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Characterization of *Lactobacillus sakei* by the type of stereoisomers of lactic acid produced

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***Lactobacillus sakei* strains were characterized by the shift of the type of stereoisomers of lactic acid produced in the presence of 50 mM sodium acetate in a medium. Of 27 *Lactobacillus sakei* strains studied, 20 strains showed high levels of DNA-DNA similarity with *L. sakei* NRIC 1071^T, and were confirmed as *L. sakei*. The three remaining strains were identified as *Lactobacillus curvatus* by DNA-DNA similarity, and three other strains were included in the cluster of *Lactobacillus plantarum*/*Lactobacillus pentosus*/*Lactobacillus paraplantarum* and one strain in the cluster of *Lactobacillus paracasei* on the basis of 16S rRNA gene sequences. Of the 20 *L. sakei* strains, 19 strains shifted the type of stereoisomers of lactic acid produced from the DL-type to the L-type in the presence of 50 mM sodium acetate. *L. curvatus* strains and strains included in the cluster of *L. plantarum*/*L. pentosus*/*L. paraplantarum* and in the cluster of *L. paracasei* did not shift the type of stereoisomers of lactic acid produced. The change of the type of stereoisomers of lactic acid from the DL-type to the L-type in the presence of sodium acetate was concluded to be species-specific for *L. sakei* and useful for identification of strains in this species.**

Key Words——effect of sodium acetate; *Lactobacillus curvatus*; *Lactobacillus sakei*; type of stereoisomers of lactic acid

Introduction

Lactic acid fermentation has been used for a long time for making dairy products and various fermented products. Lactic acid bacteria produce lactic acid in the early stage of the making of a sake starter, and this favors the sake making in Japan (Kozai, 1900). Those lactic acid bacteria involved were identified as *Leuconostoc mesenteroides* var. *sake* (sic) and *Lactobacillus sake* (sic) (Katagiri et al., 1934; Toyoda et al., 1979). Moreover, the microflora of the sake starter was reported to be divided into three stages, that is, the first stage at which nitrate reduction bacteria increase, the second stage at which lactic acid bacteria increase, and the third stage at which yeasts increase (Ashizawa, 1964; Kitahara, 1961; Saito, 1935). Furthermore, the increase of sphere-shaped lactic acid bacteria such as *Leuconostoc mesenteroides* var. *sake* was found at an early point in the second stage, and the increase of rod-shaped lactic acid bacteria such as *L. sake* was recognized after 2 or 3 days in the second stage (Kitahara, 1961; Saito, 1935). *L. sake* and *Leuconostoc mesenteroides* var. *sake*, which were able to grow at lower temperatures than the other

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lactic acid bacteria, were dominant in the sake starter because the sake making was carried out in the winter (Kitahara, 1961). Recently, the specific epithet *sake* was corrected to *sakei* according to the Latin grammar (International Code of Nomenclature of Bacteria, Rule 12c., Lapage et al., 1992; Trüper and De Clari, 1997). *Leuconostoc mesenteroides* var. *sake* was re-identified as *Leuconostoc mesenteroides* subsp. *mesenteroides* (Momose and Kamao, 1993).

Lactic acid bacteria produce two stereoisomers of lactic acid, L-form and D-form. The type of stereoisomers has been shown as the ratio of L-form to D-form of lactic acid produced, and employed for the classification and identification of lactic acid bacteria (Hammes et al., 1992; Kandler and Weiss, 1986; Kitahara, 1940; Orla-Jensen, 1919, 1942). However, *Lactobacillus sakei* was reported to change the type of stereoisomers of lactic acid under several cultural conditions (Katagiri and Kitahara, 1937), and the type shifted from the DL-type to the L-type in the presence of sodium acetate (Kitahara et al., 1957). It has been suggested that *L. sakei* produces lactate racemase (Hiyama et al., 1968; Katagiri and Kitahara, 1937; Kitahara et al., 1957; Stetter and Kandler,1973), and it has been described as producing L-lactic acid exclusively when the formation of lactate racemase was repressed by sodium acetate (Hiyama et al., 1968; Kitahara et al., 1957). Recently, lactate racemase was supposed to participate in the production of D-lactic acid by *L. sakei* because a *L. sakei* mutant that was disrupted *ldhL* gene encoded L-lactate dehydrogenase [EC 1.1.1.27, L-LDH] produced neither L-lactic acid nor D-lactic acid (Champomier-Verges et al., 2001; Marellet et al., 1998). However, the participation of lactate racemase in the production of D-lactic acid by *L. sakei* has not been clarified, and detailed studies of lactate racemase have not been reported until now. In previous papers (Iino et al., 2002, 2003), not only the resting cells of *L. sakei* strains but also the cell-free extracts of the strains were reported to produce scarcely any DL-lactic acid from L- or D-lactic acid and to show the activity of D-lactate dehydrogenase [EC 1.1.1.28, D-LDH]. In addition, the activity of L-LDH in *L. sakei* NRIC 1071^T cultivated in the presence of sodium acetate increased three times or more compared with the activity of the cells cultivated in its absence, and the activity of LDHs (the total activity of L-LDH plus D-LDH) in *L. sakei* NRIC 1071^T was retained longer under the addition of sodium acetate in the reaction mixture. Consequently, this leads to a conclusion that lactate racemase has not existed in *L. sakei* strains, and the shift of the DL-type to the L-type by *L. sakei* was explained by the fact that the ratio of L-lactic acid produced to D-lactic acid became larger by the activation of L-LDH and the stabilization of the activity of LDHs (Iino et al., 2001, 2002, 2003). The shift of stereoisomers of lactic acid by *L. sakei* strains seems interesting from the viewpoint of the delineation of this species.

This paper deals with the effect of sodium acetate on the production of stereoisomers of lactic acid produced by *L. sakei* strains and a discussion on the taxonomic significance of the change of the type in the presence of sodium acetate.

Materials and Methods

Bacterial strains. *Lactobacillus sakei* NRIC 1071^T, *Lactobacillus curvatus* NRIC 1052^T, and 29 *Lactobacillus* strains were used in this study (Table 1). Twenty-four *L. sakei* strains were isolated from sake starters in Japan. *L. sakei* TUA 2645L and TUA 2646L, and *L. curvatus* TUA 2647L, TUA 2648L, and TUA 2649L were isolated from raw sausage. *L. sakei* NRIC 1764 had been previously named *L. bavaricus.*

Cultivation. Strains were stationarily cultivated in 5 ml of GYP broth and GYP-Ac broth containing 50 mM sodium acetate at 25°C for 2 days as described previously (Iino et al., 2001). GYP medium was composed of 10 g of glucose, 10 g of yeast extract (Difco Laboratories, Detroit, MI, USA), 5 g of Polypepton (Nihon Seiyaku Ltd., Tokyo, Japan), 0.025 g of Tween 80, 5 ml of a salt solution, and 1,000 ml of distilled water, and pH was adjusted to 6.8. The salt solution contained 40 mg of $MgSO_4 \cdot 7H_2O$, 2 mg of $MnSO_4 \cdot 4H_2O$, 2 mg of $FeSO_4 \cdot 7H_2O$, and 2 mg of NaCl in 1,000 ml of distilled water. Strains were precultured in GYP broth for 2 days. Cells were collected by centrifugation at 3,500 rpm for 15 min at room temperature, and washed twice with sterile saline. The washed cells were resuspended in sterile saline, and 50 µl of the suspension was inoculated into GYP broth and GYP-Ac with a pipette.

Isolation of DNA. DNA was isolated by the methods reported by Marmur (1960) and Saito and Miura (1963). The cells were lyzed using 1 ml of a cell lysis solution containing 1 mg of lysozyme (Seikagaku Kogyo Co., Ltd., Tokyo, Japan) and 0.1 mg of Labiase (Seikagaku Kogyo Co., Ltd.) for 60 min.

Table 1. Bacterial strains used in this study.

Names received	Nos. of strains	Histories of strains	Sources
L. sakei	NRIC 1071^T	←ATCC 15521^T	Sake starter
L. sakei	NRIC 1599	←T. Uchimura (=M-6)	Sake starter
L. sakei	NRIC 1600	←T. Uchimura (=M-7)	Sake starter
L. sakei	NRIC 1601	←T. Uchimura (=M-8)	Sake starter
L. sakei	NRIC 1602	←T. Uchimura (=M-9)	Sake starter
L. sakei	NRIC 1603	←T. Uchimura (=M-10)	Sake starter
L. sakei	NRIC 1604	←T. Uchimura (=M-11)	Sake starter
L. sakei	NRIC 1606	←T. Uchimura (=M-02)	Sake starter
L. sakei	NRIC 1608	←T. Uchimura (=M-04)	Sake starter
L. sakei	NRIC 1609	←T. Uchimura (=M-05)	Sake starter
L. sakei	NRIC 1610	←T. Uchimura (=M-06)	Sake starter
L. sakei	NRIC 1611	←T. Uchimura (=M-07)	Sake starter
L. sakei	NRIC 1764	←JCM 1129 ←DSM 20269 (the former *Lactobacillus bavaricus* strain, Type strain of this species)	Sauerkraut
	IFO 12456	←S. Fukui (=G01)	Sake starter
L. sakei	TUA 2645L	←Reuter (=R 41d)	Raw sausage
L. sakei	TUA 2646L	←Reuter (=R 116/1a)	Raw sausage
L. sakei	No. 14		Sake starter
L. sakei	No. 16		Sake starter
L. sakei	No. 17		Sake starter
L. sakei	No. 18		Sake starter
L. sakei	No. 19		Sake starter
L. sakei	No. 21		Sake starter
L. sakei	No. 22		Sake starter
L. sakei	No. 23		Sake starter
L. sakei	No. 26		Sake starter
L. sakei	No. 27		Sake starter
L. sakei	No. 29		Sake starter
L. curvatus	NRIC 1052^T	←ATCC 25601^T	Milk
L. curvatus	TUA 2647L	←Reuter (=R 111i)	Raw sausage
L. curvatus	TUA 2648L	←Reuter (=R 111j)	Raw sausage
L. curvatus	TUA 2649L	←Reuter (=R 28c)	Raw sausage

^{T} Type strain.

DNA-DNA similarity. DNA-DNA similarity was determined by the fluorometric DNA-DNA hybridization in microdilution wells as described by Ezaki et al. (1989).

Sequence of 16S rRNA gene. All methods used for PCR amplification of the 16S rRNA gene and primers were those reported by Kawasaki et al. (1993) and Yamada et al. (2000). The 16S rRNA gene was amplified by PCR with the following two primers: 20F (5′-GATTTTGATCCTGGTTCAG-3′, positions 9 through 27) and 1500R (5′-GTTACCTTGTTACGACTT-3′, positions 1509 through 1492). The numbering of positions was based on the *Escherichia coli* numbering system (accession number V00348, Brosius et al., 1981). Purified PCR products were sequenced directly by using an ABI PRISM^{TM} model 310 Genetic Analyzer. The following six primers were used: 20F, 1500R, 520F (5′-CAGCAGCCGCGGTAATAC-3′, positions 519 through 536), 520R (5′-GTATTACCG-CGGCTGCTG-3′, positions 536 through 519), 920F (5′-AAACTCAAATGAATTGACGG-3′, positions 907 through 926), and 920R (5′-CCGTCAATTCATTTG-AGTTT-3′, positions 926 through 907).

Phylogenetic analysis. Published 16S rRNA gene sequences were obtained from the EMBL/GenBank/DDBJ/databases. The multiple alignments were performed by the program Clustal X (Ver. 1.8) (Thompson

et al., 1997). The distance matrixes for the aligned sequences were calculated by the two-parameter method reported by Kimura (1980) and expressed by K_{nuc}. The neighbor-joining method was used for constructing a phylogenetic tree (Saitou and Nei, 1987). The robustness for individual branches was estimated by bootstrapping with 1,000 replicates (Felsenstein, 1985). Alignment gaps and unidentified base positions were not taken into account.

Phenotypic characterization. Cell forms were examined for the cells cultivated in GYP-Ac broth for a day. GYP-Ac broth was used throughout this study because of the good growth of strains used in this medium (Iino et al., 2001, 2002). Gram staining was carried out by the method described by Hucker and Conn (1923). Catalase reaction was detected by the production of bubbles by adding a few drops of 3% hydrogen peroxide solution to living cells. The production of gas from glucose was examined using GYP-Ac broth with a Durham tube. The ratio of stereoisomers of lactic acid produced was determined by the methods described by Otsuka et al. (1994) and Manome et al. (1998), and classified into three types (L-, DL-, and D-) according to their definition (Manome et al., 1998; Otsuka et al., 1994). The growth at 10, 20, 40, and 45°C was determined using GYP-Ac broth after 7 days of cultivation. Acid production from sugars and sugar alcohols was detected using a basal medium after 3 days of incubation, and the acid produced in 4 ml of broth was titrated with 0.1 N NaOH. An indicator solution was a mixture of 0.1 g of neutral red and 0.2 g of bromothymol blue per 300 ml of ethanol. The positive reaction meant the titration value of 0.3 ml or more of 0.1 N NaOH, and the negative reaction less than 0.3 ml. The basal medium was composed of 10 g of sugar, 5 g of yeast extract (Difco Laboratories, Detroit, MI, USA), 5 g of Bactopeptone (Difco Laboratories), 2 g of sodium acetate, 0.05 g of Tween 80, 5 ml of a salt solution as described in the previous paper (Iino et al., 2001), and 1,000 ml of distilled water. L-Arabinose, D-glucose, maltose, D-mannitol, D-mannose, melibiose, D-ribose, D-sorbitol, sucrose, and D-trehalose were used for acid production.

Results

DNA-DNA similarity among L. sakei strains and L. curvatus strains

Of 27 *L. sakei* strains studied, 20 strains showed the high levels of DNA-DNA similarity with *L. sakei* NRIC 1071^T (67 to 100%), and they were confirmed as *L. sakei* (Table 2, Group A). The seven remaining *L. sakei* strains showed low levels of DNA-DNA similarity with *L. sakei* NRIC 1071^T. Four *L. curvatus* strains showed high levels of DNA-DNA similarity with *L. curvatus* NRIC 1052^T (69 to 100%), and low levels of DNA-DNA similarity with *L. sakei* NRIC 1071^T (16 to 45%) (Group B). Moreover, three *L. sakei* strains also showed high levels of DNA-DNA similarity with *L. curvatus* NRIC 1052^T (74 to 94%), and they were confirmed as *L. curvatus*. The remaining four *L. sakei* strains showed low levels of DNA-DNA similarity with both *L. sakei* NRIC 1071^T and *L. curvatus* NRIC 1052^T (Group C).

Comparison of 16S rRNA gene sequence

The 16S rRNA gene sequence was determined for four unidentified strains because these strains showed low levels of DNA-DNA similarity with *L. sakei* NRIC 1071^T and *L. curvatus* NRIC 1052^T as shown in Table 2 (Group C). NRIC 1599, NRIC 1601, and NRIC 1603 were included in the cluster with *L. plantarum* JCM 1149^T/*L. pentosus* JCM 1558^T/*L. paraplantarum* DSM 10667^T produced by 16S rRNA gene sequences (Fig. 1, Group C1). TUA 2645L was included in the cluster with *L. paracasei* subsp. *paracasei* JCM8130^T (Fig. 1, Group C2).

Phenotypic characteristics

Phenotypic characteristics of the strains studied are shown in Table 3. Cells of all strains were Gram-positive and rod-shaped. All strains were catalase negative, and did not produce gas from glucose. Twenty confirmed *L. sakei* strains grew at 15°C and produced acid from D-glucose, D-mannose, melibiose, D-ribose, and sucrose. They were variable with the growth at 40°C and the production of acid from L-arabinose, maltose, and D-trehalose (Group A). These strains did not grow at 45°C and did not produce acid from D-mannitol or D-sorbitol. Seven confirmed *L. curvatus* strains grew at 15°C and produced acid from D-glucose, maltose, D-mannose, and D-ribose (Group B). They grew at 40°C, except for NRIC 1600, and were variable with the production of acid from sucrose and D-trehalose. These strains did not grow at 45°C and did not produce acid from L-arabinose, D-mannitol, melibiose, or D-sorbitol. NRIC 1509, NRIC 1601, and NRIC 1603 grew at 15°C and 40°C, and produced acid from all sugars tested,

Table 2. DNA-DNA similarity among *L. sakei* and *L. curvatus* strains.

Groups	Names received	Nos. of strains	DNA-DNA similarity with		Confirmed as
			NRIC 1071^T	NRIC 1052^T	
	L. sakei	NRIC 1071^T	100	22	*L. sakei*
	L. sakei	NRIC 1602	103	54	*L. sakei*
	L. sakei	NRIC 1604	74	45	*L. sakei*
	L. sakei	NRIC 1606	84	41	*L. sakei*
	L. sakei	NRIC 1608	67	36	*L. sakei*
	L. sakei	NRIC 1609	70	26	*L. sakei*
	L. sakei	NRIC 1610	81	38	*L. sakei*
	L. sakei	NRIC 1611	67	24	*L. sakei*
	L. sakei	NRIC 1764	111	41	*L. sakei*
	(the former *Lactobacillus bavaricus* strain, Type strain of this species)				
Group A	*L. sakei*	No. 14	111	21	*L. sakei*
	L. sakei	No. 16	105	35	*L. sakei*
	L. sakei	No. 17	93	22	*L. sakei*
	L. sakei	No. 18	90	10	*L. sakei*
	L. sakei	No. 19	91	15	*L. sakei*
	L. sakei	No. 21	110	35	*L. sakei*
	L. sakei	No. 22	113	12	*L. sakei*
	L. sakei	No. 23	99	12	*L. sakei*
	L. sakei	No. 26	91	48	*L. sakei*
	L. sakei	No. 27	113	42	*L. sakei*
	L. sakei	No. 29	118	11	*L. sakei*
	L. curvatus	NRIC 1052^T	45	100	*L. curvatus*
	L. sakei	NRIC 1600	18	94	*L. curvatus*
	L. sakei	TUA 2646L	22	74	*L. curvatus*
Group B	*L. curvatus*	TUA 2647L	26	78	*L. curvatus*
	L. curvatus	TUA 2648L	24	80	*L. curvatus*
	L. curvatus	TUA 2649L	16	69	*L. curvatus*
	L. sakei	IFO 12456	41	93	*L. curvatus*
	L. sakei	NRIC 1599	9	19	Unidentified[a]
Group C	*L. sakei*	NRIC 1601	9	18	Unidentified[a]
	L. sakei	NRIC 1603	7	18	Unidentified[a]
	L. sakei	TUA 2645L	3	3	Unidentified[b]

^{T} Type strain.

[a] Included in the *L. plantarum*/*L. pentosus*/*L. paraplantarum* cluster deduced from 16S rRNA gene sequence (Fig. 1).

[b] Included in the *L. paracasei* subsp. *paracasei* cluster deduced from 16S rRNA gene sequence (Fig. 1).

except for L-arabinose (Group C1). These three strains produced acid weakly from L-arabinose. TUA 2645L grew at 15°C and 40°C, and produced acid from all sugars tested, except for L-arabinose and D-melibiose (Group C2). This strain produced acid weakly from melibiose.

The change of the type of stereoisomers of lactic acid produced by L. sakei strains in the presence of sodium acetate

Of 20 confirmed *L. sakei* strains, 19 produced DL-lactic acid (L-form to D-form=3 : 2) in the absence of sodium acetate, and produced L-lactic acid (L-form to D-form=9 : 1) in the presence of 50 mM sodium acetate

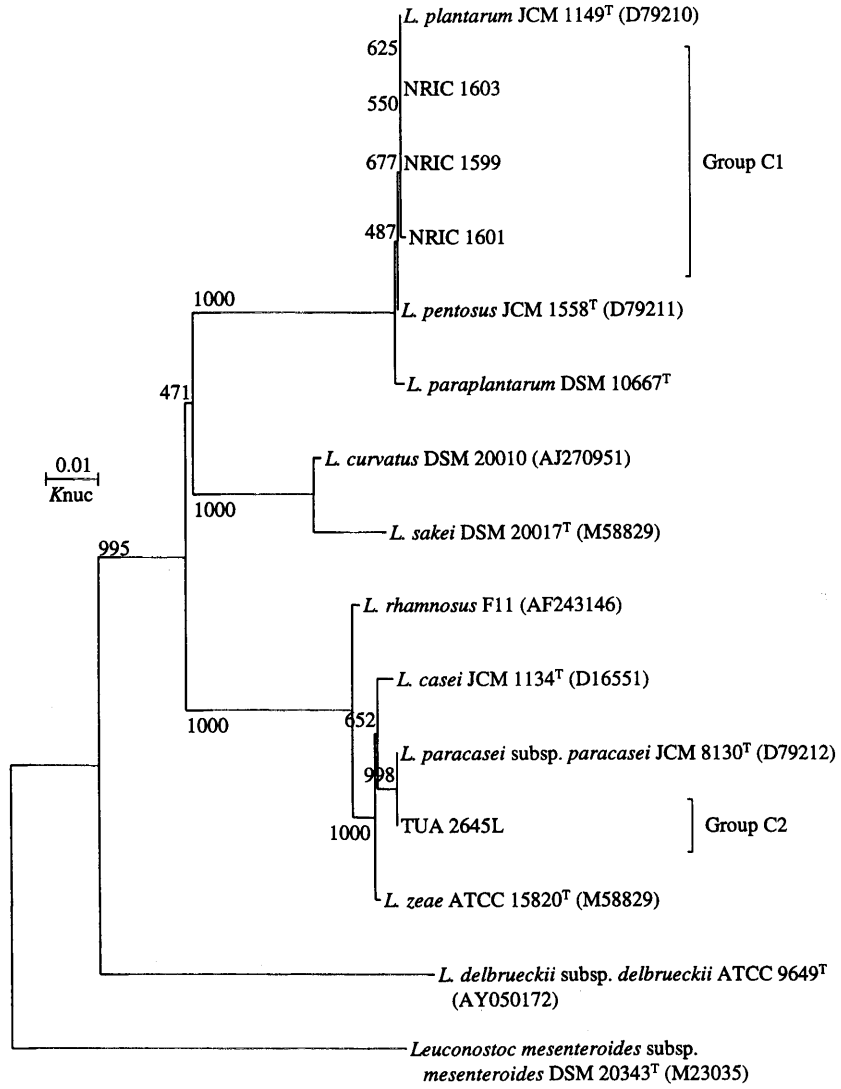

Fig. 1. Phylogenic relationships of four unidentified strains (Group C) based on 16S rRNA gene sequences.
The scale bar represents 1 nucleotide substitution per 100 nucleotides. Numericals indicate bootstrap values derived from 1,000 replications.

(Table 4). Only *L. sakei* NRIC 1764 (the former *L. bavaricus* strain) produced L-lactic acid (L-form to D-form=9:1), regardless of the presence of 50 mM sodium acetate (Group A2). Seven confirmed *L. curvatus* strains produced DL-lactic acid (L-form to D-form=1:1), regardless of the presence of 50 mM sodium acetate (Group B). NRIC 1599, NRIC 1601, and NRIC 1603 produced DL-lactic acid (L-form to D-form=2:3), regardless of the presence of 50 mM sodium acetate (Group C1), and TUA 2645L produced L-lactic acid (L-form to D-form=9:1) (Group C2).

Discussion

The strains studied were finally separated into five groups A1, A2, B, C1, and C2 by DNA-DNA similarity, 16S rRNA gene sequences, and the change of the type of stereoisomers of lactic acid produced in the presence of 50 mM sodium acetate. The strains in each group showed a good correlation with phenotypic characteristics.

DNA-DNA similarity showed that most of isolates from sake starters were confirmed as *L. sakei*, and a few isolates as *L. curvatus*. *L. sakei* has been reported to be closely related to *L. curvatus* phenotypically and phylogenetically (Collins et al., 1991; Kandler and

Table 3. Phenotypic characteristics of strains studied.

Groups	Species	Nos. of strains	Cell form	Gram staining[a]	Catalase reaction[a]	Gas from glucose[a]	Growth at[a]				Acid production from[a]									
							15°C	20°C	40°C	45°C	L-Arabinose	D-Glucose	Maltose	D-Mannose	Melibiose	D-Ribose	Sucrose	D-Trehalose	D-Mannitol	D-Sorbitol
	L. sakei	NRIC 1071^T	rod	+	−	−	+	+	+	−	+	+	+	+	+	+	+	+	−	−
	L. sakei	NRIC 1602	rod	+	−	−	+	+	−	−	−	+	w	+	+	+	+	+	−	−
	L. sakei	NRIC 1604	rod	+	−	−	+	+	−	−	−	+	−	+	+	+	+	+	−	−
	L. sakei	NRIC 1606	rod	+	−	−	+	+	−	−	+	+	+	+	+	+	+	−	−	−
	L. sakei	NRIC 1608	rod	+	−	−	+	+	−	−	+	+	+	+	+	+	+	+	−	−
	L. sakei	NRIC 1609	rod	+	−	−	+	+	−	−	+	+	−	+	+	+	+	+	−	−
	L. sakei	NRIC 1610	rod	+	−	−	+	+	−	−	+	+	+	+	+	+	+	+	−	−
	L. sakei	NRIC 1611	rod	+	−	−	+	+	−	−	+	+	+	+	+	+	+	+	−	−
	L. sakei	NRIC 1764	rod	+	−	−	+	+	+	−	−	+	−	+	+	+	+	−	−	−
	(the former *Lactobacillus bavaricus* strain, Type strain of this species)																			
Group A	*L. sakei*	No. 14	rod	+	−	−	+	+	+	−	+	+	+	+	+	+	+	+	−	−
	L. sakei	No. 16	rod	+	−	−	+	+	+	−	+	+	−	+	+	+	+	+	−	−
	L. sakei	No. 17	rod	+	−	−	+	+	+	−	+	+	−	+	+	+	+	+	−	−
	L. sakei	No. 18	rod	+	−	−	+	+	+	−	+	+	−	+	+	+	+	+	−	−
	L. sakei	No. 19	rod	+	−	−	+	+	+	−	+	+	−	+	+	+	+	+	−	−
	L. sakei	No. 21	rod	+	−	−	+	+	+	−	+	+	−	+	+	+	+	+	−	−
	L. sakei	No. 22	rod	+	−	−	+	+	+	−	+	+	−	+	+	+	+	+	−	−
	L. sakei	No. 23	rod	+	−	−	+	+	+	−	+	+	−	+	+	+	+	−	−	−
	L. sakei	No. 26	rod	+	−	−	+	+	+	−	+	+	−	+	+	+	+	+	−	−
	L. sakei	No. 27	rod	+	−	−	+	+	+	−	+	+	−	+	+	+	+	+	−	−
	L. sakei	No. 29	rod	+	−	−	+	+	+	−	+	+	−	+	+	+	+	+	−	−
	L. curvatus	NRIC 1052^T	rod	+	−	−	+	+	+	−	−	+	+	+	−	+	−	−	−	−
	L. curvatus	NRIC 1600	rod	+	−	−	+	+	−	−	−	+	+	+	−	+	−	−	−	−
	L. curvatus	IFO 12456	rod	+	−	−	+	+	+	−	−	+	+	+	−	+	+	−	−	−
Group B	*L. curvatus*	TUA 2646L	rod	+	−	−	+	+	+	−	−	+	+	+	−	+	+	+	−	−
	L. curvatus	TUA 2647L	rod	+	−	−	+	+	+	−	−	+	+	+	−	+	−	+	−	−
	L. curvatus	TUA 2648L	rod	+	−	−	+	+	+	−	−	+	+	+	−	+	−	−	−	−
	L. curvatus	TUA 2649L	rod	+	−	−	+	+	+	−	−	+	+	+	−	+	+	−	−	−
	Unidentified[b]	NRIC 1599	rod	+	−	−	+	+	+	−	w	+	+	+	+	+	+	+	+	+
Group C1	Unidentified[b]	NRIC 1601	rod	+	−	−	+	+	+	−	w	+	+	+	+	+	+	+	+	+
	Unidentified[b]	NRIC 1603	rod	+	−	−	+	+	+	−	w	+	+	+	+	+	+	+	+	+
Group C2	Unidentified[c]	TUA 2645L	rod	+	−	−	+	+	+	−	−	+	+	+	w	+	+	+	+	+

^{T} Type strain.

[a] +, positive; w, weakly positive; −, negative.

[b] Included in the *L. plantarum*/*L. pentosus*/*L. paraplantarum* cluster deduced from 16S rRNA gene sequence (Fig. 1).

[c] Included in the *L. paracasei* subsp. *paracasei* cluster deduced from 16S rRNA gene sequence (Fig. 1).

Weiss, 1986). In addition, phenotypic intermediate strains between *L. sakei* and *L. curvatus* were frequently isolated from vacuum packaged meat and dry fermented sausages. They have been referred to the *L. sakei*/*L. curvatus* group (Grant and Peterson, 1991; Hasting and Holzapfel, 1987; Hitchener et al., 1982; Hugas et al., 1993; Niemand and Holzapfel, 1984; Reuter, 1992). This indicates the difficulty in the identi-

Table 4. The change of the type of stereoisomers of lactic acid produced by strains studied in the presence of 50 mM sodium acetate.

Groups	Species	Nos. of strains	Type of stereoisomers		Sources
			GYP[a]	GYP-Ac[b]	
Group A1	*L. sakei*	NRIC 1071[T]	DL[e]	L[e]	Sake starter
	L. sakei	NRIC 1602	DL	L	Sake starter
	L. sakei	NRIC 1604	DL	L	Sake starter
	L. sakei	NRIC 1606	DL	L	Sake starter
	L. sakei	NRIC 1608	DL	L	Sake starter
	L. sakei	NRIC 1609	DL	L	Sake starter
	L. sakei	NRIC 1610	DL	L	Sake starter
	L. sakei	NRIC 1611	DL	L	Sake starter
	L. sakei	No. 14	DL[e]	L[e]	Sake starter
	L. sakei	No. 16	DL[e]	L[e]	Sake starter
	L. sakei	No. 17	DL[e]	L[e]	Sake starter
	L. sakei	No. 18	DL[e]	L[e]	Sake starter
	L. sakei	No. 19	DL[e]	L[e]	Sake starter
	L. sakei	No. 21	DL[e]	L[e]	Sake starter
	L. sakei	No. 22	DL[e]	L[e]	Sake starter
	L. sakei	No. 23	DL[e]	L[e]	Sake starter
	L. sakei	No. 26	DL[e]	L[e]	Sake starter
	L. sakei	No. 27	DL[e]	L[e]	Sake starter
	L. sakei	No. 29	DL[e]	L[e]	Sake starter
Group A2	*L. sakei* (the former *Lactobacillus bavaricus* strain, Type strain of this species)	NRIC 1764	L[e]	L[e]	Sauerkraut
Group B	*L. curvatus*	NRIC 1052[T]	DL[e]	DL[e]	Milk
	L. curvatus	NRIC 1600	DL	DL	Sake starter
	L. curvatus	IFO 12456	DL	DL	Sake starter
	L. curvatus	TUA 2646L	DL	DL	Raw sausage
	L. curvatus	TUA 2647L	DL	DL	Raw sausage
	L. curvatus	TUA 2648L	DL	DL	Raw sausage
	L. curvatus	TUA 2649L	DL	DL	Raw sausage
Group C1	Unidentified[c]	NRIC 1599	DL	DL	Sake starter
	Unidentified[c]	NRIC 1601	DL	DL	Sake starter
	Unidentified[c]	NRIC 1603	DL	DL	Sake starter
Group C2	Unidentified[d]	TUA 2645L	L	L	Raw sausage

[T] Type strain.
[a] GYP: GYP medium.
[b] GYP-Ac: GYP medium containing 50 mM sodium acetate.
[c] Included in the *L. plantarum*/*L. pentosus*/*L. paraplantarum* cluster deduced from 16S rRNA gene sequence (Fig. 1).
[d] Included in the *L. paracasei* subsp. *paracasei* cluster deduced from 16S rRNA gene sequence (Fig. 1).
[e] Cited from Iino et al., *J. Gen. Appl. Microbiol.*, **47**, 223–239 (2001).

fication of *L. sakei* and *L. curvatus.* Moreover, NRIC 1599, NRIC 1601, and NRIC 1603 were related to *L. plantarum* JCM1149^T/*L. pentosus* JCM 1558^T/*L. paraplantarum* DSM 10667^T on the basis of 16S rRNA gene sequences. *L. sakei* was described to be related to *L. plantarum* when NRIC 1599, NRIC 1601, and NRIC 1603 were isolated from sake starters (Rogosa, 1974; Toyoda et al., 1979).

L. sakei NRIC 1764 had been previously named *L. bavaricus*, which was differentiated from *L. sakei* and *L. curvatus* by the production of L-lactic acid and no production of DL-lactic acid (Kandler and Weiss, 1986). However, *L. bavaricus* was described as a junior synonym of *L. sakei* because of the high level of DNA-DNA similarity with *L. sakei* (Kagaermeir-Callaway and Lauer, 1995; Klein et al., 1996; Torriani et al., 1996). In this study, *L. sakei* NRIC 1764 (the former *L. bavaricus* strain) showed a high level of DNA-DNA similarity with *L. sakei* NRIC 1071^T, and the type of stereoisomers of lactic acid produced by this strain was the L-type, regardless of the presence of sodium acetate.

L. sakei IFO 12456 (S. Fukui, G01) was once used for the study of lactate racemase (Kitahara et al., 1957), but confirmed as *L. curvatus* by DNA-DNA similarity, not as *L. sakei* in this study. In addition, the type of stereoisomers of lactic acid produced by *L. sakei* IFO 12456 did not shift from the DL-type to the L-type in the presence of sodium acetate.

The *L. sakei* strains were separated into two groups: DL-former such as *L. sakei* NRIC 1071^T and other *L. sakei* strains from sake starters and L-former such as *L. sakei* NRIC 1764 from sauerkraut (the former *L. bavaricus* strain). The majority of *L. sakei* strains shifted the type of stereoisomers of lactic acid from the DL-type to the L-type in this study. *L. sakei* NRIC 1764 (the former *L. bavaricus* strain) was the only L-former in *L. sakei* strains. Such strains would be isolated in the future and form a substantial group in consequence. This strain is considered to be a strain for which D-LDH was inactivated or lost because this strain produced scarcely any D-lactic acid. Confirmed *L. curvatus* strains from milk, sake starters, or raw sausages, and three strains from sake starters related to *L. plantarum* JCM1149^T/*L. pentosus* JCM 1558^T/*L. paraplantarum* DSM 10667^T produced the DL-type of stereoisomers, regardless of the presence of sodium acetate. Moreover, TUA 2645L from raw sausages related to *L. paracasei* subsp. *paracasei* JCM 8130^T produced the L-type of stereoisomers, regardless the presence of sodium acetate.

The identification of *L. sakei* and *L. curvatus* by phenotypic characteristics was reported to be troublesome, and further intermediate strains between the two species were frequently isolated from vacuum packaged meat and dry fermented sausages. Therefore, various identification systems have been proposed for *L. sakei* and *L. curvatus* (Berthier and Ehrlich, 1999; Döring et al., 1988; Dykes and von Holy, 1994; Hertel et al., 1991; Kandler and Weiss, 1986; Montel et al., 1991; Schillinger and Lücke, 1987). The change of the type of stereoisomers of lactic acid from the DL-type to the L-type in the presence of sodium acetate is species-specific for *L. sakei*, except for a strain previously named *L. bavaricus*, and this characteristic is useful for identification of strains in this species.

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