**Lactobacillus curieae** sp. nov., isolated from stinky tofu brine

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A lactic acid bacterium, strain CCTCC M 2011381T, isolated from the brine of the traditional Chinese snack, stinky tofu, was studied to determine its taxonomic position. It was a Gram-stain-positive, non-motile, facultatively anaerobic rod-shaped bacterium that did not exhibit catalase activity. The DNA G+C content of the strain was 44.1 % and its peptidoglycan was characterized by the presence of meso-diaminopimelic acid. Levels of 16S rRNA gene sequence similarity between strain CCTCC M 2011381T and the most closely related species *Lactobacillus senioris* JCM 17472T, *Lactobacillus parafarraginis* JCM 14109T and *Lactobacillus diolivorans* JCM 12183T were 96.5, 96.4 and 96.4 %, respectively. Combined with data from high-resolution genomic markers *recA*, *rpoA* and *pheS*, strain CCTCC M 2011381T was classified as representing a novel species. The strain could also be distinguished from other related species of the genus *Lactobacillus* by its physiological and biochemical characteristics. Based on the phylogenetic, physiological and biochemical data, it is proposed that the new isolate can be classified as representing a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus curieae* sp. nov. (type strain CCTCC M 2011381T=S1L19T=JCM 18524T) is proposed.

Stinky tofu is one of the most popular tofu-derivative snacks in China and its manufacturing processes varies from region to region. Chao et al. (2008a) studied the diversity of lactic acid bacteria (LAB) in stinky tofu brine collected from Taiwan and isolated two novel species of the genus *Lactobacillus* (Chao et al., 2008b, 2010).

During the course of a study of the community of microbes in stinky tofu brine collected from a stinky tofu factory located in east of China, 20 strains were classified as belonging to LAB. The sample, which had been preserved for 5 years, was collected in 2009 from a local food company in Shanghai, China. 16S rRNA gene sequences of all the isolates were analysed and one strain, CCTCC M 2011381T, showed low levels of sequence similarity to all other established species. Therefore, additional tests were performed to determine whether this strain represents a novel species of the genus *Lactobacillus*.

This study presents the morphological, biochemical and molecular characterization of strain CCTCC M 2011381T, *Lactobacillus farraginis* JCM 14109T and *Lactobacillus parafarraginis* JCM 14109T were obtained from the culture collection of the Japan Collection of Microorganisms (JCM; Saitama, Japan) and used as templates of the *recA* genes. All of the strains used for further experiments were cultivated and maintained in MRS broth (de Man et al., 1960) at 30 °C for 24 or 48 h.

Chromosomal DNA used as templates for 16S rRNA, *recA*, *rpoA* and *pheS* gene sequence amplification was prepared from the isolates using a bacteria DNA extraction kit according to the manufacturer’s instructions (Sangon Biotech). The almost-complete 16S rRNA gene was amplified with primer combinations 27F and 1492R according to Marchesi et al. (1998), and the recombinase A protein (*recA*) partial gene primers described by Watanabe et al. (2009). The sequences of primers for the RNA polymerase alpha subunit (*rpoA*) and phenylalanyl-tRNA synthase alpha subunit (*pheS*) genes were based on Naser et al. (2005, 2007) and listed in Table S1, available in IJSEM Online. The PCR mix included 37.5 µl sterile water, 2.5 µl dNTP (10 mM), 5 µl 10 × PCR

†These authors contributed equally to this work.

**Abbreviations**: LAB, lactic acid bacteria; *m*-DAP, *meso*-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *recA*, *rpoA* and *pheS* gene sequences of strain CCTCC M 2011381T are JQ086550, JQ307417, JQ307418 and JQ307419, respectively. Those for the *recA* gene sequences of *Lactobacillus farraginis* JCM 14109T and *Lactobacillus parafarraginis* JCM 14109T are JQ307415 and JQ307416, respectively.

Two supplementary figures and two supplementary tables are available with the online version of this paper.
buffer, 1 µl forward primer (20 mM), 1 µl reverse primer (20 mM), 2 µl template DNA and 1 µl Taq polymerase. The PCRs were performed using a Mastercyler Pro.S PCR (Eppendorf). The programme consisted of (1) 5 min at 95 °C, (2) 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 2 min (for 16S rRNA) or 30 s (for recA) at 72 °C, or (2) 28 cycles of 30 s at 95 °C, 30 s at 50 °C, decreasing by 0.5 °C each cycle, and 45 s at 72 °C, and ten cycles of 30 s at 95 °C, 30 s at 36 °C and 45 s at 72 °C (for rpoA and pheS), and (3) 8 min at 72 °C. The closest known relatives of the novel strain were determined using FASTA and the EzTaxon server (Chun et al., 2007) and sequences of closely related species were retrieved from the DDBJ/GenBank/EMBL databases. Multiple alignments of the sequences were carried out with the CLUSTAL X program (version 1.83). Approximately 1470 bp of the 16S rRNA gene sequences (approx. 380 bp for the recA gene, 550 bp for the rpoA gene and 290 bp for the pheS gene) of this strain and related strains was used for the reconstruction of phylogenetic trees with the neighbour-joining method (Saitou & Nei, 1987). The statistical reliability of the trees was evaluated by bootstrap analysis of 1000 replicates (Felsenstein, 1985) and the tree topologies were also confirmed with the maximum-parsimony (Fitch, 1972) and maximum-likelihood (Cavalli-Sforza & Edwards, 1967) methods (data not shown), by using MEGA version 4.0.2. software (Tamura et al., 2007).

In the phylogenetic tree reconstructed on the basis of 16S rRNA gene sequences (Fig. 1), strain CCTCC M 2011381T should be placed in the Lactobacillus buchneri group. Pairwise nucleotide sequence similarity values, calculated cautiously with the web-based tool EzTaxon (Tindall et al., 2010), between strain CCTCC M 2011381T and its nearest neighbours Lactobacillus senioris JCM 17472T, L. paraffaraginis JCM 14109T and Lactobacillus doliavorans JCM 12183T were 96.5, 96.4 and 96.4 %, respectively, which were just below the cut-off line 97 % (the level generally considered as the threshold for defining bacterial species; Stackebrandt & Goebel, 1994). There is extensive evidence that two strains sharing less than 97 % 16S rRNA gene sequence similarity are not members of the same species (Tindall et al., 2010). For lactobacilli, moreover, several studies have identified novel species that exhibit more than 99 % similarity with regard to their 16S rRNA gene sequences (Bringel et al., 2001; Mañes-Lázaro et al., 2008; Watanabe et al., 2009; Chao et al., 2010). Consequently, strain CCTCC M 2011381T can be classified as representing a novel species.

In the search for a more accurate identification system, a higher degree of resolution was obtained when using recA, rpoA and pheS genes to differentiate members of the genus Lactobacillus (Eisen et al., 1995; Naser et al., 2007). Torriani et al. (2001) indicated that the clear distinction obtained with short gene sequences validated the use of the recA gene as a phylogenetic–taxonomic marker for closely related species and opened new possibilities for rapid and reliable identification of LAB of importance for food. In recent years, recA gene sequence analysis has been confirmed as an effective method for inferring relationships among very closely related species (Felis et al., 2001; Bringel et al., 2005; Watanabe et al., 2009). We amplified and sequenced a 380 bp recA DNA fragment from strain CCTCC M 2011381T, L. farraginis JCM 14108T and L. paraffaraginis JCM 14109T, with other recA genes retrieved from the DDBJ/GenBank/EMBL databases, to search for their evolutionary relationships. Sequence alignments did not introduce any gaps, as expected from closely related species. Strain CCTCC M 2011381T shared 78.5–81.6 % recA gene sequence similarity with its nearest neighbours (Table 1). Interspecies gaps within the genus Lactobacillus based on recA gene sequences normally exceed 10–15 % (Torriani et al., 2001), which further suggested that strain CCTCC M 2011381T should be classified as representing a novel species.
novel species. The neighbour-joining tree (similar topologies were obtained with the maximum-parsimony and maximum-likelihood methods, data not shown) of recA gene sequences showed that strain CCTCC M 2011381T formed a distinct subcluster in the L. buchneri cluster (Fig. 2).

The pheS and rpoA genes were also selected as biomarkers to distinguish strain CCTCC M 2011381T from its closest relatives. Naser et al. (2007) examined the differentiating power of the pheS and rpoA partial gene sequences on the basis of 201 well-characterized strains representing 98 species and 17 subspecies of the genus Lactobacillus from different origins. In the L. buchneri group, their high resolution has been verified in several different cases (Vancanneyt et al., 2005, 2006; Oki et al., 2012). With primers rpoA-21-F and rpoA-23-R, we amplified and sequenced a 821 bp rpoA gene from strain CCTCC M 2011381T. Sequence similarity values between strain CCTCC M 2011381T and its nearest neighbours ranged from 81.7 to 84.9 % (Table 1), with L. parafarraginis JCM 14109T sharing highest similarity. With primers pheS-21-F at position 557 and pheS-23-R at position 968, a 431 bp (including primers) pheS gene was amplified and sequenced, which was in accordance with the length of the referred strains. The similarities among the pheS gene sequences of the novel strain and the closest neighbouring species ranged from 76.5 to 81.6 % (Table 1). Interspecies gaps within the genus Lactobacillus based on rpoA and pheS gene sequences normally exceed 5 and 10 %, respectively (Naser et al., 2007), and this provides further data to classify strain CCTCC M 2011381T as representing a novel species. On the basis of neighbour-joining analysis of rpoA and pheS gene sequences, the novel strain did not belong to any recognized species (Figs S1 and S2). Similar topologies were obtained using the minimum-evolution and maximum-parsimony methods (data not shown).

For determining DNA G+C content, chromosomal DNA was extracted according to the method of Marmur (1961). The nucleosides were separated by HPLC with an Aglient SB-C18 column at 30 °C and 0.02 M NaH2PO4 (pH 5.6)/2.0 % (v/v) acetonitrile as solvent. Strain CCTCC M 2011381T had a DNA G+C content of 44.1 mol%, a value that is within the range (32–53 mol%) established for the genus Lactobacillus.

Morphological, physiological and biochemical characteristics of the isolate were determined according to standard techniques. MRS broth was used as the basal medium. Morphological and colony morphology were investigated after aerobic growth on MRS agar (pH 6.0) for 48 h at 30 °C unless otherwise stated. Cell shape, cell size and Gram-staining were determined by using cultures grown on MRS agar for 48 h at 30 °C. Motility was tested in MRS soft agar (0.15 %, w/w), and flagella on cells from 12 h cultures in MRS broth were observed under a transmission

Table 1. Sequence similarity values (%) between the 16S rRNA, recA, rpoA and pheS gene sequences of strain CCTCC M 2011381T and the type strains of related species.

<table>
<thead>
<tr>
<th>Type strain</th>
<th>16S rRNA</th>
<th>recA</th>
<th>rpoA</th>
<th>pheS</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. senioris JCM 17472T</td>
<td>96.5</td>
<td>ND</td>
<td>80.3</td>
<td>78.5</td>
</tr>
<tr>
<td>L. diolivorans JCM 12183T</td>
<td>96.5</td>
<td>79.3</td>
<td>84.2</td>
<td>81.6</td>
</tr>
<tr>
<td>L. parafarraginis JCM 14109T</td>
<td>96.4</td>
<td>81.64</td>
<td>85.0</td>
<td>77.9</td>
</tr>
<tr>
<td>L. hilgardii ATCC 8290T</td>
<td>96.3</td>
<td>80.73</td>
<td>81.7</td>
<td>79.4</td>
</tr>
<tr>
<td>L. rapi DSM 19907T</td>
<td>96.3</td>
<td>80.0</td>
<td>83.8</td>
<td>81.3</td>
</tr>
</tbody>
</table>

ND, No data available.

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electron microscope (Hitachi H-600). The peptidoglycan types for strain CCTCC M 2011381T were determined as described by Schleifer (1985) and Schleifer & Kandler (1972), with the modification that TLC on cellulose was used. Cellular fatty acid methyl esters were obtained from cells grown in MRS broth at 37 °C in the late-exponential growth phase. Saponification, methylation and extraction of fatty acids were performed by using the procedures described by Sasser (1990). Analysis of the fatty acid methyl esters was carried out using an Agilent 6890-5975 GC-MS system. Carbohydrate fermentation tests were conducted by using the API 50 CHL system (bioMérieux) according to the manufacturer's instructions. The isomers of lactic acid formed from glucose were determined with a DL-lactic acid test kit (R-Biopharm AG). The characteristics of the isolate were compared with those of closely related species (Table 1).

Colonies were 1–2 mm in diameter, white and circular with rough edges when grown on MRS agar at 30 °C for 48 h. Cells were rod-shaped, 0.6–0.8 μm × 1.2–3 μm and occurred singly, in pairs or in short chains comprising three to six cells. Cells were Gram-positive, non-motile, non-spore-forming and facultatively anaerobic. Catalase was not produced. The peptidoglycan of the strain was characterized by the presence of meso-diaminopimelic acid (m-DAP), consistent with Group A established on the mode of cross-linkage by Schleifer and Kandler (1972). On the basis of the results presented, it is concluded that strain CCTCC M 2011381T, a Gram-positive, facultatively anaerobic, rod-shaped, catalase-negative bacterium containing m-DAP, belongs to the genus Lactobacillus according to Holt et al. (1994).

The novel isolate could also be differentiated from phylogenetically related species by several physiological and biochemical features. The major cellular fatty acids of the strain were C18:1ω7c/C18:2ω9c (46.36 %) and C16:0 (43.77 %) and both L- (40 %) and D-lactate (60 %) were produced as the end products from glucose. The strain could be differentiated from its genetically closest relatives by its ability to grow at 45 °C, at which its growth rate is slightly lower than at 37 °C, and with 5 % NaCl, even with weak growth with 8 % NaCl. Furthermore, with the API CHL system test, there are considerable differences, such as D-galactose, maltose and melibiose, between the novel isolate and L. senioris. Table 2 also shows other differences in carbohydrate fermentation between the novel strain and related strains.

In conclusion, the data presented above show that strain CCTCC M 2011381T belongs to a novel species in the L. buchneri clade. The name Lactobacillus curiae sp. nov. is proposed for this novel species. The type strain is CCTCC M 2011381T (=SIL19T=JCM 18524T).

**Description of Lactobacillus curiae sp. nov.**

*Lactobacillus curiae* (cu.rie’ae. N.L. fem. gen. n. curiae of Curie, named after Marie Curie, a role model for female scientists).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>Growth at:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 °C</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>45 °C</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pH 4.0</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pH 8.5</td>
<td>w</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Salt tolerance</td>
<td>5%</td>
<td>w</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Galactose</td>
<td>+</td>
<td>–</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>w</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Methyl α-D-glucopyranoside</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>w</td>
<td>–</td>
<td>–</td>
<td>w</td>
</tr>
<tr>
<td>Melibiose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
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<tr>
<td>Melexitose</td>
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<td>–</td>
<td>–</td>
<td>–</td>
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<td>Raffinose</td>
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<td>–</td>
<td>w</td>
<td>+</td>
</tr>
<tr>
<td>Turanose</td>
<td>+</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>Methyl β-D-xylpyranoside</td>
<td>–</td>
<td>+</td>
<td>–</td>
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</tr>
<tr>
<td>5-Ketogluconate</td>
<td>–</td>
<td>w</td>
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<td>w</td>
</tr>
</tbody>
</table>

Cells are rod-shaped, 0.6–0.8 μm × 1.2–3 μm and occur singly, in pairs or in short chains comprising three to six cells. Cells are Gram-stain-positive, non-motile, non-spore-forming and facultatively anaerobic. Catalase is not produced. After anaerobic growth at 30 °C for 48 h, colonies on MRS agar are approximately 1–2 mm in diameter, white, with a rough surface, and circular with rough edges. In MRS broth, growth occurs at both 15 and 45 °C. Growth occurs at pH 4.0 but not at pH 8.5. Growth occurs in the presence of 8 % NaCl but not in the presence of 10 % NaCl. Gas is produced from glucose. The major cellular fatty acids are C18:1ω7c/C18:1ω9c and C16:0 and both L- and D-lactate are produced as the end products from glucose. Ammonia is not produced from arginine and nitrate is not reduced. Acid is produced from L-arabinose, D-ribose, D-xylose, D-galactose, D-fructose, maltose, melibiose, sucrose, melezitose, raffinose, turanose, methyl α-D-glucopyranoside, D-mannose (weakly), lactose (weakly), gluconate (weakly) and aesculin (weakly), but not from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-D-xylpyranoside, L-sorbose, L-rhamnose,
dulcitol, inositol, D-mannitol, D-sorbitol, methyl z-D-mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, trehalose, inulin, starch, glycogen, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 5-ketogluconate or 2-ketogluconate. Dextran is not produced from sucrose. The peptidoglycan is characterized by the presence of m-DAP.

The type strain, CCTCC M 2011381T (=S1L9T=JCM 18524T), was isolated from stinky tofu brine in Shanghai, China, in 2009 and placed in the L. buchneri species group of lactobacilli. The DNA G+C content of the type strain is 44.1 mol%.

References


