**Lactobacillus kefiranofaciens** sp. nov. Isolated from Kefir Grains

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**Lactobacillus kefiranofaciens** sp. nov. is described. Four strains of this species isolated from kefir grains are slime-forming, homofermentative, rod-shaped lactic acid bacteria. They differ from all the validly described homofermentative species of the genus *Lactobacillus* in their carbohydrate fermentation pattern. Their guanine-plus-cytosine content of deoxyribonucleic acid is 34 to 35 mol%. Deoxyribonucleic acids obtained from six other *Lactobacillus* species do not show significant relatedness to a representative strain of the new species. The type strain of *L. kefiranofaciens* sp. nov. is WT-2B (ATCC 43761).

At least 24% of the dry material of the kefir grain consists of a water-soluble polysaccharide, kefiran. La Riviere et al. (8) isolated *Lactobacillus brevis* forming kefiran and reported that the capsule formation was lost with the first transfer after the isolation of the strains. Rosi and Rossi (16) suggested that the polysaccharides of kefir grains are produced by homofermentative lactobacilli, which they called "atypical Streptobacterium." Kandler and Kunath (6) isolated heterofermentative lactobacilli from kefir grains and described a new species, *Lactobacillus kefiranofaciens*, which is not responsible for kefiran production. Thus, it remains undecided which organism is the kefiran producer.

More recently, Toba et al. (17) isolated capsule-forming homofermentative lactobacilli from kefir with the aid of a newly developed medium (KPL agar). The isolates differ sufficiently from any previously described species. In this paper, we describe the characteristics of these isolates and propose a new species, *Lactobacillus kefiranofaciens*, for them.

**MATERIALS AND METHODS**

**Bacterial strains.** Strains WT-2B, WT-8, WT-6A, and WT-7 were obtained from T. Toba, College of Agriculture, Tohoku University, Sendai, who isolated them from kefir grain purchased from the laboratory of C. Hansen, Copenhagen, Denmark. Reference *Lactobacillus* strains *L. acidophilus* ATCC 4356T (T, type strain), *L. jensenii* ATCC 25258T, *L. helveticus* ATCC 15009T, *L. gasseri* JCM 1131T, *L. crispatus* JCM 1185T, and *L. amylovorus* JCM 1126T were obtained from the American Type Culture Collection (ATCC), Rockville, Md., and Japan Collection of Microorganisms (JCM), RIKEN, Wako-shi, Saitama, Japan.

**Media.** The strains isolated from kefir grain were cultured on modified kefir grain polysaccharide-producing lactobacillus (KPL) agar (17) and in modified KPL broth (17) at 30°C in anaerobic steel wool jars in an atmosphere of 100% CO₂. The reference strains were cultured on Briggs liver broth (13) at 37°C.

**Characterization tests.** Cell shape, cell size, cell arrangement, Gram reaction, India ink staining properties, and colonial appearance were observed for cells grown on KPL agar plates after 10 days at 30°C.

For acid production from carbohydrates, lactose-digested whey (LDW) medium (17) was used as a basal medium. The carbon sources, sterilized separately by filtration, were added to the sterile basal medium to give a final concentration of 1%, except for esculin, whose final concentration was 0.25%. The pH value was measured directly in culture tubes by a long combination electrode (automatic pH-measuring system, model BIS-120; Lifetec Co. Ltd., Saitama, Japan). The final pH value was determined after incubation for 10 days. The formation of gas from glucose was detected by the appearance of bubbles in Durham tubes.

Hydrolysis of esculin and starch, catalase formation, growth at 15, 25, and 45°C, and motility were tested by methods previously described (13), except that LDW medium was used as a basal medium.

For determination of the configuration of lactic acid, whey optical rotatory (WOR) medium, adjusted to pH 5.5 and containing 20 g of glucose, 10 g of polypeptide (BBL Microbiology Systems, Cockeysville, Md.) 5 g of yeast extract (Difco Laboratories, Detroit, Mich.), and 1,000 ml of deproteinized whey, was used. The configuration was determined enzymatically by a modification of the method of Mattson (12), using D-(-)-lactate dehydrogenase (Boehringer Mannheim Yamanouchi Co., Ltd.) and L-(-)-lactate dehydrogenase (Sigma Chemical Co., St. Louis, Mo.). Fermentation products were analyzed by using the method of Holdeman et al. (4).

**Preparation of DNA.** Cells were grown in modified KPL broth, harvested in the late logarithmic phase, and washed twice with 0.15 M NaCl-0.1 M ethylenediaminetetraacetic acid (pH 8.0). DNA was isolated by a modification of the procedures of Marmur (10). The purity and the amount of DNA were estimated by measuring the hyperchromic shift during thermal denaturation (1). Tritium-labeled DNA was prepared by using a nick translation system (New England Nuclear Corp., Boston, Mass.) adapted from the procedure described by Rigby et al. (15).

**DNA base composition.** The guanine-plus-cytosine (G+C) contents of the DNA preparations were determined by the thermal melting point (T_m) method (11), using an automatic recording spectrophotometer (Komatsu Electronics, Tokyo, Japan). DNA from calf thymus containing 42 mol% G+C was included in each set as a standard.

**DNA homology.** DNA homology experiments were performed by the S1 nuclease procedure as described by...
RESULTS AND DISCUSSION

Morphology of the isolates. The cellular appearance of strains of the new species L. kefiranofaciens is shown in Fig. 1. All isolates grew on KPL agar at 30°C, but not on BL agar (14) and MRS agar (9). The cells were gram-positive, nonmotile, and nonsporeforming long rods (0.8 to 1.2 μm in width and 3.0 to 20.0 μm in length) and occurred singly, in pairs, or occasionally in short chains. India ink preparations of the isolates showed capsules surrounding the rods. After incubation on modified KPL agar plates (pH 5.5) for 10 days, colonies of all isolates were 0.5 to 3.0 mm in diameter, convex, transparent to translucent, entire, smooth to rough, rropy, and white (Fig. 2).

Biochemical and physiological characteristics. All isolates fermented glucose, fructose, galactose, sucrose, maltose, lactose, melibiose, and raffinose. Arabinose, xylose, rhamnose, ribose, cellobiose, trehalose, melezitose, dextrin, mannitol, sorbitol, esculin, salicin, and amygdalin were not fermented. Strain WT-2B failed to ferment mannose. The final pH in glucose broth was 3.5 to 3.9. The isolates produced D(-)-lactic acid, with a marked excess of D(-)-lactic acid. There was no growth observed at 15 and 45°C. All strains were catalase negative and did not produce gas from glucose.

DNA base composition and homology. Table 1 lists the G+C contents and DNA homologies of two strains of L. kefiranofaciens examined by the S1 nuclease method. Strains WT-8 and WT-2B had a high level of DNA homology with each other. The DNAs of six other Lactobacillus species had little or no homology with strain WT-2B. The G+C content of the DNAs from the isolates is 34.3 to 35.4 mol%.

A comparison of the biochemical and physiological characteristics, morphology, DNA base composition, and DNA homology of these isolates with those of the previously described species of the genus Lactobacillus indicate that these strains represent a new species, for which we propose the name Lactobacillus kefiranofaciens sp. nov. The phenotypic characteristics differentiating L. kefiranofaciens from other homofermentative Lactobacillus species having low G+C contents and not growing at 15°C are shown in Table 2.

Although the habitat of L. kefir Kandler and Kunath (6) is also kefir grains, L. kefir is heterofermentative and has a G+C content of 41 to 42 mol%. Thus, L. kefir is unlike L. kefiranofaciens, which is homofermentative and has a significantly different G+C content of 34.3 to 35.4 mol%.

Description of Lactobacillus kefiranofaciens sp. nov. Lactobacillus kefiranofaciens (ke.fir.an.o. fa.ci.ens. L. n. kefiran, a polysaccharide of kefir grain, kefiran; L. v. facio, produce; M.L. part. adj. kefiranofaciens, kefiran producing).

Gram-positive, nonmotile, capsules, nonsporeforming rods (generally 0.8 to 1.2 μm by 3.0 to 20.0 μm) that occur singly, in pairs, or occasionally in short chains.

Grows on modified KPL agar (pH 5.0) at 30°C in 10 days, producing colonies that are circular or irregular, 0.5 to 3.0 mm in diameter, convex, transparent to translucent, white, smooth to rough, and rropy. There is no growth at 15 or 45°C.

Facultatively anaerobic and produces D(-)-lactic acid, with a marked excess of D(-)-lactic acid homfermentatively.

Catalase is not produced. Escolin and starch are not hydrolyzed. Milk is curdled. Gas is not produced from glucose.

Acid is produced from glucose, fructose, galactose, sucrose, maltose, lactose, melibiose, and raffinose but not from arabinose, xylose, rhamnose, ribose, cellobiose, trehalose, melezitose, dextrin, mannitol, sorbitol, esculin, salicin, or amygdalin.

DNA G+C content is 34.3 to 35.4 mol%. The habitat of the species is kefir grains.

The type strain is strain WT-2B (ATCC 43761).

TABLE 1. DNA homologies among the strains of L. kefiranofaciens and some other Lactobacillus species having low G+C contents

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Homology with reference DNA from strain WT-2B</th>
<th>G+C content (mol%)</th>
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<tbody>
<tr>
<td>L. kefiranofaciens</td>
<td></td>
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<tr>
<td>WT-2B&lt;sup&gt;T&lt;/sup&gt;</td>
<td>100</td>
<td>34.3</td>
</tr>
<tr>
<td>WT-8</td>
<td>87</td>
<td>35.4</td>
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<tr>
<td>L. acidophilus ATCC 4356&lt;sup&gt;T&lt;/sup&gt;</td>
<td>9</td>
<td>32.3</td>
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<td>L. gasseri JCM 1131&lt;sup&gt;T&lt;/sup&gt;</td>
<td>3</td>
<td>32.9</td>
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<td>L. crispatus JCM 1185&lt;sup&gt;T&lt;/sup&gt;</td>
<td>13</td>
<td>35.4</td>
</tr>
<tr>
<td>L. amylovorans JCM 1126&lt;sup&gt;T&lt;/sup&gt;</td>
<td>11</td>
<td>NT*</td>
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<td>L. helveticus ATCC 15009&lt;sup&gt;T&lt;/sup&gt;</td>
<td>17</td>
<td>NT</td>
</tr>
<tr>
<td>L. jensenii ATCC 25258&lt;sup&gt;T&lt;/sup&gt;</td>
<td>2</td>
<td>NT</td>
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* NT, Not tested.
TABLE 2. Phenotypic characteristic differentiating *L. kefiranofaciens* from other homofermentative *Lactobacillus* species that have low G+C contents and that do not grow at 15°C

<table>
<thead>
<tr>
<th>Species</th>
<th>G+C content (mol%)</th>
<th>Isomer of lactic acid</th>
<th>Reaction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ribose</th>
<th>Saccharose</th>
<th>Cellulobiose</th>
<th>Lactose</th>
<th>Trehalose</th>
<th>Melibiose</th>
<th>Raffinose</th>
<th>Mannitol</th>
<th>Sorbitol</th>
<th>Esculin</th>
<th>Salicin</th>
<th>Amygdalin</th>
<th>Arabinose growth</th>
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<tr>
<td><em>L. kefiranofaciens</em></td>
<td>34-35</td>
<td>DL</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>+</td>
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<td></td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>34-37</td>
<td>DL</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>d</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>w</td>
<td>w</td>
<td>w</td>
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<tr>
<td><em>L. amylovorus</em></td>
<td>40-41</td>
<td>DL</td>
<td>+ + dd</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>d</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>L. crispatus</em></td>
<td>35-38</td>
<td>dl</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>w</td>
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<tr>
<td><em>L. gasseri</em></td>
<td>33-35</td>
<td>DL</td>
<td>+ +</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>-</td>
<td>+</td>
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<tr>
<td><em>L. helveticus</em></td>
<td>38-40</td>
<td>DL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>35-37</td>
<td>L</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td><em>L. salivarius</em></td>
<td>34-37</td>
<td>L</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>L. acidotolerans</em></td>
<td>35-37</td>
<td>DL</td>
<td>+ d</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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</table>

<sup>a</sup> +, 90% or more of strains positive; -, 90% or more strains negative; d, 11 to 89% of strains positive; w, positive to weakly positive.

<sup>b</sup> Based on the content of L-(+)-lactic acid as follows: D, 0 to 20%; DL, 20 to 40%; DL, 40 to 60%; L, 80 to 100%.

<sup>c</sup> Data from reference 7.

<sup>d</sup> Data from reference 5.

<sup>e</sup> Data from reference 2.

LITERATURE CITED