**Lactobacillus nagelii sp. nov., an organism isolated from a partially fermented wine**

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**INTRODUCTION**

Sluggish or stuck alcoholic fermentations are problems sometimes encountered by wine makers. These problems can be due to improper fermentation conditions or to insufficient nutrients being present in the grape must to support adequate yeast growth (Ough, 1966; Houtman et al., 1980a, b; Ingledew & Kunkee, 1985; Kunkee, 1991). Recently, Huang et al. (1996) isolated three strains of lactic acid bacteria that could slow the fermentation of a Chardonnay grape juice. One of these strains was subsequently shown to represent a novel species of Lactobacillus, Lactobacillus kunkeei (Edwards et al., 1998). Species of Lactobacillus that have been isolated previously from grapes and wines include Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus casei, Lactobacillus fermentum (Lactobacillus cellobiosus), Lactobacillus curvatus, Lactobacillus delbrueckii, Lactobacillus fructivorans (Lactobacillus trichodes), Lactobacillus hilgardii, Lactobacillus jensenii and Lactobacillus plantarum (Douglas & Cruess, 1936; Vaughn, 1955; Fornachon, 1957; Du Plessis & Van Zyl, 1963; Pilone et al., 1966; Chalfan et al., 1977; Maret & Sozzi, 1977, 1979; Costello et al., 1983; Lafon-Lafourcade et al., 1983; Wibowo et al., 1985; Davis et al., 1986a, b; Dicks & Van Vuuren, 1988; Sieiro et al., 1990).

At the present time, *L. kunkeei* is the only species of *Lactobacillus* known that has been demonstrated to slow alcoholic fermentation of grape musts (Huang et al., 1996). However, most lactobacilli found in wines are considered to be spoilage organisms, due to production of acetic acid and/or other off-flavours (Davis et al., 1985). This study presents the biochemical characteristics and the results of phylogenetic analysis of a strain of *Lactobacillus* isolated from a partially fermented wine. On the basis of phenotypic and genotypic evidence, *Lactobacillus nagelii* sp. nov. ATCC 700692T is proposed as a new species.

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**KEYWORDS:** Lactobacillus nagelii, wine, spoilage

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**METHODS**

**Bacterial strains and cultivation.** Strain LuE₁₀⁷ was isolated from a commercial red wine obtained from L. Van Der Water (The Wine Lab, Napa, CA, USA). Control bacteria used during the biochemical characterization of LuE₁₀⁷ were *L. kunkeei* ATCC 700308 and *L. plantarum* WS-16 (Edwards et al., 1993, 1998). All organisms were grown using modified Rogosa (MR) agar or broth supplemented with apple juice and adjusted to pH 4.5 (Beelman, 1982). Cultures were maintained on MR agar and in lyophilized form.

**Biochemical characterization.** Gas production from glucose was evaluated by inoculation of cultures into 10 ml MR broth and incubation at 25 °C for 3 d. Cells in exponential growth phase were harvested by centrifugation (2000 g).
washed twice in 5 ml phosphate buffer (pH 7, 0.023 M NaH₂PO₄/0.030 M Na₂HPO₄), resuspended in 0.3 ml sterile phosphate buffer and inoculated into heterofermentation-arginine broth described by Pilone et al. (1991). Tubes were overlaid with molten, sterilized vaspar (one part petroleum jelly and six parts paraffin) prior to incubation for 21 d at 25 °C. Production of ammonia from arginine, determination of optical isomers of lactic acid formed from glucose, dextran from sucrose and utilization of citric and malic acids were performed using the methods of Edwards et al. (1991). Nitrate reduction was tested as described by Carr (1970). Mannitol formation from fructose was demonstrated using the method of Pilone et al. (1991). Catalase was detected by placing drops of 3% (v/v) H₂O₂ on cultures growing on MR agar, altered by the addition of 0.5% (w/v) glucose, 20% (v/v) apple juice and 0.0005% (w/v) haematin and raising the pH from 4.5 to 5.5. Carbohydrate utilization was determined using the API Rapid CH system (bioMérieux) using the recommended CHL medium. API galleries were incubated for up to 21 d at 24–25 °C.

For pH and temperature characterizations, cultures were grown and harvested as described for biochemical characterization. MR broth was adjusted to pH 3.7 and 4.5 with 50% (v/v) H₂O₂ and pH 8.0 using 50% (w/v) KOH, inoculated with 0.1 ml of resuspended cultures and incubated at 25 °C for 7 d. Growth in MR broth (pH 4.5) incubated at 5, 15, 25, 32, 37 or 45 °C was evaluated after 7 d. MR broth (pH 4.5) containing 5% (w/v) NaCl was inoculated and was incubated at 25 °C for 7 d.

16S RNA gene sequencing. A large fragment of the 16S rRNA gene (corresponding to positions 30–1491 of the Escherichia coli 16S rRNA gene) of strain LuE₁₀ was amplified by PCR using primers close to the 3’ and 5’ ends of the gene. The PCR products were purified using a Prep-A-Gene kit (BioRad) according to the manufacturer’s instructions and sequenced directly using a Taq Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems).

Phylogenetic analysis. The closest known relatives of strain LuE₁₀ were determined by performing a sequence database search using the program FASTA (Deverereux et al., 1984). The sequences of closely related strains were retrieved from the GenBank or Ribosomal Database Project libraries and aligned with the newly determined sequence using the program PILEUP (Deverereux et al., 1984). The resulting multiple sequence alignment was corrected manually and approximately 100 bases at the 3’ end of the gene were omitted from further analyses because of alignment ambiguities and/or incomplete sequence data from some species. A distance matrix was calculated using the programs PRETTY (Deverereux et al., 1984) and DNADIST, the latter using Kimura’s two-parameter correction (Felsenstein, 1989). A phylogenetic tree was constructed according to the neighbor-joining method with the program NEIGHBOR (Felsenstein, 1989). The stability of the grouping was estimated by bootstrap analysis (200 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

RESULTS AND DISCUSSION

Strain LuE₁₀ was isolated as part of a study of lactic acid bacteria present in wines undergoing sluggish/stuck alcoholic fermentations. LuE₁₀ is a Gram-positive, rod-shaped facultative anaerobe that grew well in an atmosphere enriched with CO₂. The bacterium formed β-lactate from glucose but not gas, utilized citrate or malate in the presence of glucose and produced dextran from sucrose but did not produce mannitol from fructose or ammonia from arginine or reduce nitrate. Carbohydrates fermented by LuE₁₀ were galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, mannitol, sorbitol, α-methyl-D-glucoside, N-acetylglucosamine, amygdalin, salicin, cellobiose, maltose, sucrose, trehalose and β-gentibiose, while aesculin was hydrolysed. The strain grew in MR broth containing 5% (w/v) NaCl (pH 4.5) and at pH 3.7, 4.5 and 8.0 (25 °C). In addition, growth was observed at 15, 25, 32, 37 and 45 °C (pH 4.5).

In order to establish the phylogenetic position of strain LuE₁₀, its 16S rRNA gene was amplified by PCR and characterized by sequence analysis. The almost complete gene sequence (1451 nucleotides) was determined and sequence searches of GenBank and Ribosomal Database Project libraries revealed that the unknown bacterium was phylogenetically most closely related to species of the genus Lactobacillus. The sequences of the nearest relatives of strain LuE₁₀ were retrieved and subjected to pairwise analysis to determine its phylogenetic position. A tree depicting the phylogenetic position of strain LuE₁₀ within the Lactobacillus group of bacteria is shown in Fig. 1 and its sequence similarities with close relatives are given in Table 1. The unidentified bacterium formed a distinct line within the rRNA cluster 2 Lactobacillus (see Collins et al., 1991). No particularly close phylogenetic affinity was shown to any member of rRNA cluster 2 Lactobacillus, with sequence divergence values generally > 5%. From the branching pattern of the tree, Lactobacillus malii was the closest relative to the unknown bacterium. Bootstrap resampling, however, showed that the relationship between L. malii and LuE₁₀ was not statistically significant. In addition, LuE₁₀ fermented maltose and sorbitol, did not possess catalase and grew in 5% (w/v) NaCl and at pH 8, in contrast to L. malii (Carr et al., 1977).

On the basis of both phenotypic and phylogenetic findings, it is evident that strain LuE₁₀ constitutes a previously unknown Lactobacillus species. Thus, we propose that the bacterium isolated from partially fermented grape juice be classified as a new species, Lactobacillus nagelii sp. nov.

Description of Lactobacillus nagelii sp. nov.

Lactobacillus nagelii (na’gel.i.i. L. n. nagelii after Charles W. Nagel, Washington State University, WA, USA, for his contributions to the science of wines).

Cells are Gram-positive rods approximately 0.5 × 1–1.5 µm. Colonies on MR agar appear opaque with smooth edges and are approximately 2 mm in diameter after 4–5 d growth at 25 °C. Facultatively anaerobic. Catalase-negative. D- and L-forms of lactic acid are
**Table 1.** Percentage 16S rRNA similarities between *Lactobacillus nagelii* strain LuE10<sup>T</sup> and some closely related species

<table>
<thead>
<tr>
<th>Species</th>
<th>Similarity (%)</th>
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</thead>
<tbody>
<tr>
<td><em>Lactobacillus agilis</em></td>
<td>92.8</td>
</tr>
<tr>
<td><em>Lactobacillus animalis</em></td>
<td>94.2</td>
</tr>
<tr>
<td><em>Lactobacillus aviatorius</em></td>
<td>93.4</td>
</tr>
<tr>
<td><em>Lactobacillus mali</em></td>
<td>95.2</td>
</tr>
<tr>
<td><em>Lactobacillus murinus</em></td>
<td>94.0</td>
</tr>
<tr>
<td><em>Lactobacillus ruminis</em></td>
<td>93.5</td>
</tr>
<tr>
<td><em>Lactobacillus salivarius</em></td>
<td>93.2</td>
</tr>
</tbody>
</table>

produced from glucose without gas formation. Mannitol is not formed from fructose. Citrate or malate is utilized in the presence of glucose. Ammonia is not formed from arginine. Nitrate is not reduced. Dextran is formed from sucrose. Galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, mannitol, sorbitol, methyl α-D-glucoside, N-acetylgalactosamine, amygdalin, salicin, cellobiose, maltose, sucrose, trehalose and β-gentiobiose are fermented. Aesculin is hydrolysed. Glycerol, erythritol, D-arabinoose, L-arabinose, ribose, D-xylitol, L-xylitol, adenitol, methyl β-xyloditol, dulcitol, inositol, methyl α-D-mannoside, arbutin, lactose, melibiose, inulin, melezitose, D-raffinose, amidon, glycogen, xyliitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate are not fermented. Growth in MR broth containing 5% (w/v) NaCl (pH 4.5) and at pH 3.7–4.5 and 8.0 (25 °C). Growth at 15, 25, 32, 37 and 45 °C but weak growth at 5 °C (pH 4.5). Isolated from partially fermented grape juice. The type strain is ATCC 700692<sup>T</sup>.

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**REFERENCES**


