Lactobacillus oeni sp. nov., from wine

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Ten *Lactobacillus* strains, previously isolated from different Bobal grape wines from the Utiel-Requena Origin Denomination of Spain, were characterized phylogenetically, genotypically and phenotypically. The 16S rRNA genes were sequenced and phylogenetic analysis showed that they form a tight phylogenetic clade that is closely related to reference strains *Lactobacillus satsumensis* NRIC 0604^T, '*Lactobacillus uvarum*' 8 and *Lactobacillus mali* DSM 20444^T. DNA–DNA hybridization results confirmed the separation of the strains from other *Lactobacillus* species. Genotypically, the strains could be differentiated from their closest neighbours by 16S amplified rDNA restriction analysis and random amplified polymorphic DNA patterns. The strains were Gram-staining-positive, facultatively anaerobic rods that did not exhibit catalase activity. Phenotypically, they could be distinguished from their closest relatives by several traits such as their inability to grow at pH 3.3, to ferment sucrose, amygdalin and arbutin or to hydrolyse aesculin. The characteristics of the ten wine isolates suggest that they represent a novel species, for which the name *Lactobacillus oeni* sp. nov. is proposed. The type strain is 59b^T (=CECT 7334^T =DSM 19972^T).

Lactobacilli are Gram-positive, non-spore-forming, catalase-negative rods belonging to the group of lactic acid bacteria. They are found in a wide variety of habitats such as fermented beverages and foods, mucosal membranes and intestinal tracts of animals and humans, sewage and plant material (Bernardeau *et al.*, 2008). Lactobacilli play an important role in the winemaking process because they can transform a large number of the compounds that are present in wine to the final products that determine a wine's organoleptic properties and, hence, its quality (Lafon-Lafourcade, 1983; Lafon-Lafourcade *et al.*, 1983). Some *Lactobacillus* species are responsible for malolactic fermentation, although the species *Oenococcus oeni* is most frequently associated with this process. Malolactic fermentation is a desirable process in the production of quality wines because it reduces acidity, increases microbiological stability and improves organoleptic characteristics (Agouridis *et al.*, 2005). Nevertheless, lactobacilli can also lead to detrimental effects as they can produce acetic

acid, off-flavours, ropiness and biogenic amines that can be toxic to humans (Fleet, 1993; Landete *et al.*, 2005, 2007; Sponholz, 1993).

Because of the positive and negative effects of lactobacilli in wine, several studies have been done in recent years in order to investigate the diversity of *Lactobacillus* species that are associated with winemaking. As a consequence, some novel *Lactobacillus* species have been described (Rodas *et al.*, 2006; Mañes-Lázaro *et al.*, 2008a). Following a polyphasic study, Rodas *et al.* (2005) found a group of ten strains (59b^T, 54, 59c, 80, 81, 82, 103, 209g, 376 and 420) that did not cluster with any of the known *Lactobacillus* species. The 16S rRNA gene of strain 59b^T was sequenced and phylogenetic analysis showed that the closest reference strain was *Lactobacillus mali* DSM 20444^T, with 97.8 % similarity.

The phylogenetic relationships of wine isolates and reference strains have been studied in depth, which include the latest descriptions of *Lactobacillus satsumensis* (Endo & Okada, 2005), *Lactobacillus vini* (Rodas *et al.*, 2006) and '*Lactobacillus uvarum*' (Mañes-Lázaro *et al.*, 2008b). There was some evidence to suggest that the ten strains characterized by Rodas *et al.* (2005) may represent a novel species. For this report, the ten strains and their nearest relatives were characterized phylogenetically, genotypically and phenotypically.

Strains were isolated by Rodas *et al.* (2005) from different Bobal wines produced in five different wineries from the

Abbreviations: ARDRA, amplified rDNA restriction analysis; RAPD, random amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 59b^T, 54, 59c, 80, 81, 82, 103, 209g, 376 and 420 are AY681127 and EU821345–EU821353, respectively.

Phylogenetic trees based on 16S rRNA gene sequences and constructed using maximum-parsimony and neighbour-joining methods are available as supplementary figures with the online version of this paper.

Utiel-Requena Origin Denomination of Spain: strains 59b^T, 54 and 59c from Sinarcas winery; strains 80, 81 and 82 from Cuevas de Utiel winery; strain 103 from Fuenterrabias winery; 209g from Casas de Prada winery; and strains 376 and 420 from Requena winery. Reference strains *L. mali* DSM 20444^T, *L. mali* CECT 4149, *L. mali* CECT 7382, *L. mali* Lb44, *L. mali* Lb206, *L. nagelii* DSM 13675^T, *L. satsumensis* DSM 16230^T, *L. satsumensis* CECT 7371, *L. satsumensis* 4555, '*L. uvarum*' CECT 7335, '*L. uvarum*' 24, '*L. uvarum*' 68, *L. vini* CECT 5924^T, *L. vini* Lb154, *L. vini* Lb116 and the wine isolates were grown in MRS broth (Scharlab) supplemented with 0.5 g l-cysteine hydrochloride l⁻¹ (mMRS) under the conditions described by Rodas *et al.* (2003).

Sequences of the 16S rRNA genes from strains 59b^T, 54, 59c, 80, 81, 82, 103, 209g, 376 and 420 were obtained in this work by applying the methods described by Rodas *et al.* (2005). Although the 16S rRNA sequence of strain 59b^T was already available in GenBank, we sequenced this gene again and the deposited sequence has been updated. Almost-complete 16S rRNA gene sequences from the wine isolates and their nearest relatives were subjected to phylogenetic analysis. Neighbour-joining with Kimura's two-parameter model of nucleotide substitution, maximum-parsimony and maximum-likelihood methods were applied in the BioNumerics version 2.5 software package to infer the phylogeny of those strains. Fig. 1 shows the tree that was constructed using maximum likelihood. The

topology of the branches was the same when maximum parsimony was applied and slightly different when neighbour joining was applied (see Supplementary Figs S1 and S2, available in IJSEM Online). The similarity of the 16S rRNA gene sequences between the ten wine isolates was at least 99.83 %. The 16S rRNA gene of strain 59b^T showed 99.1 % sequence similarity with *L. satsumensis* NRIC 0604^T, 97.5 % with '*L. uvarum*' 8 (=CECT 7335), 97.2 % with *L. mali* DSM 20444^T and *L. hordei* UCC 128^T, 96.6 % with *L. cacaonum* LMG 24285^T, 96.5 % with *L. ghanensis* L489^T and 96.2 % with *L. vini* CECT 5924^T and *L. nagelii* LuE₁₀^T. All of these species belong to the *L. salivarius* phylogenetic group (Felis & Dellaglio, 2007).

DNA–DNA hybridization experiments were performed as described by Urdiain *et al.* (2008) to ascertain whether the wine strains represented a novel species. DNA–DNA hybridization was first performed between strains 59b^T and 54. The result, expressed as a mean percentage value based on two independent hybridization experiments, was 100 %, which indicates that these two strains belong to the same species. Hybridizations between strain 59b^T and *L. mali* DSM 20444^T, *L. mali* CECT 7382, *L. nagelii* CECT 5983^T, '*L. uvarum*' CECT 7335 and *L. vini* CECT 5924^T produced mean results of 30.7, 38.3, 32.1, 35.5 and 34.2 %, respectively. Finally, two reciprocal hybridization experiments using genomic DNA from strains *L. satsumensis* CECT 7371 and *L. mali* CECT 7382 as templates against strain 59b^T resulted in mean values of 47.6 and 55.1 %, respectively. All of these values are below 70 %, which confirms that 59b^T is a member of a novel species (Stackebrandt & Goebel, 1994).

16S amplified rDNA restriction analysis (ARDRA) and random amplified polymorphic DNA (RAPD) analysis were applied to characterize these strains genotypically. Restriction of the amplified 16S rRNA genes with *Bfa*I distinguished the wine isolates from *L. nagelii* CECT 5983^T and *L. vini* strains but not from *L. mali*, *L. satsumensis* or '*L. uvarum*' strains, whereas restriction with *Mse*I distinguished the wine isolates from all of the other strains used. These results confirm the higher discriminatory power of *Mse*I compared with *Bfa*I that was noted by Rodas *et al.* (2003). RAPD analysis with primers COC and 17R differentiated the wine isolates from strains belonging to other species, forming a cluster of the ten isolates with 78.53 and 77.25 % similarity, respectively. As shown in the dendrogram derived from the combined analysis (Fig. 2), the wine isolates formed a tight cluster with 89.87 % similarity. COC fingerprinting was useful to discriminate all of the *L. vini*, '*L. uvarum*', *L. mali* and *L. satsumensis* strains, but only some of the wine isolates. RAPD with primer 17R had less discriminatory power than COC at the strain level.

The G+C content of strain 59b^T was determined by hydrolysing the DNA enzymically and quantifying the nucleosides by HPLC as described by Tamaoka & Komagata (1984) and Ziemke *et al.* (1998). The G+C

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences and constructed using the maximum-likelihood method. Bootstrap values (≥ 50 %) based on 1000 resamplings are shown at branch nodes. *Oenococcus oeni* DSM 20252^T was used as the outgroup. Bar, 10 % nucleotide sequence divergence.

Fig. 2. Dendrogram derived from comparison of the combined RAPD (COC and 17R) and 16S ARDR (*MseI* and *BfaI*) patterns obtained from strains 59b^T, 54, 59c, 80, 81, 82, 103, 209g, 376 and 420 and reference strains. Levels of similarity between the patterns were calculated by using similarity coefficients from each technique and the clustering is based on the UPGMA method. The vertical dotted line indicates the 78 % similarity value for delineating clusters. The calculated global co-phenetic correlation value for the global analysis was 98.

content is 37.17 ± 0.16 mol%, a value that is within the range (32–53 mol%) established for the genus *Lactobacillus* and similar to the G+C contents of *L. mali* and *L. satsumensis*, 32–34 and 39–41 mol%, respectively.

To test for the presence of D-meso-diaminopimelic acid in strain 59b^T, whole cells were hydrolysed with 4 M HCl at 100 °C for 15 h and the hydrolysates were subjected to thin-layer chromatography on cellulose plates using the solvent system of Rhuland *et al.* (1955). D-meso-Diaminopimelic acid was detected in strain 59b^T; similarly, it has been detected in *L. mali* (<http://www.dsmz.de>) and *L. satsumensis* (Endo & Okada, 2005; Felis & Dellaglio, 2007).

The phenotypic traits of the wine isolates are given in Table 1 and the species description. Strains 59b^T, 54, 59c, 80, 81, 82, 103, 209g, 376 and 420 are Gram-positive, catalase-negative and microaerophilic lactobacilli. They form L-lactate from glucose and do not ferment gluconate or ribose: thus, they are considered as homofermentative. Colonies measure 0.8–1.2 mm in diameter and are white, smooth, circular and regular when grown on mMRS agar at 28 °C for 4 days. Cells are rods, 0.63–0.92 µm wide and 1.38–3.41 µm long (mean size 0.80×2.36 µm). Cells are motile (except strain 376) and non-spore-forming and occur mainly in pairs but also in short chains of up to five cells. All strains grow at 15, 25, 37 and 45 °C but not at 5 °C. They grow at pH 4.5 and 8.0, but not at pH 3.3, and

with 5 % (w/v) NaCl (except strain 80) and 10 % (v/v) ethanol, but not with 10 % (w/v) NaCl. They produce exopolysaccharide from sucrose but do not produce ammonia from arginine or mannitol from fructose. They are able to transform L-malic acid into L-lactic acid in MRS broth supplemented with 5 g malic acid l⁻¹. This characteristic, as well as the ability to grow with 10 % ethanol, is seen in other species that have been isolated from wine (Henick-Kling, 1993; Pardo & Zúñiga, 1992).

The abilities of the wine isolates and the reference strains to ferment carbohydrates were tested with the API 50 CH system (bioMérieux) according to the manufacturer's instructions. All of the wine isolates ferment N-acetylglucosamine, fructose, glucose, methyl α-D-glucoside, mannitol, mannose, sorbitol, L-sorbose and trehalose. They do not ferment D-adonitol, amygdalin, D- or L-arabinose, D- or L-arabitol, arbutin, cellobiose, dulcitol, erythritol, D- or L-fucose, galactose, gluconate, 2- or 5-ketogluconate, glycogen, inositol, inulin, lactose, D-lyxose, maltose, melezitose, melibiose, raffinose, rhamnose, D-ribose, starch, sucrose, D-tagatose, turanose, xylitol, D- or L-xylose, methyl α-D-mannoside or methyl β-xyloside and do not hydrolyse aesculin. Strains 54, 81, 80 and 376 ferment glycerol weakly but the other strains do not. Strain 103 ferments salicin and β-gentiobiose but the other strains do not.

Table 1. Differential traits between *Lactobacillus oeni* sp. nov. strains and their closest phylogenetic neighbours

Reference strains: 1, *L. mali* CECT 4149 (data from Rodas *et al.*, 2006); 2, *L. satsumensis* DSM 16230^T (Rodas *et al.*, 2006); 3, '*L. uvarum*' CECT 7335 (Mañes-Lázaro *et al.*, 2008b). All strains produce L-lactic acid isomer. All strains are positive for growth at pH 4.5 and 8.0, fermentation of N-acetylglucosamine, D-fructose, D-glucose, D-mannitol, D-mannose and trehalose and production of exopolysaccharide from sucrose. All strains are negative for growth with 10 % NaCl, production of ammonia from arginine and mannitol from fructose and fermentation of D-adonitol, D- and L-arabinose, D- and L-arabitol, dulcitol, erythritol, D- and L-fucose, gluconate, 2- and 5-ketogluconate, glycogen, inositol, inulin, lactose, D-lyxose, melezitose, melibiose, raffinose, D-ribose, starch, D- and L-xylose, xylitol and methyl β -D-xyloside. +, Positive; –, negative; W, weak; ND, no data available.

Characteristic	<i>Lactobacillus oeni</i> sp. nov.										1	2	3
	59b ^T	54	59c	80	81	82	103	209g	376	420			
Motility	+	+	+	+	+	+	+	+	–	+	+	+	+
Growth at/with:													
pH 3.3	–	–	–	–	–	–	–	–	–	–	+	+	–
5 % NaCl	+	+	+	–	+	+	+	+	+	+	–	+	+
10 % Ethanol	+	+	+	+	+	+	+	+	W	+	ND	ND	ND
Malolactic fermentation	+	+	+	+	+	+	+	+	+	+	ND	ND	ND
Fermentation of:													
Aesculin ferric citrate	–	–	–	–	–	–	–	–	–	–	+	+	+
Amygdalin	–	–	–	–	–	–	–	–	–	–	+	+	+
Arbutin	–	–	–	–	–	–	–	–	–	–	+	+	+
Cellobiose	–	–	–	–	–	–	–	–	–	–	+	–	–
D-Galactose	–	–	–	–	–	–	–	–	–	–	–	+	–
β -Gentiobiose	–	–	–	–	–	–	+	–	–	–	+	+	+
Methyl α -D-glucoside	+	+	+	+	+	+	+	+	+	+	–	+	+
Glycerol	–	W	–	W	W	–	–	–	W	–	–	–	–
Maltose	–	–	–	–	–	–	–	–	–	–	–	+	+
Methyl α -D-mannoside	–	–	–	–	–	–	–	–	–	–	–	+	–
L-Rhamnose	–	–	–	–	–	–	–	–	–	–	–	+	–
Salicin	–	–	–	–	–	–	+	–	–	–	–	+	+
D-Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	–
L-Sorbose	+	+	+	+	+	+	+	+	+	+	–	+	–
Sucrose	–	–	–	–	–	–	–	–	–	–	+	+	+
D-Tagatose	–	–	–	–	–	–	–	–	–	–	–	+	–
Turanose	–	–	–	–	–	–	–	–	–	–	–	+	+

All of these analyses confirm that status should be given to the wine isolates 59b^T, 54, 59c, 80, 81, 82, 103, 209g, 376 and 420 at the species level and, therefore, the novel species *Lactobacillus oeni* sp. nov. is proposed.

Description of *Lactobacillus oeni* sp. nov.

Lactobacillus oeni (oe'ni. N.L. gen. n. *oeni* of wine).

Gram-positive, non-spore-forming rods, 0.63–0.92 μ m wide by 1.38–3.41 μ m long. Nine of ten known strains are motile. Cells are found singly, in pairs and in short chains. Microaerophilic. Colonies on MRS agar after 4 days of incubation at 28 °C are 0.8–1.2 mm in diameter, smooth, circular, regular and white. Catalase-negative. Growth occurs at 15–45 °C but not at 5 °C, at pH 4.5–8.0 but not at pH 3.3 and with 10 % ethanol. Transforms L-malic acid into L-lactic acid. Homofermentative: does not ferment gluconate or ribose. L-Lactate is produced as the end product from hexoses. Ammonia is not produced from arginine and mannitol is not produced from fructose.

Exopolysaccharide is produced from sucrose. Acid is produced from N-acetylglucosamine, fructose, glucose, mannose, mannitol, sorbitol, L-sorbose, methyl α -D-glucoside and trehalose but not from adonitol, amygdalin, D- or L-arabinose, D- or L-arabitol, arbutin, cellobiose, dulcitol, erythritol, D- or L-fucose, galactose, gluconate, 2- or 5-ketogluconate, glycogen, inositol, inulin, D-lyxose, lactose, maltose, melezitose, melibiose, raffinose, rhamnose, ribose, starch, sucrose, D-tagatose, turanose, xylitol, D- or L-xylose, methyl α -D-mannoside or methyl β -xyloside. Aesculin is not hydrolysed. Acid production from glycerol is strain dependent. Nine of ten known strains ferment salicin and β -gentiobiose. The cell wall contains peptidoglycan of the D-meso-diaminopimelic acid type. The DNA G+C content of the type strain is 37.17 ± 0.16 mol%.

The type strain is 59b^T (=CECT 7334^T =DSM 19972^T). The type strain and additional strains of the species, 54, 59c, 80, 81, 82, 103, 209g, 376 and 420, were isolated in 1997 by A. M. Rodas from Bobal wine.

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