Lactobacillus kefirgranum sp. nov. and *Lactobacillus parakefir* sp. nov., Two New Species from Kefir Grains

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Twelve strains of homofermentative lactobacilli and two strains of heterofermentative lactobacilli were isolated from kefir grains by using R-CW agar medium. The physiological and biochemical characteristics, DNA guanine-plus-cytosine contents, and levels of DNA-DNA relatedness of these isolates and previously described lactobacilli were compared. Our results indicated that two new species, *Lactobacillus kefirgranum* and *Lactobacillus parakefir*, could be distinguished. The type strain of *L. kefirgranum* sp. nov. is GCL 1701 (= JCM 8572), and the type strain of *L. parakefir* sp. nov. is GCL 1731 (= JCM 8573).

The microorganisms in kefir grains have been studied by many workers (6, 11, 14, 16–18). Kandler and Kunath (7) described *Lactobacillus kefir*, a new species of heterofermentative lactobacilli isolated from kefir grains and kefir. Toba et al. (21) developed a medium for isolation of bacteria which produce a capsular polysaccharide that previously had been named kefiran by Kooiman (10) and La Riviere and Kooiman (11). Fujisawa et al. (4) also studied bacteria isolated from kefir grains and described a new species, *Lactobacillus kefirnofaciens*. The fact that there have been many studies shows

MATERIALS AND METHODS

Bacterial strains. Twelve homofermentative strains and two heterofermentative strains were isolated from kefir grains obtained from E. C. Hansen's Laboratory in Copenhagen, Denmark. Reference *Lactobacillus* strains were obtained from the Japan Collection of Microorganisms, Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama, Japan.

Isolation of bacteria from kefir grains. Active kefir grains were rinsed and diluted with sterile anaerobic diluting solution

FIG. 1. Photomicrographs of two new species of the genus *Lactobacillus* isolated from kefir grains and grown on R-CW agar. (A) *L. kefirgranum* GCL 1701^T. (B) *L. parakefir* GCL 1731^T. Bar = 10 µm.

that the taxonomic relationships among bacterial species in kefir grains have not been investigated completely.

In this paper, we describe the characteristics of bacteria isolated from kefir grains on R-CW medium (9) and propose two new species, *Lactobacillus kefirgranum* and *Lactobacillus parakefir*.

and homogenized with a Teflon homogenizer. Diluted homogenates were plated on R-CW agar (9) and incubated at 30°C for 5 days in an atmosphere containing 100% CO₂. The strains that grew on the plates were subcultured to obtain pure-colony growth and were stored at –80°C after they were subcultured in R-CW broth.

Culture conditions. The strains isolated from kefir grains were cultured on R-CW agar and in R-CW broth at 30°C in an atmosphere containing 100% CO₂. The reference strains were cultured on MRS agar and in MRS broth (2).

Determination of taxonomic characteristics. Cell shape, cell size, cell arrangement, the Gram stain reaction, the India

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FIG. 2. Colony morphology of two new species of the genus *Lactobacillus* isolated from kefir grains and grown at 30°C for 5 days on R-CW agar. (A) *L. kefirgranum* GCL 1701^T. (B) *L. parakefir* GCL 1731^T.

ink-staining properties, and colonial appearance were determined by using cells grown on R-CW agar plates for 5 days at 30°C.

To determine acid production from carbohydrates, R-CW broth (in which lactose in cheese whey was digested by *Candida kefir* GCL 5401) was used as the basal medium. The carbon sources were added to the sterile basal medium as filter-sterilized solutions to a final concentration of 0.5%. The pH of each preparation was measured after incubation at 30°C for 10 days by using an automatic pH-measuring system (model BIS-120; Lifetec Co., Ltd., Saitama, Japan). Production of gas from glucose and gluconate was examined with Durham tubes.

Hydrolysis of esculin, catalase formation, growth at 15 and 45°C, and motility were tested by methods previously described by Mitsuoka (15), except that lactose-digested R-CW medium was used as the basal medium. To determine production of ammonia from arginine, we used medium containing 10 g of L-arginine monohydrochloride, 0.5 g of tryptone (Difco), 0.5 g of Trypticase (BBL), 5 g of sodium chloride, 0.3 g of KH₂PO₄, 0.01 g of phenol red, 3 g of Bacto Agar (Difco) and 1,000 ml of cheese whey.

An F-Kit with L-lactate and D-lactate dehydrogenases (Boehringer Mannheim Yamanouchi Ltd., Tokyo, Japan) was used to determine L-(+)- and D-(-)-lactic acids.

TABLE 1. Biochemical and physiological characteristics of *L. kefirgranum* sp. nov. and *L. parakefir* sp. nov. isolated from kefir grains^a

Strain	Growth at 15°C	Gas production from glucose	NH ₃ production from arginine	Hydrolysis of esculin	Acid production from:													Stereoisomer of lactic acid
					Amygdalin	Arabinose	Cellobiose	Esculin	Fructose	Mannose	Melezitose	Melibiose	Raffinose	Ribose	Salicin	Sucrose	Trehalose	
<i>L. kefirgranum</i> strains ^c																		
GCL 1701	w	-	-	+	+	-	+	-	+	+	+	-	+	+	-	+	+	D (L)
GCL 1702	w	-	-	+	-	-	-	+	+	+	+	-	+	+	-	+	+	D (L)
GCL 1703	w	-	-	+	-	-	-	+	+	+	+	-	+	+	-	+	+	D (L)
GCL 1704	w	-	-	+	-	-	-	+	-	+	+	-	+	+	-	+	+	D
GCL 1705 ^T	w	-	-	+	-	-	-	+	-	+	+	-	+	+	-	+	+	D
GCL 1706	w	-	-	+	-	-	-	+	-	+	+	-	+	+	-	+	+	D (L)
GCL 1707	w	-	-	+	-	-	-	+	-	+	+	-	+	+	-	+	+	D (L)
GCL 1708	w	-	-	+	-	-	-	-	-	+	+	-	+	+	-	+	+	D (L)
GCL 1709	w	-	-	+	-	-	-	-	-	+	+	-	+	+	-	+	+	D (L)
GCL 1710	w	-	-	+	-	-	-	-	-	+	+	-	+	+	-	+	+	D (L)
GCL 1711	w	-	-	+	-	-	-	-	-	+	+	-	+	+	-	+	+	D (L)
GCL 1712	w	-	-	+	-	-	-	-	-	+	+	-	+	-	-	+	+	L (D)
<i>L. parakefir</i> strains																		
GCL 1731 ^T	+	+	+	-	-	+	-	-	-	-	-	+	+	-	+	-	-	L
GCL 1732	+	+	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	L

^a +, positive reaction; -, negative reaction; w, weakly positive reaction. All strains produced acid from galactose, glucose, maltose, and lactose. No acidification occurred on gluconate, mannitol, rhamnose, sorbitol, and xylose. No isolate produced gas from gluconate. No growth occurred at 45°C.

^b Based on the content of L-(+)-lactic acid: D, 0 to 20%; D(L), 20 to 40%; L(D), 60 to 80%; L, 80 to 100%.

^c *L. kefirgranum* GCL 1701, GCL 1703, GCL 1705^T, GCL 1708, GCL 1709, and GCL 1712 formed a flocculent sediment. Strains GCL 1702, GCL 1704, GCL 1706, GCL 1707, GCL 1710, and GCL 1711 formed a powdery sediment in broth cultures.

TABLE 2. DNA G+C contents and levels of DNA-DNA relatedness for *L. kefirgranum* strains and some reference *Lactobacillus* strains

Strain	G+C content (mol%)	% DNA-DNA relatedness to:		
		<i>L. kefirgranum</i> GCL 1701 ^T	<i>L. amylovorus</i> JCM 1126 ^T	<i>L. helveticus</i> JCM 1120 ^T
<i>L. kefirgranum</i>				
GCL 1701 ^T	38.1	100	35	42
GCL 1702	38.1	103		
GCL 1703	38.4	89		
GCL 1704	37.9	111		
GCL 1705	38.6	105		
GCL 1706	34.3	101		
GCL 1707	37.5	100		
GCL 1708	37.4	98		
GCL 1709	38.6	98		
GCL 1710	37.7	104		
GCL 1711	38.2	106		
GCL 1712		106		
<i>L. acidophilus</i> JCM 1132 ^T	32.6	26	30	
<i>L. acidophilus</i> JCM 2009	34.3	38		
<i>L. amylovorus</i> JCM 1126 ^T	38.6	51	100	41
<i>L. amylovorus</i> JCM 2010	37.1	14	31	
<i>L. animalis</i> JCM 5670 ^T	39.7	2		
<i>L. crispatus</i> JCM 1185 ^T	37.0	24		
<i>L. delbrueckii</i> subsp. <i>delbrueckii</i> JCM 1012 ^T	48.9	3		
<i>L. delbrueckii</i> subsp. <i>lactis</i> JCM 1248 ^T	50.2	5		
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> JCM 1002 ^T	51.6	4		
<i>L. gallinarum</i> JCM 2011 ^T	35.1	13	15	
<i>L. gasseri</i> JCM 1131 ^T	34.8	10	11	
<i>L. helveticus</i> JCM 1120 ^T	35.5	42		100
<i>L. helveticus</i> JCM 1003	37.8	43		92
<i>L. helveticus</i> JCM 1008	37.0	32		
<i>L. jensenii</i> JCM 1146 ^T	42.7	5		
<i>L. johnsonii</i> JCM 2012 ^T	32.4	3	5	
<i>L. kefiranoferiens</i> JCM 6985 ^T	36.0	30		
<i>L. salivarius</i> JCM 1231 ^T	30.4	3		

Cell wall analysis. The presence of diaminopimelic acid isomers in the cell wall was examined by the method of Stanek and Roberts (19).

DNA base composition and DNA-DNA hybridization. DNA was isolated and purified by the method of Marmur (12) by using cells grown for 12 h in R-CW medium. DNA base composition was calculated from the melting temperature (13) obtained with a spectrophotometer (model DU-8B; Beckman

Instruments, Inc., Fullerton, Calif.). DNA from calf thymus (G+C content, 42 mol%) was used as the reference DNA. DNA-DNA hybridization experiments were performed by using the procedure described by Suzuki et al. (20). DNAs were labeled with a model 5500 nick translation kit (Amersham).

RESULTS

Morphology of the isolates. The cells of all of the isolates obtained from kefir grains were gram-positive, nonmotile, non-spore-forming rods. These cells occurred singly, in pairs, or occasionally in short chains (Fig. 1). After incubation on R-CW agar plates for 5 days, colonies of *L. kefirgranum* were 0.5 to 3.0 mm in diameter, circular to irregular, convex, opaque, smooth to rough, and white, and colonies of *L. parakefir* were 0.5 to 2.0 mm in diameter, circular to irregular, flat, opaque, and white (Fig. 2).

Biochemical and physiological characteristics. Characteristics of the isolates are shown in Table 1. *L. kefirgranum* was similar to species belonging to the *Lactobacillus acidophilus* group, such as *Lactobacillus gallinarum* and *Lactobacillus johnsonii*, with respect to G+C content and physiological characteristics, but did not grow in the presence of 4% NaCl. *L. parakefir* was similar to *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus parabuchneri*, and *L. kefir* in its pattern of acid production from carbohydrates, but differed from these taxa by producing the L-(+) isomer of lactic acid and by not fermenting gluconate.

Chemotaxonomic characteristics. The isolates which we

TABLE 3. DNA G+C contents and levels of DNA-DNA relatedness for *L. parakefir* strains and some reference *Lactobacillus* strains

Strain	G+C content (mol%)	% DNA-DNA relatedness to:	
		<i>L. parakefir</i> GCL 1731 ^T	<i>L. kefir</i> JCM 5818 ^T
<i>L. parakefir</i>			
GCL 1731 ^T	42.0	100	35
GCL 1732	41.4	100	
<i>L. brevis</i> JCM 1059 ^T	45.0	4	
<i>L. buchneri</i> JCM 1115 ^T	42.7	36	
<i>L. collinoides</i> JCM 1123 ^T	44.7	16	
<i>L. fermentum</i> JCM 1173 ^T	49.4	7	
<i>L. hilgardii</i> JCM 1155 ^T	39.1	29	
<i>L. kefir</i> JCM 5818 ^T	39.7	38	100
<i>L. malefermentans</i> JCM 1167 ^T	39.9	25	
<i>L. reuteri</i> JCM 1112 ^T	38.0	39	

TABLE 4. Phenotypic characteristics that differentiate *L. kefirgranum* sp. nov. and *L. parakefir* sp. nov. from closely related *Lactobacillus* species^a

Species	Growth at 15°C	Growth at 45°C	Gas production from glucose	Gas production from gluconate	NH ₃ from arginine	Hydrolysis of esculin	Tolerance to 4% NaCl	Acid production from:																Stereoisomer of lactic acid ^b						
								Amygdalin	Arabinose	Cellobiose	Esculin	Fructose	Galactose	Glucose	Gluconate	Lactose	Maltose	Mannitol	Mannose	Melezitose	Melibiose	Raffinose	Rhamnose		Ribose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose
Homofermentative																														
<i>Lactobacillus</i> spp.																														
<i>L. kefirgranum</i>	w	-	-	-	-	+	-	-	-	d	d	+	+	+	-	+	+	-	+	+	-	-	d	-	d	d	-	D (L)		
<i>L. gallinarum</i> ^c	+	-	-	-	ND	ND	+	+	-	+	+	+	+	+	ND	d	+	-	+	+	-	-	+	-	+	-	-	L		
<i>L. johnsonii</i> ^c	+	-	-	-	ND	ND	+	+	-	+	+	+	+	+	ND	d	+	-	+	+	-	-	+	-	+	+	-	L		
<i>L. farciminis</i> ^d	+	-	-	-	+	ND	+	+	-	+	+	+	+	+	-	+	+	-	+	-	-	-	+	-	+	+	-	L (D)		
<i>L. acidophilus</i> ^d	-	+	-	-	-	+	-	+	-	+	+	+	+	+	-	+	+	-	d	d	-	-	+	-	+	d	-	DL		
<i>L. amylovorus</i> ^d	-	+	-	-	-	+	-	+	-	+	+	+	+	+	-	+	+	-	-	-	-	-	+	-	+	+	-	DL		
<i>L. crispatus</i> ^d	-	+	-	-	-	+	ND	+	-	+	+	+	+	+	-	+	+	-	-	-	-	-	+	-	+	-	-	DL		
<i>L. gasseri</i> ^d	-	+	-	-	-	+	ND	+	-	+	+	+	+	+	-	d	d	-	-	d	d	-	-	+	-	d	-	DL		
<i>L. helveticus</i> ^d	-	+	-	-	-	-	ND	-	-	-	-	d	+	+	-	+	d	-	d	-	-	-	-	-	+	d	-	DL		
<i>L. jensenii</i> ^d	-	+	-	-	-	+	ND	+	-	+	+	+	+	+	-	-	d	d	+	-	-	-	-	+	-	+	+	D		
<i>L. salivarius</i> ^d	-	+	-	-	-	d	ND	-	-	-	d	+	+	+	-	+	+	+	-	+	+	d	-	d	-	+	+	L		
<i>L. animalis</i> ^d	-	+	-	-	-	+	ND	d	d	+	+	+	+	+	-	+	+	-	+	+	-	-	+	-	+	+	-	L		
<i>L. kefirnofaciens</i> ^e	-	-	-	-	-	-	ND	-	-	-	-	+	+	+	-	+	+	-	+	+	-	-	-	-	+	-	-	D (L)		
Heterofermentative																														
<i>Lactobacillus</i> spp.																														
<i>L. parakefir</i>	+	-	+	-	+	-	ND	-	+	-	-	-	+	+	-	+	+	-	-	d	d	-	-	+	-	-	-	L		
<i>L. brevis</i> ^d	+	-	+	+	+	d	ND	-	+	-	d	+	d	+	+	d	+	-	-	+	d	-	+	-	-	d	-	d	DL	
<i>L. buchneri</i> ^d	+	-	+	+	+	d	ND	-	+	-	d	+	d	+	+	d	+	-	-	+	+	d	-	+	-	-	d	-	d	DL
<i>L. parabuchneri</i> ^f	+ND	+	+	ND	+	-	ND	-	+	-	ND	+	+	+	+	V	V	ND	+	+	+	-	+	-	+	-	-	-	ND	
<i>L. kefir</i> ^d	+	-	+	+	+	-	ND	-	d	-	-	-	+	+	+	+	+	+	-	-	+	+	-	+	-	-	-	-	DL	
<i>L. collinoides</i> ^d	+	-	+	+	+	+	ND	-	+	-	+	+	+	+	+	d	+	-	-	+	-	-	+	d	-	-	-	+	DL	
<i>L. fermentum</i> ^d	-	-	+	+	+	-	ND	-	d	d	-	+	+	+	+	+	+	-	+	+	+	-	+	-	-	+	d	d	DL	
<i>L. hilgardii</i> ^d	+	-	+	+	+	-	ND	-	-	-	-	+	d	+	+	d	+	-	-	d	-	-	+	-	-	d	-	+	DL	
<i>L. reuteri</i> ^d	-	-	+	+	+	ND	ND	ND	+	-	ND	+	+	+	+	+	+	-	-	+	+	-	+	-	-	+	-	-	DL	
<i>L. malefermentans</i> ^f	+	-	+	d	+	-	ND	-	-	-	-	-	-	+	d	-	+	-	-	-	-	-	+	-	-	-	-	-	ND	

^a +, 90% or more of the strains are positive; -, 90% or more of the strains are negative; d, 11 to 89% of the strains are positive; ND, not done; V, variable reaction; w, weak reaction.

^b Based on the content of L-(+)-lactic acid: D, 0 to 20%; D(L), 20 to 40%; DL, 40 to 60%; L(D), 60 to 80%; L, 80 to 100%.

^c Data from reference 5.

^d Data from reference 8.

^e Data from reference 4.

^f Data from reference 3.

studied did not contain diaminopimelic acid isomers. This indicates that *L. parakefir*, which produces L-(+)-lactic acid, also differs from *Carnobacterium* spp. (1), which contain meso-diaminopimelic acid.

DNA base composition and DNA-DNA hybridization. Tables 2 and 3 show the G+C contents and DNA-DNA hybridization data for the isolates which we studied and some reference species. *L. kefirgranum* GCL 1701^T (T = type strain) exhibited high levels of DNA relatedness to all other strains of *L. kefirgranum*, but little or no relatedness to reference strains. The level of DNA relatedness between *L. parakefir* GCL 1731^T and strain GCL 1732 was 100%, whereas all of the reference *Lactobacillus* strains used for comparison exhibited only very low levels of relatedness to *L. parakefir* GCL 1731^T.

DISCUSSION

The morphological, biochemical, and physiological characteristics of the strains isolated from kefir grains indicate that these organisms belong to the genus *Lactobacillus* (8). DNA hybridization experiments revealed two separate genotypic

groups among the isolates obtained from kefir grains when they were compared with authentic strains of lactobacilli having similar DNA base compositions. All of the previously described strains which we studied exhibited relatively low levels of DNA relatedness to the isolates obtained in this study.

On the basis of our results, we propose that the isolates obtained from kefir grains should be placed in the following two new species of the genus *Lactobacillus*: *Lactobacillus kefirgranum*, a homofermentative *Lactobacillus* species; and *Lactobacillus parakefir*, a heterofermentative *Lactobacillus* species. The characteristics that differentiate these new species from *L. acidophilus*, *Lactobacillus amylovorus*, *Lactobacillus helveticus*, and *L. kefirnofaciens* are shown in Table 4.

Description of *Lactobacillus kefirgranum* sp. nov. *Lactobacillus kefirgranum* (ke.fir.gra'num. Turkish n. kefir, Caucasian sour milk; L. n. granum, grain; M.L. adj. kefirgranum, kefir grain). Cells are gram-positive, nonmotile, non-spore-forming rods that are generally 0.5 to 1.2 by 5.0 to 15.0 µm and occur singly, in pairs, or occasionally in short chains. When the organism is grown on R-CW agar at 30°C for 5 days, colonies are 0.5 to 3.0 mm in diameter, circular to irregular, convex,

opaque, white, and smooth to rough. There is no growth at 45°C, and weak growth occurs at 15°C.

Facultatively anaerobic, producing DL-lactic acid homofermentatively. Hydrogen sulfide, catalase, and oxidase are not produced. Esculin is hydrolyzed by almost all strains. Arginine is not deaminated. No gas is produced from glucose or gluconate. Milk is curdled. Acid is produced from galactose, glucose, fructose, lactose, maltose, mannose, and melibiose but not from L-arabinose, gluconate, mannitol, melezitose, L-rhamnose, D-ribose, sorbitol, and D-xylose. Acid production from amygdalin, cellobiose, esculin, raffinose, salicin, sucrose, and trehalose varies with the strain.

The DNA G+C content is 34.3 to 38.6 mol%; the DNA G+C content of the type strain is 38.1 mol%. The habitat is kefir grains.

The type strain is strain GCL 1701 (= JCM 8572).

Description of *Lactobacillus parakefir* sp. nov. *Lactobacillus parakefir* (pa.ra.ke'fir. Gr. prep. *para*, resembling; M.L. n. *kefir*, the specific epithet of *L. kefir*; M.L. adj. *parakefir*, resembling *L. kefir*). Cells are gram-positive, nonmotile, non-spore-forming rods that are generally 0.5 to 1.2 by 1.0 to 3.5 µm and occur singly, in pairs, or occasionally in short chains. When the organism is grown on R-CW agar at 30°C for 5 days, colonies are 0.5 to 2.0 mm in diameter, circular to irregular, flat, opaque, white, and rough. There is no growth at 45°C, and good growth occurs at 15°C.

Facultatively anaerobic. Produces L-lactic acid and CO₂ by heterofermentation. Hydrogen sulfide, catalase, and oxidase are not produced. Arginine is deaminated. Esculin is not hydrolyzed. Gas is produced from glucose, but not from gluconate. Milk is curdled.

Acid is produced from L-arabinose, galactose, glucose, lactose, maltose, and D-ribose but not from amygdalin, cellobiose, esculin, fructose, gluconate, mannose, mannitol, raffinose, L-rhamnose, salicin, sorbitol, sucrose, trehalose, and D-xylose. Acid production from melezitose and melibiose varies with the strain.

The DNA G+C content is 41.4 to 42.0 mol%; the DNA G+C content of the type strain is 42.0 mol%. The habitat is kefir grains.

The type strain is strain GCL 1731 (= JCM 8573).

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