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

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Minimum-evolution phylogenetic trees based on 16S rRNA, *rpoA* and *pheS* gene sequences demonstrated that the novel strains were members of the genus *Lactobacillus*. On the basis of 16S rRNA gene sequence similarity, the type strains of *Lactobacillus collinoides* (98.6%) and *Lactobacillus paracollinoides* (98.6%) and *Lactobacillus similis* (99.6%) were the closest neighbours. However, DNA–DNA reassociation values with these strains were less than 10%. The phenotypic and genotypic features demonstrated that these isolates represent a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus odoratitofui* sp. nov. is proposed. The type strain is YIT 11304T (≡JCM 15043T =BCRC 17810T =DSM 19909T).

Three Gram-positive-staining strains isolated from fermented stinky tofu brine were rod-shaped, non-motile, asporogenous, facultatively anaerobic, heterofermentative and did not exhibit catalase activity. Comparative analyses of 16S rRNA, *rpoA* and *pheS* gene sequences demonstrated that the novel strains were members of the genus *Lactobacillus*. On the basis of 16S rRNA gene sequence similarity, the type strains of *Lactobacillus collinoides* (98.6%) and *Lactobacillus paracollinoides* (98.6%) were the closest neighbours. However, DNA–DNA reassociation values with these strains were less than 10%. The phenotypic and genotypic features demonstrated that these isolates represent a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus odoratitofui* sp. nov. is proposed. The type strain is YIT 11304T (≡JCM 15043T =BCRC 17810T =DSM 19909T).

Stinky tofu is made by immersing tofu in fermented stinky brine, thus permeating the tofu with the odour of the brine. The fermented brine used for stinky tofu production is from the liquid part of decomposed raw materials, such as various vegetables or animal proteins (shrimp or fish). Samples of fermented stinky brines were obtained in May 2005 from a factory in Taipei County, Taiwan, and analysed as described previously (Chao et al., 2008b). During a study on the biodiversity of lactic acid bacteria in stinky tofu brines, three of the *Lactobacillus* strains [S-4-5 (=YIT 11304T), S-4-6 and S-4-7] isolated could not be clearly placed within any recognized species of the genus by means of 16S rRNA gene sequence similarity. Levels of DNA–DNA relatedness also separated the strains from existing related species.

This study presents the morphological, biochemical and molecular characterization of strain YIT 11304T, *L. collinoides* YIT 0263T, *L. paracollinoides* YIT 10360T and *L. similis* YIT 12117T were obtained from the culture collection of Yakult Central Institute (YIT; Tokyo, Japan) and were used as reference strains. The strains used for further experiments were cultivated and maintained in MRS broth (BD, Difco; pH 6.8) (de Man et al., 1960) at 30 °C for 1 or 2 days, unless indicated otherwise. Chromosomal DNA used as a template for 16S rRNA, *rpoA* and *pheS* gene sequence amplification was prepared from the isolates according to the method of Watanabe et al. (2008). The conditions for PCR amplification of the partial 16S rRNA gene and subsequent DNA sequencing have been described previously (Chao et al., 2008a). The *rpoA* and *pheS* gene sequences for strains YIT 11304T and *L. similis* YIT 12117T were amplified by PCR with primers rpoA-F2 (5′-GTGGATGGCCGTGTCGTTGARGA-3′) and rpoA-R2 (5′-TTGATTGACRTCCHGTCGTGTCGTTGARGA-3′) (Chao et al., 2009), and pheS-21-F (5′-CYCCNCGGCHGAYGATGCG-3′) and pheS-23-R (5′-GGGCTGACCATVCCNCGHCCCC-3′) (Naser et al., 2005), respectively. The PCR mixture (25 μl) contained 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 200 μM of each dNTP, 1 mM MgCl2, 1 μg BSA, 0.5 U Taq DNA polymerase (Takara Bio), 0.1 μM each primer and 10 ng template DNA. The amplification program consisted of 1 cycle of 94 °C for 2 min; 30 cycles of 94 °C for 20s, 55 °C for 30s (for 16S rRNA gene) or 48 °C for 20s (for *rpoA* and *pheS* genes), 72 °C for 20s; and finally 1 cycle of 72 °C for 3 min. The PCR-amplified 16S rRNA, *rpoA* and *pheS* genes from these strains were purified using a Montage PCR Filter unit (Millipore) according to the manufacturer’s instructions. Cycle-sequencing reactions
were performed using an ABI PRISM BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). Sequence assembly was performed by using the programs Autoassembler version 2.1 (Applied Biosystems) and GENETYX-MAC version 13.0.1 (Software Development). The closest known relatives of the strains were determined by using FASTA, and sequences of closely related species were retrieved from the GenBank/EMBL/DDBJ databases. Multiple alignment of the sequences was carried out with the CLUSTAL X program (version 1.82) (Thompson et al., 1997). Approximately 1490 bp of the 16S rRNA gene sequences (approx. 400 bp for the rpoA gene and 390 bp for the pheS gene) of the strains and related species were used for reconstructing phylogenetic trees with the neighbour-joining method (Saitou & Nei, 1987). The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Felsenstein, 1985) and the tree topology was also confirmed with the minimum-evolution (Rzhetsky & Nei, 1992) and maximum-parsimony (Fitch, 1971) methods, by using MEGA4 software (Tamura et al., 2007), according to the Kimura two-parameter model (Kimura, 1980). The sequence similarity of 16S rRNA gene sequences between strain YIT 11304T and its nearest neighbours, L. collinoides YIT 0263T, L. paracollinoides YIT 10360T and L. similis YIT 12117T was high, with values of 98.6, 98.6 and 99.6% respectively. The novel strain formed a subcluster with L. collinoides, L. paracollinoides and L. similis on the basis of neighbour-joining analysis (Fig. 1). Similar topologies were obtained by the minimum evolution (Supplementary Figs S2 and S3) and maximum-parsimony (data not shown) methods. The similarities among the rpoA and pheS gene sequences of the novel strain and the closest three neighbouring species ranged from 86.1 to 86.6%, and from 80.3 to 85.3%, respectively (Figs 2 and 3). Interspecies gaps within the genus Lactobacillus based on rpoA and pheS gene sequences normally exceed 5% and 10%, respectively (Naser et al., 2007), which suggested that the novel strain should be classified in a novel species. On the basis of the neighbour-joining analysis of the rpoA and pheS gene sequences, the novel strain did not belong to any species with a validly published name (Figs 2 and 3). Similar topologies were obtained by the minimum evolution (Supplementary Figs S2 and S3) and maximum-parsimony methods (data not shown).

For determining DNA G+C content, chromosomal DNA was extracted according to the method of Marmur (1961). DNA–DNA reassociation analyses were performed according to the microdilution well technique, using photobiotin for DNA labelling (Ezaki et al., 1989). Reciprocal hybridization experiments were performed for every pair of strains at 43°C for 15 min in the presence of 50% formamide. DNA–DNA relatedness values between strain YIT 11304T and L. collinoides YIT 0263T, L. paracollinoides YIT 10360T and L. similis YIT 12117T were 5–6%, 3–6% and 7–8%, respectively (Supplementary Table S1). These values were well below the 70% cut-off value that indicates separate species (Stackebrandt & Goebel, 1994). To assess DNA base composition (G+C content), DNA was enzymically degraded into nucleosides as previously described (Mesbah et al., 1989) and then separated by HPLC. The DNA G+C content of strain YIT 11304T was 43.7 mol%, which is within the range for the genus Lactobacillus (32–53 mol%) (Kandler & Weiss, 1986).

Morphological, cultural, and biochemical testing according to standard techniques was performed at 30°C unless otherwise stated. Cell shape, cell size and Gram staining were determined by using cultures grown in MRS broth at 30°C for 24 h. Catalase and pseudocatalase activities were determined by using cells grown on MRS agar and MRS agar supplemented with 5% defibrinated horse blood, respectively. Gas production from glucose was determined with a Durham tube in MRS broth. Production of dextran was assessed on MRS agar in which glucose was replaced with formate and succinate, respectively. Gas production from formate and succinate was determined using a Durham tube in MRS broth.

![Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain YIT 11304T with closely related species. Tree reconstructed by the neighbour-joining method on the basis of a comparison of approximately 1490 nt. Lactobacillus acidophilus YIT 0070T was used as an outgroup. Bootstrap values (%) based on 1000 replicates are given at nodes. Bar, 1% sequence divergence.](image-url)
with 2% (w/v) sucrose. Growth at various temperatures and pH, and in the presence of NaCl, reduction of nitrate, and production of ammonia from arginine were determined according to Barrow & Feltham (1993). Carbohydrate fermentation tests were carried out by using the API 50 CHL system (bioMérieux) according to the manufacturer’s instructions. The presence of diaminopimelic acid in the cell wall peptidoglycan was determined according to Kandler & Weiss (1986). The isomers of lactic acid formed from glucose were determined with a D-/L-lactic acid enzymic kit (R-Biopharm).

Various phenotypic characteristics of the novel strains differed from those of L. collinoides, L. paracollinoides and L. similis (Kitahara et al., 2010), which are the phylogenetically closest related species. Among them, acid production from methyl β-xyloside, D-arabitol and 5-ketogluconate, and aesculin hydrolysis differentiated the novel strains from the closest species, L. similis (Table 1). According to the data obtained, the three strains are genetically distinguishable from recognized species of lactic acid bacteria and thus represent a novel species, for which we suggest the name Lactobacillus odoratitofui sp. nov.

Description of Lactobacillus odoratitofui sp. nov.

Lactobacillus odoratitofui (o.do.ra.ti.to’fu.i. L. part adj. odoratus -a -um that has a smell, fragrant; N.L. n. tofuum tofu; N.L. gen. n. tofui of tofu; N.L. gen. n. odoratitofui pertaining to the stinky tofu, a kind of fermented tofu in Taiwan, from which the type strain was isolated). Cells are rod-shaped, 0.3–0.4 × 1–3 μm and occur singly, in pairs or in short chains comprising three or four cells. Gram-positive-staining, non-motile, asporogenous and aesculin hydrolysis differentiated the novel strains from the closest species, L. similis (Table 1). According to the data obtained, the three strains are genetically distinguishable from recognized species of lactic acid bacteria and thus represent a novel species, for which we suggest the name Lactobacillus odoratitofui sp. nov.

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Table 1. Major differential characteristics among the novel strain and genetically closely related lactobacilli

<table>
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<tr>
<th>Characteristic</th>
<th>1</th>
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<th>4</th>
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<tr>
<td>Acid production from:</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Methyl β-xyloside</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>W</td>
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<tr>
<td>D-Galactose</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>+</td>
<td>W</td>
<td>–</td>
<td>W</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-Arabitol</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Gluconate</td>
<td>+</td>
<td>W</td>
<td>–</td>
<td>W</td>
</tr>
<tr>
<td>5-Ketogluconate</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Aesculin hydrolysis</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>43.7</td>
<td>47.1</td>
<td>46.4</td>
<td>47.1</td>
</tr>
</tbody>
</table>

facultatively anaerobic. Catalal and pseudocatalase are not produced. After anaerobic growth at 30 °C for 48 h, colonies on MRS agar are 1–1.5 mm in diameter, milk white, translucent, smooth and circular with entire edges. In MRS broth, growth occurs at 15 °C but not at 45 °C. Growth occurs at pH 4.0 and pH 8.5. No growth occurs with 8% NaCl. Gas is produced from glucose. Both L- (15%) and D-lactate (75%) are produced as end products from glucose. Ammonia is produced from arginine. Nitrate is not reduced. Acid is produced from L-arabinose, ribose, D-xylose, galactose, D-glucose, N-acetylgalactosamine, maltose, lactose, melibiose, D-arabitol and glucuronate. Acid is not produced from glycerol, erythritol, D-arabinose, L-xylose, adonitol, D-mannose, L-sorbos, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl β-D-mannoside, methyl α-D-glucoside, amygdalin, arbutin, salacin, cellobiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, β-gentiobiose, turanose, D-lyxose, D-tagatose, D- or L-fucose, or 2-ketogluconate. +, Positive; –, negative; W, weakly positive reaction.

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References


Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the

