International Journal of Systematic and Evolutionary Microbiology (2000), **50**, 699–702 Printed in Great Britain

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

Charles G. Edwards,[1] Matthew D. Collins,[2] Paul A. Lawson[2] and Ana V. Rodriguez[1]†

Author for correspondence: Charles Edwards. Tel: +1 509 335 6612. Fax: +1 509 335 4815. e-mail: edwardsc@wsu.edu

[1] Department of Food Science and Human Nutrition, Washington State University, Pullman, WA 99164-6376, USA

[2] Department of Food Science and Technology, University of Reading, Reading RG6 6AP, UK

A Gram-positive rod was isolated from a commercial grape wine undergoing a sluggish/stuck alcoholic fermentation. The organism produced DL-lactic acid from glucose without gas formation, produced dextran from sucrose, hydrolysed aesculin and fermented galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, mannitol, sorbitol, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, salicin, cellobiose, maltose, sucrose, trehalose and β-gentiobiose. 16S rRNA gene sequence analysis revealed that the isolate was phylogenetically a member of the genus *Lactobacillus* and formed a distinct subline within the *Lactobacillus casei* cluster of species. On the basis of phenotypic and phylogenetic evidence, *Lactobacillus nagelii* sp. nov. ATCC 700692^T is proposed as a new species.

Keywords: *Lactobacillus nagelii*, wine, spoilage

INTRODUCTION

Sluggish or stuck alcoholic fermentations are problems sometimes encountered by wine makers. These problem fermentations 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 analysis of this micro-organism, a new species of *Lactobacillus*, *Lactobacillus nagelii* sp. nov., is proposed.

METHODS

Bacterial strains and cultivation. Strain LuE_{10}^T 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 LuE_{10}^T 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***),

†**Permanent address:** Centro de Referencia para Lactobacilos (CERELA), Chacabuco 145, 4000 Tucumán, Argentina.

The GenBank accession number for the 16S rRNA sequence of strain LuE_{10}^T is Y17500.

washed twice in 5 ml phosphate buffer (pH 7, 0·023 M NaH_2PO_4/0·030 M Na_2HPO_4), 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 % (w/v) H_2O_2 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) H_3PO_4 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 incubated at 25 °C for 7 d.

16S rRNA gene sequencing. A large fragment of the 16S rRNA gene (corresponding to positions 30–1491 of the *Escherichia coli* 16S rRNA gene) of strain LuE_{10}^T 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 LuE_{10}^T were determined by performing a sequence database search using the program FASTA (Devereux *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 (Devereux *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 (Devereux *et al.*, 1984) and DNADIST, the latter using Kimura's two-parameter correction (Felsenstein, 1989). A phylogenetic tree was constructed according to the neighbour-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 LuE_{10}^T was isolated as part of a study of lactic acid bacteria present in wines undergoing sluggish/stuck alcoholic fermentations. LuE_{10}^T is a Gram-positive, rod-shaped facultative anaerobe that grew well in an atmosphere enriched with CO_2. The bacterium formed DL-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 LuE_{10}^T 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*-gentiobiose, 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 LuE_{10}^T, 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 LuE_{10}^T were retrieved and subjected to pairwise analysis to determine its phylogenetic position. A tree depicting the phylogenetic position of strain LuE_{10}^T 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 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 mali* was the closest relative to the unknown bacterium. Bootstrap resampling, however, showed that the relationship between *L. mali* and LuE_{10}^T was not statistically significant. In addition, LuE_{10}^T fermented maltose and sorbitol, did not possess catalase and grew in 5 % (w/v) NaCl and at pH 8, in contrast to *L. mali* (Carr *et al.*, 1977).

On the basis of both phenotypic and phylogenetic findings, it is evident that strain LuE_{10}^T 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

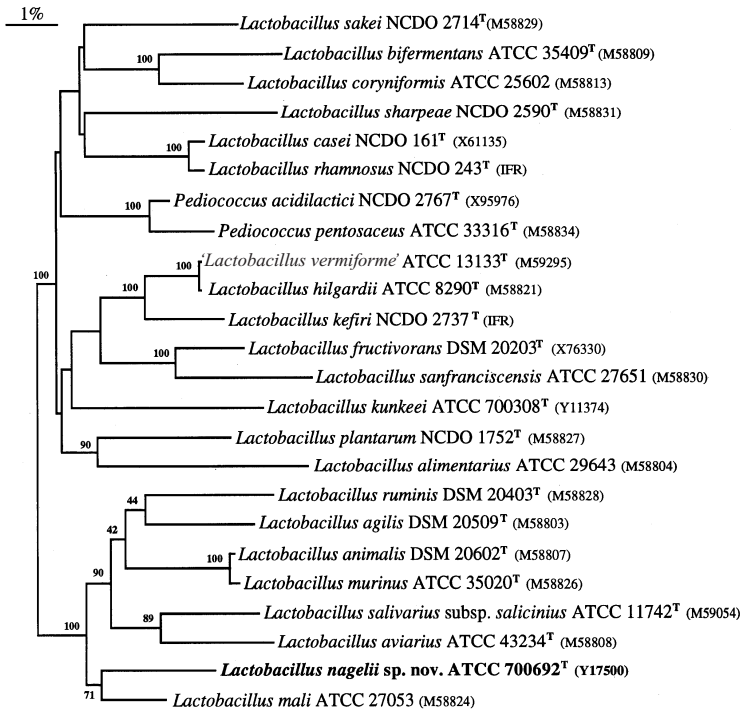

Fig. 1. Dendrogram showing the phylogenetic position of *Lactobacillus nagelii* strain LuE_{10}^T within the *L. casei* cluster of micro-organisms (see Collins *et al.*, 1991). The dendrogram was constructed using the neighbour-joining method. Bootstrap values were calculated from 200 replications. Values $\geqslant 90\%$ are considered statistically significant.

Table 1. Percentage 16S rRNA similarities between *Lactobacillus nagelii* strain LuE_{10}^T and some closely related species

Species	Similarity (%)
Lactobacillus agilis	92·8
Lactobacillus animalis	94·2
Lactobacillus aviarius	93·4
Lactobacillus mali	95·2
Lactobacillus murinus	94·0
Lactobacillus ruminis	93·5
Lactobacillus salivarius	93·2

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*-acetylglucosamine, amygdalin, salicin, cellobiose, maltose, sucrose, trehalose and β-gentiobiose are fermented. Aesculin is hydrolysed. Glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-xyloside, dulcitol, inositol, methyl α-D-mannoside, arbutin, lactose, melibiose, inulin, melezitose, D-raffinose, amidon, glycogen, xylitol, 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^T.

ACKNOWLEDGEMENTS

The authors would like to thank the Washington Wine Grape Advisory Board and the Northwest Center for Small Fruit Research for financial support of this project. The laboratory assistance of C. E. Sachs and B. Knabe-Manwaring is gratefully acknowledged. The authors also thank L. Van Der Water (The Wine Lab, Napa, CA) for providing commercial wines for analysis.

REFERENCES

Beelman, R. B. (1982). Development and utilization of starter cultures to induce malolactic fermentation in red table wines. In *Proceedings of the University of California, Davis, Grape and Wine Centennial Symposium*, pp. 109–117. Edited by A. D. Webb. Davis, CA: University of California.

Carr, J. G. (1970). Tetrad-forming cocci in ciders. *J Appl Bacteriol* **33**, 371–379.

Carr, J. G., Davies, P. A., Dellaglio, F., Vescovo, M. & Williams, R. A. D. (1977). The relationship between *Lactobacillus mali* from cider and *Lactobacillus yamanashiensis* from wine. *J Appl Bacteriol* **42**, 219–228.

Chalfan, Y., Goldberg, I. & Mateles, R. I. (1977). Isolation and characterization of malo-lactic bacteria from Israeli red wines. *J Food Sci* **42**, 939–943, 968.

Collins, M. D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J. A. E., Martinez-Murcia, A., Phillips, B. A., Williams, A. M. & Wallbanks, S. (1991). Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse tran-

scriptase sequencing of 16S rRNA. *FEMS Microbiol Lett* **77**, 5–12.

Costello, P. J., Morrison, G. J., Lee, T. H. & Fleet, G. H. (1983). Numbers and species of lactic acid bacteria in wines during vinification. *Food Technol Aust* **35**, 14–18.

Davis, C. R., Wibowo, D., Eschenbruch, R., Lee, T. H. & Fleet, G. H. (1985). Practical implications of malolactic fermentation: a review. *Am J Enol Vitic* **36**, 290–301.

Davis, C. R., Wibowo, D. J., Lee, T. H. & Fleet, G. H. (1986a). Growth and metabolism of lactic acid bacteria during and after malolactic fermentations of wines at different pH. *Appl Environ Microbiol* **51**, 539–545.

Davis, C. R., Wibowo, D. J., Lee, T. H. & Fleet, G. H. (1986b). Growth and metabolism of lactic acid bacteria during fermentation and conservation of some Australian wines. *Food Technol Aust* **38**, 35–40.

Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.

Dicks, L. M. T. & Van Vuuren, H. J. J. (1988). Identification and physiological characteristics of heterofermentative strains of *Lactobacillus* from South African red wines. *J Appl Bacteriol* **64**, 505–513.

Douglas, H. C. & Cruess, W. V. (1936). A *Lactobacillus* from California wine: *Lactobacillus hilgardii*. *Food Res* **1**, 113–119.

Du Plessis, L. D. W. & Van Zyl, J. A. (1963). The microbiology of South African winemaking. Part IV. The taxonomy and incidence of lactic acid bacteria from dry wines. *South Afr J Agric Sci* **6**, 261–273.

Edwards, C. G., Jensen, K. A., Spayd, S. E. & Seymour, B. J. (1991). Isolation and characterization of native strains of *Leuconostoc oenos* from Washington state wines. *Am J Enol Vitic* **42**, 219–226.

Edwards, C. G., Powers, J. R., Jensen, K. A., Weller, K. M. & Peterson, J. C. (1993). *Lactobacillus* spp. from Washington state wines: isolation and characterization. *J Food Sci* **58**, 453–458.

Edwards, C. G., Haag, K. M., Collins, M. D., Hutson, R. A. & Huang, Y. C. (1998). *Lactobacillus kunkeei* sp. nov.: a spoilage organism associated with grape juice fermentations. *J Appl Microbiol* **84**, 698–702.

Felsenstein, J. (1989). PHYLIP – Phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.

Fornachon, J. C. M. (1957). The occurrence of malo-lactic fermentation in Australian wines. *Aust J Appl Sci* **8**, 120–129.

Houtman, A. C., Marais, J. & Du Plessis, C. S. (1980a). The possibilities of applying present-day knowledge of wine aroma components: influence of several juice factors on fermentation rate and ester production during fermentation. *South Afr J Enol Vitic* **1**, 27–33.

Houtman, A. C., Marais, J. & Du Plessis, C. S. (1980b). Factors affecting the reproducibility of fermentation of grape juice and of the aroma composition of wines. I. Grape maturity, sugar, inoculum concentration, aeration, juice turbidity and ergosterol. *Vitis* **19**, 37–54.

Huang, Y.-C., Edwards, C. G., Peterson, J. C. & Haag, K. M. (1996). Relationship between sluggish fermentations and the antagonism of yeast by lactic acid bacteria. *Am J Enol Vitic* **47**, 1–10.

Ingledew, W. M. & Kunkee, R. E. (1985). Factors influencing sluggish fermentations of grape juice. *Am J Enol Vitic* **36**, 65–76.

Kunkee, R. E. (1991). Relationship between nitrogen content of must and sluggish fermentation. In *Proceedings of the International Symposium on Nitrogen in Grapes and Wine*, pp. 148–155. Edited by J. M. Rantz. Davis, CA: American Society for Enology and Viticulture.

Lafon-Lafourcade, S., Carre, E. & Ribéreau-Gayon, P. (1983). Occurrence of lactic acid bacteria during the different stages of vinification and conservation of wines. *Appl Environ Microbiol* **46**, 874–880.

Maret, R. & Sozzi, T. (1977). Flore malolactique de moûts et de vins du Canton du Valais (Suisse). I. Lactobacilles et pédiocoques. *Ann Technol Agricole* **27**, 255–273.

Maret, R. & Sozzi, T. (1979). Flore malolactique de moûts et de vins du Canton du Valais (Suisse). II. Evolution des populations de lactobacilles et de pédiocoques au cours de la vinification d'un vin blanc (un Fendant) et d'un vin rouge (une Dole). *Ann Technol Agricole* **28**, 31–40.

Ough, C. S. (1966). Fermentation rates of grape juice. II. Effect of initial °Brix, pH, and fermentation temperature. *Am J Enol Vitic* **17**, 20–25.

Pilone, G. J., Kunkee, R. E. & Webb, A. D. (1966). Chemical characterization of wines fermented with various malo-lactic bacteria. *Appl Microbiol* **14**, 608–615.

Pilone, G. J., Clayton, M. G. & Van Duivenboden, R. J. (1991). Characterization of wine lactic acid bacteria: single broth culture for tests of heterofermentation, mannitol from fructose, and ammonia from arginine. *Am J Enol Vitic* **42**, 153–157.

Sieiro, C., Cansado, J., Agrelo, D., Velázquez, J. B. & Villa, T. G. (1990). Isolation and enological characterization of malolactic bacteria from the vineyards of northwestern Spain. *Appl Environ Microbiol* **56**, 2936–2938.

Vaughn, R. H. (1955). Bacterial spoilage of wines with special reference to California conditions. *Adv Food Res* **6**, 67–108.

Wibowo, D., Eschenbruch, R., Davis, C. R., Fleet, G. H. & Lee, T. H. (1985). Occurrence and growth of lactic acid bacteria in wine: a review. *Am J Enol Vitic* **36**, 302–313.