Description of *Paralactobacillus selangorensis* gen. nov., sp. nov., a new lactic acid bacterium isolated from chili bo, a Malaysian food ingredient

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***Paralactobacillus selangorensis* gen. nov., sp. nov. is described. This organism, isolated from a Malaysian food ingredient called chili bo, is an obligatory homofermentative, rod-shaped lactic acid bacterium. The G+C content is 46·1–46·2±0·3 mol%. Earlier 16S rRNA studies showed that this organism constitutes a new taxon distantly related to the *Lactobacillus casei–Pediococcus* group. A phenotypic description that distinguishes *Paralactobacillus selangorensis* from other genera of lactic acid bacteria is presented. The type strain of *Paralactobacillus selangorensis* is LMG 17710^T.**

Keywords: chili bo, Malaysia, lactic acid bacteria, *Paralactobacillus selangorensis* gen. nov., sp. nov.

INTRODUCTION

During studies on lactic acid bacteria (LAB) isolated from a traditional Malaysian food ingredient, chili bo, isolates belonging to a new group of LAB were obtained (Leisner *et al.*, 1997). These isolates exhibited phenotypic characteristics, including SDS-PAGE whole-cell profiles, which excluded them from any described species of LAB. 16S rRNA sequencing data demonstrated that they represent a new group of LAB distantly related to the *Lactobacillus casei–Pediococcus*, *Weissella* and *Carnobacterium* groups (Leisner *et al.*, 1999). No genus or species names were designated to the group.

In this study, G+C content, DNA–DNA hybridization studies, cellular fatty acids analysis, and biochemical and physiological reactions were used to classify these isolates as a new taxon of LAB.

METHODS

Bacterial strains and growth media. Strains were isolated from two distinct batches of chili bo obtained from a commercial manufacturer located in Pataling Jaya, Selangor DE, Malaysia. The processing of chili bo and the procedure for obtaining the isolates have been described previously (Leisner *et al.*, 1997, 1999). Initially, 22 strains belonging to this group were obtained and characterized by a battery of traditional phenotypic tests. The results of these tests showed that all 22 strains shared very similar phenotypic traits (Leisner *et al.*, 1999; unpublished data). Five strains, three of which (LMG 17710^T–17712) were isolated from one batch of chili bo and two (LMG 17713–17714) which were isolated from the other batch, were selected as representative isolates for additional SDS-PAGE whole-cell protein electrophoresis and deposited with the Belgian Coordinated Collections of Microorganisms (BCCM/LMG) (Leisner *et al.*, 1999). These five strains were included in this study.

Biochemical and physiological tests. Strains were tested for the ability to grow in Man–Rogosa–Sharpe broth (MRS; Merck) at 7, 10, 15, 21, 25, 30, 37, 42 and 45 °C. Production of acids from carbohydrates and related compounds was tested by using API 50 CH strips and API CHL medium (bioMérieux). Tests were done according to the manufacturer's instructions and the results were read after incubation at 30 °C for 2, 4, 7 and 14 d. Production of lactic acid isomers, acetic acid and ethanol was determined enzymically using D- and L-lactate dehydrogenase, acetyl-CoA synthetase and alcohol dehydrogenase (all Boehringer Mannheim), respectively, for cultures grown in MRS broth for 45 h or in All Purpose Tween (APT; Difco) broth for 73 h. Incubation was at 30 °C.

Fatty acid methyl ester analysis. Strains were grown for 24 h on MRS agar plates. A loopful of well grown cells was

Abbreviation: LAB, lactic acid bacteria.

harvested. Preparation, separation, identification and numerical comparison of the fatty acid methyl esters was performed using the Microbial Identification System (Microbial ID) as described by Vancanneyt *et al*. (1996).

DNA base composition. DNA was enzymically degraded into nucleosides as described by Mesbah *et al*. (1989). The nucleoside mixture obtained was then separated by HPLC using a Waters SymmetryShield C8 column with the thermostat set at 37 °C. The solvent was 0·02 M $NH_4H_2PO_4$ (pH 4·0) with 1·5% acetonitrile. Non-methylated λ phage DNA (Sigma) was used as the calibration reference.

DNA–DNA hybridization. DNA–DNA hybridizations were performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al*. (1989) using an HTS7000 Bio Assay Reader (Perkin Elmer) for fluorescence measurements. The hybridization temperature was 40 °C.

RESULTS AND DISCUSSION

All five strains of *Paralactobacillus selangorensis* produced acid from D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine and salicin within 4 d of incubation in API 50 CH medium at 30 °C. Some strains also produced acid within 4 d or more (up to 2 weeks) from galactose, rhamnose, methyl α-D-glucoside, amygdalin, arbutin, cellobiose, maltose, sucrose, trehalose, β-gentiobiose, D-turanose and D-tagatose. Aesculin was hydrolysed. No acid was produced by any of the five strains from glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β-methylxyloside, L-sorbose, mannitol, dulcitol, inositol, sorbitol, methyl α-D-mannoside, lactose, melibiose, melizitose, inulin, D-raffinose, starch, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. API 50 CH reactions useful in differentiating *Paralactobacillus selangorensis* from homofermentative *Lactobacillus* spp. of the *Lactobacillus casei–Pediococcus* group are shown in Table 1 and from homofermentative *Lactobacillus* spp. of the *Lactobacillus delbrueckii* group in Table 2.

All of the strains, excluding LMG 17711, were tested for production of lactic acid isomers, acetic acid and ethanol. Both D- and L-lactic acid were produced from MRS broth in the approximate ratio 1·6–2:1 (D:L).The yield of D- and L-lactic acid was 50·1–76·1 and 30·9–41·2 mM, respectively, after 44 h incubation at 30 °C in MRS broth. Only trace amounts of acetic acid (<3 mM) and ethanol (<0·35 mM) were produced under these conditions. LMG 17710^T was examined for production of acetic acid, ethanol and lactic acid during growth in APT broth. Both D- and L-lactic acid were produced, whereas only trace amounts of acetic acid and ethanol were observed (results not shown).

All five strains exhibited visible growth within 2–3 d in MRS broth incubated at 21–30 °C. Variation in growth was observed at 37 °C. Visible growth at 15 °C was observed only after 5–10 d incubation. No strains grew above 37 °C or below 15 °C.

The G+C content of the DNA was 46·1 ± 0·3 mol% for LMG 17710^T and 17112, and 46·2 mol% for LMG 17714. DNA–DNA hybridization gave similarity

Table 1. Differential phenotypic characteristics of *Paralactobacillus* and obligatory homofermentative *Lactobacillus* spp. of the *Lactobacillus casei–Pediococcus* group

Data for homofermentative *Lactobacillus* spp. are from Hammes & Vogel (1995) except for *Lactobacillus aviarius* (inulin data from Fujisawa *et al*., 1984), *Lactobacillus manihotivorans* (all data from Morlon-Guyot *et al*., 1998), *Lactobacillus farciminis* (some of the data from Reuter, 1983), *Lactobacillus mali* (some of the data from Kaneuchi *et al*., 1988), and *Lactobacillus salivarius* and *Lactobacillus ruminis* (data for anaerobic growth from Fujisawa *et al*., 1990). For data given by Hammes & Vogel (1995): +, ⩾ 90% of strains are positive, −, ⩾ 90% of strains are negative, d, 11–89% of strains are positive.

P. selangorensis or *Lactobacillus* spp.	G+C (mol%)	Growth at 45 °C	Strictly anaerobic	Acid from:				Lactic acid isomer*
				Raffinose	Lactose	Mannitol	Inulin	
P. selangorensis	45–47	−	−	−	−	−	−	DL
L. aviarius								
subsp. *aviarius*	39–43		+	+	d	−	−	DL
subsp. *araffinosus*	39–43		+	−	−	−	−	L(D)
L. farciminis	34–36	−	−	−	+	−	−	L(D)
L. mali	32–34	d	−	−	−	+	+	DL
L. manihotivorans	48–49	+	−	+	+	−	−	L
L. ruminis	44–47	d	+	+	d	−		L
L. salivarius†	34–36	+	−	+	+	+		L
L. sharpeae	53	−		−	+	−		L

* Isomers in parentheses indicate <15–20% of total acid.

† Includes both subspecies: *Lactobacillus salivarius* subsp. *salicinius* and subsp. *salivarius*.

Table 2. Differential phenotypic characteristics of *Paralactobacillus* and obligatory homofermentative *Lactobacillus* spp. of the *Lactobacillus delbrueckii* group

Data for homofermentative *Lactobacillus* spp. are from Hammes & Vogel (1995) except for *Lactobacillus amylolyticus*, *Lactobacillus iners* and *Lactobacillus kefirgranum* for which the data are from Bohak *et al*. (1998), Falsen *et al*. (1999) and Takizawa *et al*. (1994), respectively. For data given by Hammes & Vogel (1995): +, ⩾ 90 % of strains are positive, −, ⩾ 90 % of strains are negative, d, 11–89 % of strains are positive.

P. selangorensis **or *Lactobacillus* spp.**	**G + C (mol %)**	**Growth at 45 °C**	**Acid from:**			**Lactic acid isomer**
			Raffinose	**Lactose**	**Starch**	
P. selangorensis	45–47	−	−	−	−	DL
L. acidophilus	34–37	+	d	+		DL
L. amylolyticus	39	+	d	−	+	DL
L. amylophilus	44–46	−	−	−	+	L
L. amylovorus	40–41	+	−	−	+	DL
L. crispatus	35–38	+	−	+		DL
L. delbrueckii						
subsp. *bulgaricus*	49–51	+	−	+		D
subsp. *delbrueckii*	49–51	+	−	−		D
subsp. *lactis*	49–51	+	−	+		D
L. gallinarum	36–37	+	+	d		DL
L. gasseri	33–35	+	d	d		DL
L. helveticus	38–40	+	−	+		DL
L. iners	34–35		−	−		L
L. jensenii	35–37	+	−	−		D
L. johnsonii	33–35	+	d	d		DL
L. kefiranofaciens	34–35	−	+	+		DL
L. kefirgranum	34–39	−	+	+		DL

Table 3. Mean values of fatty acid profiles ± SD for *Paralactobacillus selangorensis* strains LMG 17710ᵀ–17714

Fatty acid	**Incidence (%)**
14:0	4·1 ± 1·1
16:0	12·1 ± 2·0
16:1ω7*c*	6·1 ± 0·8
17:1ω8*c*	1·1 ± 0·1
18:0	1·5 ± 0·1
18:1ω9*c*	57·3 ± 2·2
Summed feature 7*	7·9 ± 0·3
Summed feature 9*	8·1 ± 1·8

* Summed feature 7 contained one or more of the following isomers: 18:1 47*c*, 18:1 49*t* and/or 18:1 412*t* (*cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively). Summed feature 9 contained one or more of the following fatty acids: 19:0 cyclo 410*c*, unknown 18·846 and/or unknown 18·858.

values of 99 ± 5 % between LMG 17710ᵀ and 17712, 100 ± 6 % between LMG 17710ᵀ and 17714, and 104 ± 11 % between LMG 17712 and 17714.

The cellular fatty acid composition for the five strains examined is shown in Table 3. All of the strains possessed predominantly straight-chain saturated and monounsaturated types. This combination of major fatty acids is similar to that for the genus *Lactobacillus* (Kandler & Weiss, 1986).

Recently, *Paralactobacillus selangorensis* was found to be distantly related to other LAB taxa by phylogenetic analysis of 16S rRNA sequences (Leisner *et al*., 1999). In Fig. 1 the phylogenetic relationship between *Paralactobacillus selangorensis* and a representative collection of low-G + C Gram-positive bacteria has been established on the basis of 16S rRNA sequence comparison by maximum-likelihood analysis as described previously (Leisner *et al*., 1999). Thirty-nine taxa were aligned, resulting in 1457 nt positions being compared covering the region 33–1472 of the 16S rRNA sequence (*Escherichia coli* numbering). The phylogenetic relationship between *Paralactobacillus selangorensis* and the *Lactobacillus casei–Pediococcus* group was repeatedly verified by maximum-likelihood analysis. The position of the node joining *Paralactobacillus selangorensis* with the *Lactobacillus casei–Pediococcus* group was, however, not well supported by bootstrap analysis (48 %). The highest similarity in 16S rRNA sequences between *Paralactobacillus selangorensis* and the *Lactobacillus casei–Pediococcus* group was between 90·1 and 91·7 %. These values are similar to or lower than those reported for similarities in 16S rRNA sequences for other LAB

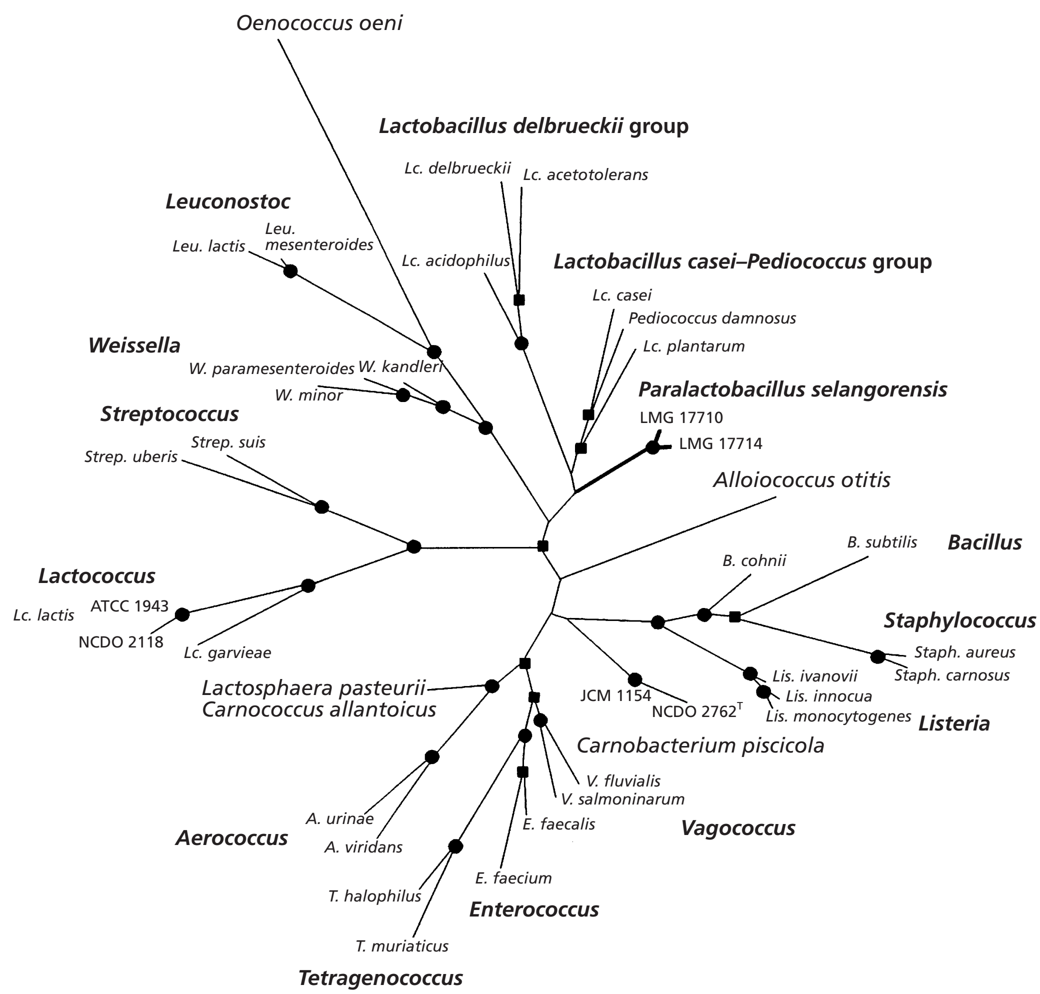

Fig. 1. Phylogenetic relationship between *Paralactobacillus selangorensis* and a representative collection of low-G+C Gram-positive bacteria established by maximum-likelihood analysis of 16S rRNA sequences. Nodes strongly supported by bootstrap analysis (75–100 %) are marked by circles and nodes moderately supported by bootstrap analysis (50–74 %) are marked by squares.

genera described recently and accepted LAB genera. Examples include comparisons of 16S rRNA sequences for *Vagococcus* and *Enterococcus* (Collins *et al.*, 1989), *Weissella* and *Leuconostoc* (Collins *et al.*, 1993) and *Lactosphaera* and *Carnobacterium* (Janssen *et al.*, 1995). It requires a combination of several phenotypical tests to differentiate *Paralactobacillus selangorensis* from homofermentative *Lactobacillus* spp., including species of the *Lactobacillus casei–Pediococcus* group (Table 1 and 2), similar to what has been demonstrated for the phenotypic differentiation of *Weissella* and *Leuconostoc* (Collins *et al.*, 1993). However, 16S rRNA sequences clearly demonstrate that *Paralactobacillus selangorensis* represents a new group of LAB, distantly related to the *Lactobacillus casei–Pediococcus* group (Leisner *et al.*, 1999). It is therefore formally proposed that these organisms are given independent genus rank within the LAB. Analyses of G+C content, DNA–DNA similarity and fatty acid content all demonstrate that the new taxon is very homogeneous and constitutes a single genomic species.

Description of *Paralactobacillus* gen. nov.

Paralactobacillus (pa.ra.lac.to.ba.cill′us. Gr. prep. *para* resembling; M.L. n. *Lactobacillus* a bacterial genus; *Paralactobacillus* resembling the genus *Lactobacillus*).

Surface colonies on MRS agar after 3 d aerobic incubation at 30 °C are <2–3 mm in diameter, round and with smooth surfaces. Non-spore-forming, straight, slender rods 2·5–6·5 µm long and 1·0 µm wide, usually occurring singly or as pairs but sometimes in older cultures as short chains. Cells are Gram-positive and non-motile. Homofermentative, producing D-(−)- as well as L-(+)-lactic acid from glucose. No gas is produced from glucose and growth is not observed with gluconic acid as substrate. Acid is produced from mannose and salicin but not from lactose, melibiose, raffinose, ribose or xylose. No ammonia is produced from arginine (Leisner *et al.*, 1999). Growth is slow in sterilized chili bo (Leisner *et al.*, 1997) as well as in MRS broth at 30 °C (unpublished data). Growth occurs in MRS broth at 15 °C but not at 45 °C. No growth with 6·5 % NaCl. Able to grow on acetate agar and to lower pH to <4·15 in La-broth (Leisner *et al.*, 1999). Resistant towards 30 µg vancomycin g^{-1} (Leisner *et al.*, 1999). Catalase-negative. Nitrate is not reduced (Leisner *et al.*, 1999). G+C content of DNA is 46 mol%. Isolated from a Malaysian food ingredient, chili bo (Leisner *et al.*, 1997). Readily distinguished from obligatory heterofermentative *Lactobacillus*, *Weissella* and *Leuconostoc* by lack of production of gas from glucose and from facultative heterofermentative *Lactobacillus* by lack of production of gas and acid from gluconic acid and lack of production of acid from ribose or xylose. Two species of facultative heterofermentative *Lactobacillus*, *Lactobacillus acetotolerans* and *Lactobacillus homohiochii*, may contain strains that also show negative reactions in all three tests (Hammes & Vogel, 1995). *Lactobacillus acetotolerans* may be differentiated from *Paralactobacillus selangorensis* by its G+C content of 35–37 mol% and lack of growth at 15 °C, and *Lactobacillus homohiochii* may be differentiated by a G+C content of 35–38 mol% and lack of growth in MRS broth (Hammes & Vogel, 1995). *Paralactobacillus* may be distinguished from the homofermentative rod-shaped *Carnobacterium* by the ability to grow on acetate agar and the lowering of pH to below 4·15 during growth in La-broth, and from the homofermentative *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Lactosphaera*, *Vagococcus* and *Tetragenococcus* by possessing a distinctive rod-shaped cell morphology. The differentiation of *Paralactobacillus* from homofermentative *Lactobacillus* is more problematic and requires the use of a combination of characters for particular species. The differentiation of *Paralactobacillus* from homofermentative *Lactobacillus* spp. of the *Lactobacillus casei–Pediococcus* group is given by the tests listed in Table 1 and from homofermentative *Lactobacillus* spp. of the *Lactobacillus delbrueckii* group by the tests listed in Table 2.

Description of *Paralactobacillus selangorensis* sp. nov.

Paralactobacillus selangorensis (sel.an.gor′en.sis. M. L. adj. *selangorensis* belonging to the province of Selangor, Malaysia).

Description of species as for genus. The type strain is LMG 17710^T. The characteristics of LMG 17710^T correspond to those of the species except that acid is produced from D-tagatose, in contrast to other strains.

ACKNOWLEDGEMENTS

Dr Sten Ebbesen, Institute for Greek and Latin, University of Copenhagen, Denmark, is thanked for his help in naming *Paralactobacillus selangorensis*.

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