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

Yi-Sheng Chen,^{1,2} Mika Miyashita,³ Ken-ichiro Suzuki,³ Hajime Sato,³ Jar-Sheng Hsu⁴ and Fujitoshi Yanagida²

Correspondence

Yi-Sheng Chen

yisheng@mail.mcu.edu.tw

¹Department of Biotechnology, Ming Chuan University, No. 5, De-Ming Rd, Gui-Shan Township, Taoyuan County 333, Taiwan, ROC

²The Institute of Enology and Viticulture, Yamanashi University, 1-13-1, Kitashin, Kofu, Yamanashi 400-0005, Japan

³NITE Biological Resource Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba 292-0818, Japan

⁴Kokumori Foods Co. Ltd, No. 18, Lane 10, Sec. 3, Jhongsiao E. Rd, Da-an District, Taipei City 106, Taiwan, ROC

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 102163^T 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 pobuzihii* sp. nov. is proposed. The type strain is E100301^T (=RIFY 6501^T =NBRC 103219^T =KCTC 13174^T).

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*, *Tetragenococcus 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 102163^T and NBRC 102164 were used as reference strains. *L. acidipiscis* NBRC 102163^T 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

Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of isolates E100301^T, E100309, E100311, E100313, E100318, E100326 and E100329 are AB326358–AB326364, respectively.

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-ATACCCTGGTAGTC-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 pobuzihi 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 *AccII*, *HaeIII*, *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, *AccII* digests; H, *HaeIII* 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 pobuzihi 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 aviarius* subsp. *aviarius* 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 pobuzihi 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 pobuzihi 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 pobuzihi 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 pobuzihi isolates in the genus *Lactobacillus*. Based on the results of DNA–DNA hybridization, the 21 pobuzihi isolates represent a species that is clearly separated from its

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 *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.

closest phylogenetic neighbours and has phenotypic characteristics that clearly distinguish it from *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 pobuzihi are considered to represent a single novel species of the genus *Lactobacillus*, for which the name *Lactobacillus pobuzihii* sp. nov. is proposed.

Description of *Lactobacillus pobuzihii* sp. nov.

Lactobacillus pobuzihii [po.bu.zi'hi.i. N.L. gen. n. *pobuzihii* referring to the isolation of the type strain from pobuzihi (fermented cummingcordia)].

Cells are Gram-stain-positive rods, approximately $0.5 \times 2.2\text{--}3.9\ \mu\text{m}$. 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 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 β -xyloside, L-sorbose, dulcitol, inositol, sorbitol, methyl α -D-mannoside, amygdalin, melibiose, inulin, melezitose, raffinose, glycogen, xylitol, D-arabitol,

Table 1. Physiological characteristics of the pobuzihi isolates and *L. acidipiscis* strains

All strains produce L-lactic acid homofermentatively. +, Positive; w, weakly positive; –, negative; ND, not determined. Numbers indicate the number of isolates that show a positive reaction.

Characteristic	<i>L. pobuzihii</i> sp. nov.		<i>L. acidipiscis</i>	
	E100301 ^T	Twenty other isolates	NBRC 102163 ^T	NBRC 102164
DNA G + C content (mol%)	37.2	ND	38.9	38.9
Acid production from:				
Glycerol	+	18	–	+
L-Arabinose	+	+	–	–
Rhamnose	+	+	–	–
Mannitol	–	–	+	–
Maltose	+	+	+	+
Lactose	+	+	–	–
D-Turanose	–	–	+	–
5-Ketogluconate	+	+	–	–
D-Tagatose	+	18	–	–
Amygdalin	–	–	–	w
Arbutin	–	5	w	+
Cellobiose	+	19	–	+

Table 2. Levels of DNA–DNA relatedness between selected pobuzihi isolates and *L. acidipiscis* strains

Source of unlabelled DNA	DNA–DNA hybridization (%) with labelled DNA from:			
	1	2	3	4
1. E100301 ^T	100	86	13	16
2. E100311	86	100	11	17
3. <i>L. acidipiscis</i> NBRC 102163 ^T	10	15	100	107
4. <i>L. acidipiscis</i> NBRC 102164	11	15	91	100

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^T (=RIFY 6501^T =NBRC 103219^T =KCTC 13174^T), was isolated from fermented cumming-cordia (pobuzihi). The species includes another 20 isolates from pobuzihi.

Acknowledgements

We thank the Hua-tai Sauce Co., Ltd (Yunlin county, Taiwan), for supplying pobuzihi samples.

References

- Chen, Y.-S., Yanagida, F. & Shinohara, T. (2005). Isolation and identification of lactic acid bacteria from soil using an enrichment procedure. *Lett Appl Microbiol* **40**, 195–200.
- Chen, Y.-S., Yanagida, F. & Hsu, J.-S. (2006a). Isolation and characterization of lactic acid bacteria from suan-tsai (fermented mustard), a traditional fermented food in Taiwan. *J Appl Microbiol* **101**, 125–130.
- Chen, Y.-S., Yanagida, F. & Hsu, J.-S. (2006b). Isolation and characterization of lactic acid bacteria from *dochi* (fermented black beans), a traditional fermented food in Taiwan. *Lett Appl Microbiol* **43**, 229–235.
- Christensen, J. E., Reynolds, C. E., Shukla, S. K. & Reed, K. D. (2004). Rapid molecular diagnosis of *Lactobacillus* bacteremia by terminal restriction fragment length polymorphism analysis of the 16S rRNA gene. *Clin Med Res* **2**, 37–45.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.
- Fitch, W. M. (1977). On the problem of discovering the most parsimonious tree. *Am Nat* **111**, 223–257.
- Gurtler, V., Wilson, V. A. & Mayall, C. (1991). Classification of medically important clostridia using restriction endonuclease site differences of PCR-amplified 16S rDNA. *J Gen Microbiol* **137**, 2673–2679.
- Johansson, M. L., Quednau, M., Ahrne, S. & Molin, G. (1995). Classification of *Lactobacillus plantarum* by restriction endonuclease analysis of total chromosomal DNA using conventional agarose gel electrophoresis. *Int J Syst Bacteriol* **45**, 670–675.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Lawson, P. A., Papademas, P., Wachter, C., Falsen, E., Robinson, R. & Collins, M. D. (2001). *Lactobacillus cypricasei* sp. nov., isolated from Halloumi cheese. *Int J Syst Evol Microbiol* **51**, 45–49.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Nakagawa, Y., Sakane, T., Suzuki, M. & Hatano, K. (2002). Phylogenetic structure of the genera *Flexibacter*, *Flexithrix*, and *Microscilla* deduced from 16S rRNA sequence analysis. *J Gen Appl Microbiol* **48**, 155–165.
- Naser, S. M., Vancanneyt, M., Hoste, B., Snauwaert, C. & Swings, J. (2006). *Lactobacillus cypricasei* Lawson *et al.* 2001 is a later heterotypic synonym of *Lactobacillus acidipiscis* Tanasupawat *et al.* 2000. *Int J Syst Evol Microbiol* **56**, 1681–1683.
- Rambaut, A. (1996). Se-Al: Sequence Alignment Editor version 1.0α1. Distributed by the author. Department of Zoology, University of Oxford, Oxford, UK. <http://evolve.zoo.ox.ac.uk/>
- Ramos, M. S. & Harlander, S. K. (1990). DNA fingerprinting of lactococci and streptococci used in dairy fermentations. *Appl Microbiol Biotechnol* **34**, 368–374.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sato, H., Yanagida, F., Shinohara, T. & Yokotsuka, K. (2000). Restriction fragment length polymorphism analysis of 16S rRNA genes in lactic acid bacteria isolated from red wine. *J Biosci Bioeng* **90**, 335–337.
- Tanasupawat, S., Shida, O., Okada, S. & Komagata, K. (2000). *Lactobacillus acidipiscis* sp. nov. and *Weissella thailandensis* sp. nov., isolated from fermented fish in Thailand. *Int J Syst Evol Microbiol* **50**, 1479–1485.
- Tanasupawat, S., Thongsanit, J., Okada, S. & Komagata, K. (2002). Lactic acid bacteria isolated from soy sauce mash in Thailand. *J Gen Appl Microbiol* **48**, 201–209.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.