. Aminolipids were determined using ninhydrin reagent and phospholipids were identified with Zinadze...
reagent. The results were analysed as described by Fang et al. (2012). Analysis of major fatty acids was performed on strain LAM201<sup>T</sup> and <i>R. lituseburensis</i> DSM 797<sup>T</sup>. The fatty acids were extracted from fresh cells of the two strains, which had been incubated in TSB medium for 48 h at 35 °C. Identification and quantification of the major acids were performed using the Sherlock Microbial Identification System with the standard MIS Library Generation Software (Version 6.0 and Date 4, Microbial ID). The isoprenoid quinones of strain LAM201<sup>T</sup> were extracted with the method described by Minnikin et al. (1984) and analysed by HPLC as described by Tindall (1990). The cell-wall peptidoglycan was obtained and purified after 48 h of incubation in TSB medium. Quantitative analysis of the peptidoglycan amino acids was performed by GC (MacKenzie, 1987) and the whole-cell sugars in the hydrolysate were analysed by TLC, according to Staneck & Roberts (1974).

Colonies of strain LAM201<sup>T</sup> grown on BHI agar in Hungate tubes were round, convex, white and 0.5 mm to 1 mm in diameter after anaerobic incubation for 36 h at 35 °C. Cells were non-motile and spore-forming and 1.2 μm–2 μm in width × 2.3 μm–10 μm in length (Figs 1 and S1, available in the online Supplementary Material). Strain LAM201<sup>T</sup> was obligately anaerobic and formed free round spores after heat treatment at 80 °C for 10 min (Fig. S2). The pH and temperature ranges for growth were pH 6.0–9.0 (optimum: pH 7.0) and 10 °C–40 °C (optimum: 30 °C), respectively. The strain grew in medium without NaCl and tolerated up to 5 % (w/v) NaCl. Strain LAM201<sup>T</sup> was able to utilize glucose, fructose, maltose, trehalose and sorbitol as the sole carbon source. Xylose, arabinose, lactose, rhamnose, sucrose, mannose, ribose, galactose, cellobose and mannotol could not be utilized by strain LAM201<sup>T</sup>. In the API ZYM system, strain LAM201<sup>T</sup> gave positive reactions for alkaline phosphatase, cystine arylamidase and α-chymotrypsin; weakly positive reactions for α-galactosidase, α-glucosidase and α-mannosidase, and negative reactions for esterase (C4),

![Fig. 1. Transmission electron micrograph of cells of strain LAM201<sup>T</sup>. Bar, 0.5 μm.](image)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3†</th>
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<tr>
<td>Cell width (μm)</td>
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<td>1.4–1.7*</td>
<td>1.0–2.0</td>
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<tr>
<td>Cell length (μm)</td>
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<td>3.1–6.5*</td>
<td>1.0–5.3</td>
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<tr>
<td>Motility</td>
<td>–</td>
<td>+*</td>
<td>–</td>
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<tr>
<td>Gelatin hydrolysis</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Utilization of:</td>
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<tr>
<td>Arabinose</td>
<td>–</td>
<td>–†</td>
<td>w</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>+†</td>
<td>–</td>
</tr>
<tr>
<td>Galactose</td>
<td>–</td>
<td>–†</td>
<td>w</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+†</td>
<td>–</td>
</tr>
<tr>
<td>Ribose</td>
<td>–</td>
<td>+†</td>
<td>–</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>+</td>
<td>–†</td>
<td>–</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
<td>+†</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>–†</td>
<td>–</td>
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<td>Polar lipids</td>
<td>4 GL, 5 PL</td>
<td>8GL, 5PL*</td>
<td>6 GL, 4 PL, L</td>
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<td>DNA G+C content (mol%)</td>
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<td>30†</td>
<td>28.1§</td>
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<td>97.3 %</td>
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<td>DNA–DNA reassociation</td>
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<td>37 %</td>
<td>31 %</td>
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</table>

*Data from this study.
†Data from Gerritsen et al. (2014).
§DNA G+C content was determined by the T<sub>m</sub> method.
§DNA G+C content was determined by HPLC.

Table 1. Differential phenotypic, physiological and genotypic characteristics of strain LAM201<sup>T</sup> and related strains

Strains: 1, LAM201<sup>T</sup>; 2, <i>R. lituseburensis</i> DSM 797<sup>T</sup>; 3, <i>R. ilealis</i> CRIB<sup>T</sup>. Data for strain LAM201<sup>T</sup> were all obtained from this study. +, Positive; –, negative; W, moderately positive; ND, not determined; GL, glycolipid; PL, phospholipid; L, lipid.
esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase and x-fucosidase. In the API 20A system, positive reactions to maltose, trehalose, sorbitol and glucose were observed. Reactions to lactose, salicin, xylose, arabinose, glycerol, cellobiose, mannose, melezitose, raffinose, rhamnose, urea, tryptophan, gelatin and ascinul were negative. Strain LAM201T could not hydrolyse casein, while R. lituseburensis DSM 797T could. A comparison between the physiological characteristics of strain LAM201T and its related reference strains is shown in Table 1. Sodium sulfite was used as an electron acceptor. The main end products of glucose fermentation in the basal medium were acetic acid (65.5%), ethanol (16.8%), iso-butanoic acid (6%) and iso-valeric acid (4%). The 16S rRNA gene sequence (1339 nt) was obtained from strain LAM201T. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain LAM201T is a member of the genus Romboutsia and is most closely related to R. lituseburensis DSM 797T and R. ilealis CRIBT with sequence similarities of 97.3% and 97.2%, respectively (Fig. 2). The DNA–DNA hybridization values between strain LAM201T and the two reference strains were 37% and 31%, respectively. The topologies of the phylogenetic trees reconstructed by using the maximum-parsimony method support strain LAM201T forming a stable clade with related species (Fig. S3). The genomic DNA G+C content of strain LAM201T was 32 ± 0.8 mol%, as determined by the Tm method; this value is higher than those of R. lituseburensis DSM 797T and R. ilealis CRIBT.

The major fatty acids of strain LAM201T were C16:0 (26.7%) and C18:0 (11.2%). The C16:0 content of strain LAM201T was lower than those of R. lituseburensis DSM 797T (29.2%) and R. ilealis CRIBT (31.3%), while the C18:0 content was higher than those of R. lituseburensis DSM 797T (8.5%) and R. ilealis CRIBT (6.6%). The detailed fatty acid compositions of strain LAM201T, R. lituseburensis DSM 797T and R. ilealis CRIBT are shown in Table 2. The main polar lipids of strain LAM201T were four unknown glycolipids and five unknown phospholipids (Figs S4, S5 and S6). The main polar lipids of strain LAM201T and R. lituseburensis DSM 797T were similar, while differences existed in their relative contents and other minor compositions (Figs. S4 and S7). Respiratory quinones were not found in strain LAM201T, while its predominant cell-wall sugars were ribose and galactose. The peptidoglycan of strain LAM201T contained alanine, glycine, glutamic acid and aspartic acid (Fig. S8).

On the basis of this phenotypic, phylogenetic and chemotaxonomic characterization, strain LAM201T is considered to represent a novel species within the genus Romboutsia, for which the name Romboutsia sedimentorum sp. nov. is proposed.

![Fig. 2. Neighbour-joining phylogenetic tree based on a comparison of the 16S rRNA gene sequences of strain LAM201T and its closest relatives. GenBank accession numbers are given in parentheses. Bootstrap values (percentages) based on 1000 replications are shown at nodes. Bar, 0.01 nucleotide substitutions per nucleotide position.](image-url)
The type strain is LAM201\(^T\) (=ACCCC 00717\(^T\)=JCM 19607\(^T\)), which was isolated from sediment samples of an alkaline-saline lake located in the Daqing oilfield, Daqing City, PR China. The genomic DNA G+C content of the type strain is 32±0.8 mol%, as determined by the \(T_m\) method.

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## References


