Lactobacillus pobuzhiii sp. nov., isolated from pobuzihi (fermented cummingcordia)

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Twenty-one homofermentative lactic acid bacteria were isolated from fermented cummingcordia (pobuzihi), a traditional food in Taiwan. The isolates had identical 16S rRNA gene sequences that were distinct from those of other lactobacilli, and their closest neighbours in the 16S rRNA gene sequence phylogenetic tree were strains of Lactobacillus acidipiscis. Levels of DNA–DNA relatedness between representative pobuzihi isolates and strains of L. acidipiscis were 17 % and below. Furthermore, the new isolates could be differentiated clearly from L. acidipiscis NBRC 102163T and NBRC 102164 in terms of acid production from L-arabinose, rhamnose, mannitol, lactose and 5-ketogluconate. It was concluded that the new isolates represent a single novel species of the genus Lactobacillus, for which the name Lactobacillus pobuzhiii sp. nov. is proposed. The type strain is E100301T (=RIFY 6501T =NBRC 103219T =KCTC 13174T).

Lactic acid bacteria (LAB) are widely distributed in foods in Taiwan such as suan-tsai (fermented mustard) (Chen et al., 2006a) and dochi (fermented black beans) (Chen et al., 2006b). Pediococcus pentosaceus, Tetragnococcus halophilus and Enterococcus faecium have been isolated from these foods and possibly play important roles in their souring and ripening. Pobuzihi is a popular traditional food sold in both traditional markets and supermarkets in Taiwan. It is produced by fermenting cummingcordia, a grain product. Pobuzihi is produced by two different methods that yield different flavours. In one method (method A), harvested cummingcordia is washed, boiled, mixed with salt (NaCl) and then packed in containers while hot. After cooling, congealed cummingcordia is removed from the containers and placed in urns containing a salt solution. Usually, fermentation continues for at least 10 days, but some producers maintain a longer fermentation time. In the other method (method B), cummingcordia is boiled and dried, and then mixed with sugar, salt, soy and sake (rice wine). Finally, the mixture is packed in bottles, sealed and fermented for 15 days or longer. In both methods A and B, the fermentations are carried out at room temperature. Pobuzihi is used both as a food and as a seasoning. The fermentation process of pobuzihi has not been studied in detail, despite the popularity of the product. The present study deals with the isolation of LAB predominating in pobuzihi and their taxonomic characterization as representing a novel species of the genus Lactobacillus.

Twelve pobuzihi samples were collected from one factory (Yunlin county, Taiwan), traditional markets and supermarkets. Eight of the samples were fermented with method A and four with method B. LAB were isolated from pobuzihi by using the methods described by Chen et al. (2006a). MRS broth (Difco) containing 6 % NaCl was used for isolation. Cultivation for isolation was carried out anaerobically at 37 °C. The purity of the isolates was confirmed by spreading on an MRS agar plate containing 6 % NaCl. Lactobacillus acidipiscis strains NBRC 102163T and NBRC 102164 were used as reference strains. L. acidipiscis NBRC 102163T was isolated from fermented fish in Thailand (Tanasupawat et al., 2000, 2002). Strain NBRC 102164 was isolated from halloumi cheese produced in Cyprus (Lawson et al., 2001) and is the type strain of Lactobacillus cypricasei, which is considered a heterotypic
Synonym of *L. acidipiscis* (Naser et al., 2006). These two strains were cultured for 2 days in MRS broth at 37 °C. All strains were stored at −80 °C in 10% skimmed milk.

Tests of phenotypic characteristics such as isomers of lactic acid produced, lactic acid fermentation type, salt tolerance and growth temperature range were carried out based on the procedures described by Chen et al. (2006a, b). Tests of acid production from carbohydrates were performed by using the API 50CHL fermentation kit (bioMérieux).

Restriction fragment length polymorphism (RFLP) analysis of 16S rRNA genes was used for preliminary classification of the novel bacterial isolates (Ramos & Harlander, 1990; Gurtler et al., 1991; Johansson et al., 1995). PCR was carried out by using a TaKaRa Ex Taq gene amplification PCR kit (Takara Bio Inc.) and was performed on a Gene Amp PCR System 9700 (Perkin Elmer) following the methods described by Chen et al. (2005). RFLP analysis of the 16S rRNA gene was carried out according to the methods described by Chen et al. (2005). In the present study, four restriction enzymes were used: AccI (CG/CG), HaeIII (GG/CC), AluI (AG/CT) and MspI (C/CGG) (Sato et al., 2000; Christensen et al. 2004; Chen et al., 2006b).

Sequencing of 16S rRNA genes was carried out as described by Nakagawa et al. (2002) by using the following primers: 5′-GAGTTTGATCCTGGCTCAG-3′ (9F), 5′-GGATTAG-ATACCTGGTAGTC-3′ (785F), 5′-TACCAGGGTATC- TAATCC-3′ (802R) and 5′-GGCTACCTTGTTACGA-3′ (1510R). DNA sequencing was performed on a model ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Multiple sequence alignments and construction of phylogenetic trees were performed by using the CLUSTAL X version 1.83 software package (Thompson et al., 1997) based on the neighbour-joining method (Saitou & Nei, 1987) and K_{nuc} values (Kimura, 1980). Alignments were subsequently modified manually with Se-Al version 2.0 (Rambaut, 1996). Alignment gaps and unidentified base positions were not taken into consideration in the calculations. The topological robustness of the phylogenetic trees was evaluated by a bootstrap resampling method based on 1000 replicates (Felsenstein, 1985).

Inter- and intraspecific DNA–DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells, as described by Ezaki et al. (1989). Fluoroskan Ascent (Thermo Fisher Scientific, Inc.) was used for fluorescence measurements. Hybridization was carried out at 49.5 °C in 2× SSC buffer containing 25% formamide. The DNA G+C content was subsequently determined according to Mesbah et al. (1989).

Twenty-one isolates were obtained from the pobuzhi samples. These isolates showed the same 16S rRNA gene RFLP patterns when digested with AccII, HaeIII, AluI and MspI. However, the patterns when digested with HaeIII, AluI and MspI were different from those of *L. acidipiscis* NBRC 102163^T and NBRC 102164 (Fig. 1).

**Fig. 1.** RFLP patterns of AccI, HaeII, AluI and MspI digests of the 16S rRNA genes of isolates E100301^T and E100311 and *L. acidipiscis* strains NBRC 102163^T and NBRC 102164. Lanes: M, size marker; A, AccI digests; H, HaeII digests; U, AluI digests; P, MspI digests.

16S rRNA gene sequences (approximately 1489 nt) were determined for seven randomly selected isolates: E100301^T, E100309, E100311, E100313, E100318, E100326 and E100329. The seven pobuzhi isolates shared 100% 16S rRNA gene sequence similarity. As shown in Fig. 2, results of phylogenetic analysis confirmed the position of the seven isolates as closely related to *L. acidipiscis* NBRC 102163^T (98.46% 16S rRNA gene sequence similarity). Lower levels of 16S rRNA gene sequence similarity were obtained with *Lactobacillus salivarius* NBRC 102160^T (93.42%) and *Lactobacillus aviarus* subsp. *aviarus* NBRC 102162^T (93.15%). The 16S rRNA gene sequence was also analysed by using the maximum-parsimony (Fitch, 1971, 1977) and maximum-likelihood (Felsenstein, 1981) methods. Results similar to those with the neighbour-joining method were obtained (Fig. 2).

All 21 pobuzhi isolates grew in a medium with 10% (w/v) NaCl and showed homolactic fermentation and production of l-lactic acid. The results obtained with the API50 CHL fermentation kit indicated that all the pobuzhi isolates were clearly different from *L. acidipiscis* strains NBRC 102163^T and NBRC 102164 in terms of acid production from L-arabinose, rhamnose, mannitol, lactose and 5-ketogluconate (Table 1). The DNA G+C content of strain E100301^T was 37.2 mol%, slightly lower than that of *L. acidipiscis* NBRC 102163^T (38.9 mol%).

DNA–DNA hybridization experiments were performed by using DNA derived from isolates E100301^T and E100311 and *L. acidipiscis* strains NBRC 102163^T and NBRC 102164. Levels of DNA–DNA relatedness between the two pobuzhi isolates and the *L. acidipiscis* strains were 17% and below, indicating that the new isolates are not members of *L. acidipiscis* (Table 2), supporting the result of 16S rRNA gene sequence analyses.

The data presented here indicate the independent status of the pobuzhi isolates in the genus *Lactobacillus*. Based on the results of DNA–DNA hybridization, the 21 pobuzhi isolates represent a species that is clearly separated from its

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closest phylogenetic neighbours and has phenotypic characteristics that clearly distinguish it from \textit{L. acidipiscis} (strains NBRC 102163$^T$ and NBRC 102164). More detailed characteristics are given in the species description below and in Table 1.

In conclusion, the 21 isolates from pobuzhi are considered to represent a single novel species of the genus \textit{Lactobacillus}, for which the name \textit{Lactobacillus pobuzihii} sp. nov. is proposed.

**Description of \textit{Lactobacillus pobuzihii} sp. nov.**

\textit{Lactobacillus pobuzihii} [po.bu.zi’hi.i. N.L. gen. n. pobuzihii referring to the isolation of the type strain from pobuzhi (fermented cummingcordia)].

Cells are Gram-stain-positive rods, approximately 0.5–2.2–3.9 μm. Colonies on MRS agar containing 6% NaCl are opaque with smooth edges and approximately 2 mm in diameter after 4–5 days of growth at 37°C. Facultatively anaerobic. Catalase-negative. Utilizes D-glucose homofermentatively but does not produce gas from glucose. Produces L-lactic acid from glucose. Grows at 30–37°C but not at 15 or 45°C. Grows in the presence of 10% NaCl. Acid is produced from D-glucose, ribose, D-fructose, L-arabinose, trehalose, rhamnose, maltose, lactose, 5-ketogluconate and \textit{N}-acetylglucosamine. Most known isolates produce acid from glycerol (19/21), D-tagatose (19/21) and cellobiose (20/21). Acid is not produced from mannitol, erythritol, D-xylose, L-xylose, adonitol, D-arabinose, methyl \(\beta\)-xyloside, L-sorbose, dulcitol, inositol, sorbitol, methyl \(\alpha\)-D-mannoside, amygdalin, melibiose, inulin, melezitose, raffinose, glycogen, xylitol, D-arabitol.

![Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence comparisons showing the position of strain E100301$^T$ and related lactobacilli. Bootstrap values are indicated at branch points based on 1000 replications. The sequence of \textit{Lactobacillus plantarum} NBRC 15891$^T$ was used as an outgroup. GenBank accession numbers are given in parentheses. Filled circles indicate nodes that were also recovered in maximum-parsimony and maximum-likelihood analyses, and open circles indicate nodes that were also recovered in the maximum-likelihood analysis. Bar, 1 substitution per 100 nucleotide positions.](image)

**Table 1.** Physiological characteristics of the pobuzhi isolates and \textit{L. acidipiscis} strains

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>\textit{L. pobuzihii} sp. nov.</th>
<th>\textit{L. acidipiscis}</th>
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<tbody>
<tr>
<td></td>
<td>E100301$^T$</td>
<td>Twenty other isolates</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>37.2</td>
<td>ND</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>18</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Turanose</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5-Ketogluconate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Tagatose</td>
<td>+</td>
<td>18</td>
</tr>
<tr>
<td>Amygdalin</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Arbutin</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>+</td>
<td>19</td>
</tr>
</tbody>
</table>
Table 2. Levels of DNA–DNA relatedness between selected pobuzhi isolates and *L. acidipiscis* strains

<table>
<thead>
<tr>
<th>Source of unlabelled DNA</th>
<th>DNA–DNA hybridization (%) with labelled DNA from:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1. E100301&lt;sup&gt;T&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>2. E100311</td>
<td>86</td>
</tr>
<tr>
<td>3. <em>L. acidipiscis</em> NBRC 102163&lt;sup&gt;T&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>4. <em>L. acidipiscis</em> NBRC 102164</td>
<td>11</td>
</tr>
</tbody>
</table>

L-arabitol, D-fucose, L-fucose or D-turanose. The DNA G+C content of the type strain is 37.2 mol%.

The type strain, E100301<sup>T</sup> (= RIFY 6501<sup>T</sup> = NBRC 103219<sup>T</sup> = KCTC 13174<sup>T</sup>), was isolated from fermented cummington-cordia (pobuzhi). The species includes another 20 isolates from pobuzhi.

Acknowledgements

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References


