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 (≥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 D(-)-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 Agroalimentaries, 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, 1 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*

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Strain(s)</th>
<th>% DNA-DNA homology with DNA froma:</th>
<th>Biotyped</th>
<th>Protein groupc</th>
<th>RAPD-PCR cluster</th>
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<td></td>
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<td><em>L. curvatus</em> ATCC 20019T</td>
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<td></td>
</tr>
<tr>
<td><em>L. curvatus</em> subgroup I*</td>
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<td>45</td>
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<td>A8</td>
<td>CI</td>
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<td>ND</td>
<td>ND</td>
<td>A4</td>
<td>CI</td>
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<td>100</td>
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<td>CI</td>
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<td>39</td>
<td>87</td>
<td>A7</td>
<td>I</td>
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<td>W 21N</td>
<td>40</td>
<td>91</td>
<td>C2a</td>
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<td>DF 131</td>
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<td>79</td>
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<td>A2</td>
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<td>A2</td>
<td>CII</td>
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<td>SII</td>
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<td>46</td>
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<td>I</td>
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<td>C2</td>
<td>I</td>
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<td></td>
<td>Nr. 440/2151, Rv 2f/1a</td>
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<td>ND</td>
<td>C</td>
<td>I</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tbody>
</table>

*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).
* ND, not determined.

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deoxynucleoside triphosphate at a concentration of 200 μM, 5 pmol of a 10-base primer (Operon Kit I, 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, OPL-04, OPL-05, OPL-06) of T7 phage were used. Each 25-μl mixture was covered by 30 μl 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 EcoRI and HindIII (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). Dendograms were constructed from the normalized average-linkage cluster analysis data. Distances between clusters were expressed in R² 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 R² 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 R² 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 strains 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...
we describe the subspecies \textit{L. curvatus} subsp. \textit{curvatus} subsp. nov. (type strain, DSM 20019) and \textit{L. curvatus} subsp. \textit{melibiosus} subsp. nov. (type strain, R 60) for the species \textit{L. curvatus} Abo-Elnaga and Kandler 1965 (Klein et al. 1996, emended description).

\textit{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 \textit{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 \textit{L. sake} strains grouped with strain R 14b/a, the proposed type strain of \textit{L. sake} subgroup II (7), at $R^2$ values of $\geq 0.60$ (Fig. 1, cluster III). Four \textit{L. sake} strains, seven strains previously classified as \textit{L. bavaricus}, and the type strain of \textit{L. sake} (strain NCFB 2714) grouped in cluster IV at $R^2$ values of $\geq 0.44$ (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 \textit{L. bavaricus} were not included in the study of Klein et al. (7). However, the RAPD-PCR profiles of the seven \textit{L. bavaricus} strains, including type strain LMG 9844, corresponded well
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 μm) 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. A few strains produce acid from lactose and salicin. Acid is not produced.
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 μm) 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, and often there is an excess of the L-(-) isomer. A few strains produce inactive lactic acid. Acid is produced from fructose, galactose, gluconate, mannose, melibiose, ribose, sucrose, 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 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 μm) 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. Most 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.

ACKNOWLEDGMENT

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REFERENCES