Lactobacillus curvatus subsp. *curvatus* subsp. nov. and
Lactobacillus curvatus subsp. *melibiosus* subsp. nov. and
Lactobacillus sake subsp. *sake* subsp. nov. and *Lactobacillus sake*
subsp. *carnosus* subsp. nov., New Subspecies of *Lactobacillus curvatus*
Abo-Elnaga and Kandler 1965 and *Lactobacillus sake* Katagiri,
Kitahara, and Fukami 1934 (Klein et al. 1996, Emended
Descriptions), Respectively

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Lactobacillus curvatus and *Lactobacillus sake* are each genetically homogeneous species, as indicated by the high levels of DNA homology ($\geq 76\%$) exhibited by strains of these taxa. However, the results of a numerical analysis of total soluble cell protein patterns and biochemical test data revealed that there are two phenotypic subgroups within *L. curvatus* and two phenotypic subgroups within *L. sake*. The overall randomly amplified polymorphic DNA (RAPD)-PCR band patterns obtained for the majority of *L. curvatus* strains corresponded well to the pattern obtained for the type strain of *L. curvatus* (strain DSM 20019). However, six strains of *L. curvatus* had different, but similar, RAPD-PCR profiles and grouped in a separate genetic cluster, which was linked to one of the clusters of *L. sake* strains. On the basis of these results, differences in biochemical and physiological characteristics, and total soluble cell protein profiles, we describe the subspecies *L. curvatus* subsp. *curvatus* subsp. nov. and *L. curvatus* subsp. *melibiosus* subsp. nov. for *L. curvatus* Abo-Elnaga and Kandler 1965 (Klein et al. 1996, emended description). Strains of *L. sake* grouped in two RAPD-PCR clusters, which was consistent with previous reports of phenotypic heterogeneity. Strains of *Lactobacillus bavaricus*, including type strain LMG 9844, clustered with the type strain of *L. sake* (strain NCFB 2714), indicating that these organisms belong to the same genetic group. We propose that strains of *L. sake* Katagiri, Kitahara, and Fukami 1934 (Klein et al. 1996, emended description) should be reclassified as members of *L. sake* subsp. *sake* subsp. nov. and *L. sake* subsp. *carnosus* subsp. nov. Strains of *L. bavaricus* are reclassified as members of *L. sake* subsp. *sake*, and the name *L. bavaricus* Stetter and Stetter 1980 is rejected.

The species *Lactobacillus curvatus* and *Lactobacillus sake* are not closely related genetically, as indicated by the low levels of DNA homology (40 to 50%) between members of the two species (5, 6). However, strains of *L. curvatus* and *L. sake* are closely related phenotypically and differ only in the fermentation of a few carbohydrates (6, 7). Identification of these organisms is also hampered by the phenotypic diversity of strains within *L. curvatus* and *L. sake* (4, 7, 9, 11, 12). Numerical analysis of total soluble cell protein patterns (7) has proven to be the only reliable phenotypic method for differentiating *L. curvatus* from *L. sake*. Two phenotypic subgroups have been described for *L. curvatus* and *L. sake* (7).

Lactobacillus bavaricus is differentiated from *L. curvatus* and *L. sake* by the production of L-(+)-lactic acid and the lack of production of DL-lactic acid (6). Champomier et al. (1) reported low levels of DNA homology between strains of *L. bavaricus* and strains of *L. sake* and *L. curvatus*. However, in the study of Kagermeier-Callaway and Lauer (5), all *L. bavaricus* strains except one exhibited high levels of DNA homology (80 to 100%) with *L. sake*. These results confirmed previous suggestions (6) that *L. bavaricus* strains should be considered

members of a racemase-free subspecies of *L. sake* (or *L. curvatus* in the case of one of the strains). The name *L. bavaricus* has recently been described as a junior synonym of *L. sake* (5).

In this study we determined the genetic relatedness among strains of *L. curvatus*, *L. sake*, and *L. bavaricus* by performing a numerical analysis of randomly amplified polymorphic DNA (RAPD)-PCR profiles.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains included in this study are listed in Table 1. The strains whose designations begin with CTC were received from M. Hugas (Institut de Recerca i Tecnologia Agroalimentàries, Monells, Spain), and the strains whose designations begin with DF were received from F. Dellaglio (Istituto Policattedra, Università degli Studi di Verona, Verona, Italy). The remaining strains were received from G. Reuter (Institute of Meat Hygiene and Technology, Free University of Berlin, Berlin, Germany); the majority of these strains were included in the study of Klein et al. (7). Reference strains were obtained from the culture collection of the Laboratorium voor Mikrobiologie in Ghent, Belgium, the Deutsche Sammlung von Mikroorganismen, and the National Collection of Food Bacteria. All of the strains were grown in MRS broth (3) at 30°C.

DNA-DNA hybridizations. Cultures were grown in MRS broth (3) for 48 h at 30°C. The DNAs were isolated and hybridized and the DNA homology values were calculated as described by Dellaglio et al. (2).

RAPD-PCR analysis. Total bacterial DNAs were isolated from strains by the method of Dellaglio et al. (2). DNA was amplified with a Biometra Trio-thermoblock apparatus. Each reaction mixture contained 25 µl of PCR buffer (50 mM KCl, 2 mM MgCl₂, 0.1% Triton X-100, 10 mM Tris-HCl [pH 8.3]), each

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TABLE 1. Phenotypic and genetic relatedness among *L. curvatus*, *L. sake*, and *L. bavaricus*

Taxon	Strain(s)	% DNA-DNA homology with DNA from ^a :		Biotype ^b	Protein group ^c	RAPD-PCR cluster
		<i>L. sake</i> DSM 20017 ^T	<i>L. curvatus</i> ATCC 20019 ^T			
<i>L. curvatus</i> subgroup I ^d	LMG 9198 ^T (= DSM 20019 ^T)	45	100	A8	CI	I
	W 55d	40	89	A5	CI	I
	W 3	ND ^e	ND	A4	CI	I
	R 28c	41	100	A5	CI	I
	R 60a	39	87	A7	CI	I
	W 21N	40	91	C2a	CI	I
	DF 131	30	79	ND	ND	I
	DF 58	48	75	ND	ND	I
	DF 100	50	81	ND	ND	I
	Rv 2eI	40	89	A6	CI	I
	Rv 40 a	ND	ND	A8	CI	I
<i>L. curvatus</i> subgroup II	R 60 ^T	50	84	A2	C II	II
	R 17b, R 7g/b, R 116/3a, R 111g	ND	ND	A2	C II	II
	DF 126	46	81	ND	ND	II
<i>L. sake</i> subgroup II	DF 60	76	20	ND	ND	III
	DF 109	94	40	ND	ND	III
	R 28d/b, R 6a/a	ND	ND	A1	S II	III
	R116/1a	98	51	C1	S II	III
	W 8N	ND	ND	A1	S II	III
	CCUG 30939, CCUG 32077	ND	ND	C	S II	III
	R 14 b/a ^T	92	54	C3	S II	III
<i>L. sake</i> subgroup I	NCFB 2714 ^T	100	39	ND	ND	IV
	R 61b	98	46	A1	S I	IV
	R 41d	84	45	C2	S I	IV
	Nr. 440/2151, Rv 2f/1a	ND	ND	C	S I	IV
<i>L. bavaricus</i>	LMG 9844 ^T , CTC 407, CTC 488, C21W 22N, W 23Nlab, A5W 55, A21R 9g/b	ND	ND	ND	ND	IV

^a Data from this study and reference 7.^b Data from references 7 through 9.^c As determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie blue staining (7).^d Subgroups as defined by Klein et al. (7).^e ND, not determined.

deoxynucleoside triphosphate at a concentration of 200 μ M, 5 pmol of a 10-base primer (Operon Kit L; Operon Technologies, Alameda, Calif.), 40 ng of genomic DNA, and 2.5 U of *Taq* polymerase (Advanced Biotechnologies, West Hampstead, England). Four primers (primers OPL-01 [GGCATGACCT], OPL-02 [TGGGCGTCAA], OPL-04 [GACTGCACAC], and OPL-05, [ACGCAGGCA C]) were used. Each 25- μ l mixture was covered by 30 μ l of light mineral oil. The cycling program used was 45 cycles consisting of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. The final incubation step consisted of 72°C for 5 min, and this was followed by cooling to 4°C until samples were retrieved. Amplification products were analyzed by electrophoresis in 1.4% agarose gels by using TAE buffer (10). Lambda DNA digested with *Eco*RI and *Hind*III (Boehringer Mannheim) was used as the molecular weight marker.

Numerical analysis of RAPD-PCR profiles. A numerical analysis of the RAPD-PCR profiles was performed as described by Van Reenen and Dicks (14), using the CLUSTER program of SAS Institute, Inc. (13). Dendrograms were constructed from the normalized average-linkage cluster analysis data. Distances between clusters were expressed in R^2 values.

RESULTS AND DISCUSSION

The RAPD-PCR profiles obtained for *L. curvatus*, *L. sake*, and *L. bavaricus* are shown in Fig. 1. Eighteen *L. curvatus* strains grouped into two well-defined RAPD-PCR clusters (clusters I and II), which were distinct from *L. sake* strains (clusters III and IV) and *L. bavaricus* strains in cluster IV (Fig. 2).

Different RAPD-PCR profiles were obtained for strains which grouped in clusters I and II, as shown in Fig. 1. Ten *L.*

curvatus strains grouped at R^2 values of ≥ 0.50 with the type strain of *L. curvatus* (strain LMG 9198 [= DSM 20019]) in cluster I (Fig. 2). Five *L. curvatus* strains formed a tight cluster at R^2 values of ≥ 0.80 with strain R 60 (Fig. 2, cluster II), the type strain of *L. curvatus* subgroup II (7), and this cluster was linked to one of the *L. sake* clusters (cluster III). The overall RAPD-PCR profiles of cluster I strains were similar, but they were not as similar to each other as the profiles obtained for cluster II strains were, indicating that cluster I is a genetically less homogeneous group than cluster II. Furthermore, cluster I contained strains belonging to six biotypes (biotypes A8, A5, A4, A7, C2a, and A6) (Table 1). All of the cluster II strains belonged to the same biotype (biotype A2). Cluster I strains did not ferment melibiose, whereas cluster II strains were melibiose positive (7).

The grouping of *L. curvatus* strains in two separate RAPD-PCR clusters (Fig. 2) confirmed the results obtained when a numerical analysis of total soluble cell protein patterns was performed (7). *L. curvatus* strains exhibit high levels of DNA homology (Table 1), indicating that this species is genetically homogeneous. However, on the basis of the results of biochemical tests, sugar fermentation reactions, and numerical analyses of RAPD-PCR profiles and total soluble cell protein patterns, *L. curvatus* should be divided into subspecies. Below

FIG. 1. (A and B) DNA fragments obtained after RAPD-PCR analyses of the genomic DNAs of *L. curvatus*, *L. sake*, and *L. bavaricus* strains performed with primers OPL-01 (GGCATGACCT) (A) and OPL-02 (TGGGCGTCAA) (B). (C and D) DNA fragments obtained after RAPD-PCR analyses of the genomic DNAs of *L. curvatus*, *L. sake*, and *L. bavaricus* strains performed with primers OPL-04 (GACTGCACAC) (C) and OPL-05 (ACGCAGGCAC) (D).

we describe the subspecies *L. curvatus* subsp. *curvatus* subsp. nov. (type strain, DSM 20019) and *L. curvatus* subsp. *melibiosus* subsp. nov. (type strain, R 60) for the species *L. curvatus* Abo-Elnaga and Kandler 1965 (Klein et al. 1996, emended description).

L. sake is a genetically homogeneous species, as revealed by the high levels of DNA homology (76 to 100%) obtained for strains belonging to clusters III and IV (Table 1). However, 14 *L. sake* strains grouped into two well-defined RAPD-PCR profile clusters (Fig. 2), confirming the groups obtained by a numerical analysis of total soluble cell protein profiles (7). Eight

L. sake strains grouped with strain R 14b/a, the proposed type strain of *L. sake* subgroup II (7), at R^2 values of ≥ 0.60 (Fig. 1, cluster III). Four *L. sake* strains, seven strains previously classified as *L. bavaricus*, and the type strain of *L. sake* (strain NCFB 2714) grouped in cluster IV at R^2 values of ≥ 0.44 (Fig. 2). The cluster IV strains were previously classified in one phenotypic subgroup on the basis of sugar fermentation reactions and total soluble cell protein profiles (7). Strains of *L. bavaricus* were not included in the study of Klein et al. (7). However, the RAPD-PCR profiles of the seven *L. bavaricus* strains, including type strain LMG 9844, corresponded well

FIG. 1. Continued.

with the profiles obtained for *L. sake* NCFB 2714^T (T = type strain) and the other *L. sake* strains in cluster IV (Fig. 1), confirming the suggestion of Kagermeier-Callaway and Lauer (5) that *L. bavaricus* is a junior synonym of *L. sake*.

On the basis of the results of biochemical tests, sugar fermentation reactions, and numerical analyses of RAPD-PCR profiles and total soluble cell protein patterns, we propose that *L. sake* Katagiri, Kitahara, and Fukami 1934 (Klein et al. 1996, emended description) should be divided into two subspecies, *L. sake* subsp. *sake* subsp. nov. (type strain, NCFB 2714) and *L. sake* subsp. *carnosus* subsp. nov. (type strain, R 14b/a). Furthermore, we propose that the name *L. bavaricus* Stetter and Stetter 1980 should be rejected.

Description of *Lactobacillus curvatus* subsp. *curvatus* subsp. nov. *Lactobacillus curvatus* subsp. *curvatus* (cur.va'tus. L. adj. *curvatus*, curved). Cells are curved, bean-shaped rods that have rounded ends (0.7 to 0.9 by 1 to 2 μ m) and occur in pairs or short chains; horseshoe-shaped or closed rings are frequently observed. Usually nonmotile. Some strains are motile, but lose their motility when they are subcultured. No growth occurs at 45°C, but most strains grow at 42 and 4°C; some strains grow at 2°C. Facultatively heterofermentative. DL-Lactic acid is produced. Acid is produced from fructose, galactose, glucose, maltose, mannose, and ribose. Most strains produce acid from amygdalin, cellobiose, sucrose, and trehalose. A few strains produce acid from lactose and salicin. Acid is not produced

FIG. 2. Dendrogram showing the clustering of *L. curvatus*, *L. sake*, and *L. bavaricus* strains based on the results of a numerical analysis of RAPD-PCR profiles (Fig. 1). Clustering was accomplished by performing a normalized average-linkage analysis. The distances between clusters are expressed in R^2 values.

from arabinose, gluconate, inositol, mannitol, melezitose, melibiose, raffinose, rhamnose, sorbitol, and xylose. Ammonia is not produced from arginine. Most strains do not grow in the presence of 10% NaCl and do not produce acetoin from glucose. The guanine-plus-cytosine content of the DNA ranges from 42 to 44 mol%. Isolated from fermented meat products, vacuum-packaged meat, sauerkraut, and silage.

The type strain is strain ATCC 15601 (= DSM 20019). In most respects the description of the type strain resembles the description of the species. The type strain produces acid from cellobiose. Acid is not produced from amygdalin, sucrose, and trehalose.

Description of *Lactobacillus curvatus* subsp. melibiosus subsp. nov. *Lactobacillus curvatus* subsp. *melibiosus* (me.li.bi. o'sus. M. L. adj. *melibiosus*, pertaining to melibiose). Cells are curved, bean-shaped rods that have rounded ends (0.7 to 0.9 by 1 to 2 μ m) and occur in pairs or short chains; horseshoe-shaped or closed rings are frequently observed. Usually non-motile. Some strains are motile, but lose their motility when they are subcultured. No growth occurs at 45°C, but most strains grow at 42 and 4°C; some strains grow at 2°C. Facultatively heterofermentative. DL-Lactic acid is produced, and often there is an excess of the L-(+) isomer. Acid is produced from fructose, galactose, gluconate, glucose, mannose, melibiose, ribose, sucrose, and trehalose. Most strains produce acid from salicin. A few strains produce acid from cellobiose. Acid is not produced from amygdalin, arabinose, inositol, lactose, maltose, mannitol, melezitose, raffinose, rhamnose, sorbitol, and xylose. Ammonia is not produced from arginine. No growth occurs in the presence of 10% NaCl. Acetoin is not produced from glucose. The guanine-plus-cytosine content of the DNA ranges from 42 to 44 mol%. Isolated from fermented meat products, vacuum-packaged meat, sauerkraut, and silage.

The type strain is strain R 60 (= CCUG 34545). In most respects the description of the type strain resembles the description of the subspecies. The type strain produces acid from cellobiose.

Description of *Lactobacillus sake* subsp. sake subsp. nov. *Lactobacillus sake* subsp. *sake* (Sa'ke. M. L. n. *sake*, Japanese alcoholic beverage made by fermenting rice). Cells are rod shaped with rounded ends (0.6 to 0.8 by 2 to 3 μ m); cells are often slightly curved or irregular, especially during the stationary growth phase, and occur in short chains or singly. Non-motile. No growth occurs at 45°C, but most strains grow at 42 and 4°C, and some strains grow at 2°C. Facultatively heterofermentative. DL-Lactic acid is produced, and often there is an excess

of the L-(+) isomer. A few strains produce inactive lactic acid. Acid is produced from arabinose, fructose, galactose, gluconate, glucose, mannose, melibiose, ribose, and sucrose. Most strains produce acid from cellobiose, salicin, and trehalose. A few strains produce acid from lactose and maltose. A few strains do not produce acid from arabinose. Acid is not produced from amygdalin, inositol, mannitol, melezitose, raffinose, rhamnose, sorbitol, and xylose. Ammonia is always produced from arginine. Most strains grow in the presence of 10% NaCl and produce acetoin from glucose. The guanine-plus-cytosine content of the DNA ranges from 42 to 44 mol%. Originally isolated from sake starter. Regularly found in fermented meat products, vacuum-packaged meat, sauerkraut, and other fermented plant material.

The type strain is strain ATCC 15521 (= DSM 20017). In most respects the description of the type strain resembles the description of the species. The type strain produces acid from lactose, maltose, salicin, and trehalose. Acid is not produced from cellobiose.

Description of *Lactobacillus sake* subsp. carnosus subsp. nov. *Lactobacillus sake* subsp. *carnosus* (car.no'sus. L. adj. *carnosus*, pertaining to meat). Cells are rods with rounded ends (0.6 to 0.8 by 2 to 3 μ m) and often are slightly curved or irregular, especially during the stationary growth phase; the cells occur in short chains or singly. Nonmotile. No growth occurs at 45°C, but most strains grow at 42 and 4°C and some strains grow at 2°C. Facultatively heterofermentative. DL-Lactic acid is produced, and often there is an excess of the L-(+) isomer. A few strains produce inactive lactic acid. Acid is produced from fructose, galactose, gluconate, glucose, mannose, melibiose, ribose, salicin, sucrose, and trehalose. Many strains do not produce acid from arabinose. A few strains produce acid from cellobiose. Acid is not produced from amygdalin, inositol, lactose, maltose, mannitol, melezitose, raffinose, rhamnose, sorbitol, and xylose. Ammonia is produced from arginine. Most strains grow in the presence of 10% NaCl and produce acetoin from glucose. The guanine-plus-cytosine content of the DNA ranges from 42 to 44 mol%. Originally isolated from fermented meat products. Regularly found in vacuum-packaged meat, sauerkraut, and other fermented plant material.

The type strain is strain R 14b/a (= CCUG 31331). The description of the type strain corresponds to the description of the subspecies, and the type strain produces acid from arabinose.

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REFERENCES

1. Champomier, M.-C., M.-C. Montel, F. Grimont, and P. A. D. Grimond. 1987. Genomic identification of meat lactobacilli as *Lactobacillus sake*. *Ann. Inst. Pasteur/Microbiol.* (Paris) **138**:751-758.
2. Dellaglio, F., V. Bottazzi, and L. D. Trovatielli. 1973. Deoxyribonucleic acid homology and base composition in some thermophilic lactobacilli. *J. Gen. Microbiol.* **74**:289-297.
3. De Man, J. C., M. Rogosa, and M. E. Sharpe. 1960. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* **23**:130-135.
4. Dykes, G. A., and A. von Holy. 1994. Taxonomic status of atypical *Lactobacillus sake* and *Lactobacillus curvatus* strains associated with vacuum-packaged meat spoilage. *Curr. Microbiol.* **28**:197-200.
5. Kagermeier-Callaway, A. S., and E. Lauer. 1995. *Lactobacillus sake* Katagiri, Kitahara, and Fukami 1934 is the senior synonym for *Lactobacillus bavaricus* Stetter and Stetter 1980. *Int. J. Syst. Bacteriol.* **45**:398-399.
6. Kandler, O., and N. Weiss. 1986. Genus *Lactobacillus* Beijerinck 1901, 212^{AL}, p. 1209-1234. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 2. The Williams & Wilkins Co., Baltimore.

7. Klein, G., L. M. T. Dicks, A. Pack, K. Zimmermann, F. Dellaglio, and G. Reuter. 1996. Emended description of *Lactobacillus sake* (Katagiri, Kitahara, and Fukami) and *Lactobacillus curvatus* (Abo-Elnaga and Kandler): numerical classification revealed by protein fingerprinting and identification based on biochemical patterns and DNA-DNA hybridizations. *Int. J. Syst. Bacteriol.* **46**:367–376.
8. Reuter, G. 1970. Laktobazillen und eng verwandte Mikroorganismen in Fleisch und Fleischerzeugnissen. 2. Mitteilung: Die Charakterisierung der isolierten Laktobazillenstämme. *Fleischwirtschaft*. **50**:954–962.
9. Reuter, G. 1992. The heterogeneity of the *Lactobacillus sake-curvatus* group, p. 17. *In* Abstracts of the Conference on Taxonomy and Automated Identification of Bacteria of the Federation of European Microbiological Societies. Czechoslovak Society for Microbiology, Prague.
10. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
11. Samelis, J., F. Maurogenakis, and J. Metaxopoulos. 1994. Characterization of lactic acid bacteria isolated from naturally fermented Greek dry salami. *Int. J. Food Microbiol.* **23**:179–196.
12. Samelis, J., E. Tsakalidou, J. Metaxopoulos, and G. Kalantzopoulos. 1995. Differentiation of *Lactobacillus sake* and *Lact. curvatus* isolated from naturally fermented dry salami by SDS-PAGE of whole-cell protein. *J. Appl. Bacteriol.* **78**:157–163.
13. SAS Institute, Inc. 1989. SAS/STAT user's guide: statistics, version 6, 4th ed., vol. 2. SAS Institute, Inc., Cary, N.C.
14. Van Reenen, C. A., and L. M. T. Dicks. 1996. Evaluation of numerical analysis of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus plantarum* and *Lactobacillus pentosus*. *Curr. Microbiol.* **32**:1–5.