Lactobacillus bobalius sp. nov., a lactic acid bacterium isolated from Spanish Bobal grape must

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A *Lactobacillus* strain, designated 203^T, previously isolated from Bobal grape must was characterized phylogenetically, genotypically and phenotypically in order to establish whether it represents a novel species. On the basis of the 16S rRNA gene sequence, strain 203^T was shown to belong to the genus *Lactobacillus*, falling within the *Lactobacillus alimentarius*–*Lactobacillus farciminis* group and being closely related to the type strains of *L. alimentarius*, *Lactobacillus kimchii* and *Lactobacillus paralimentarius*. DNA–DNA hybridization results confirmed the separate status of strain 203^T at the species level. To establish the similarities and differences between 203^T and the three aforementioned closest species, the following methods were used: amplified rDNA restriction analysis, analysis of the 16S–23S rDNA intergenic spacer region, random amplification of polymorphic DNA (RAPD) profiling, ribotyping, carbohydrate fermentation and physiological tests. Strain 203^T could be differentiated genetically using RAPD analysis and ribotyping. Phenotypically, it can be distinguished from its closest relatives by its ability to grow at pH 3.3, by gas production from gluconate and by certain carbohydrate fermentations. On the basis of these data, strain 203^T represents a novel species of the genus *Lactobacillus*, for which the name *Lactobacillus bobalius* sp. nov. is proposed. The type strain is 203^T (=CECT 7310^T =DSM 19674^T).

Eighteen different species of the genus *Lactobacillus* have been reported to be involved in the winemaking process (Chalfan *et al.*, 1977; Costello *et al.*, 1983; Davis *et al.*, 1986; Lafon-Lafourcade, 1983; Lafon-Lafourcade *et al.*, 1983; Maret & Sozzi, 1977, 1979; Ribéreau-Gayon *et al.*, 2000b; Rodas *et al.*, 2005; Wibowo *et al.*, 1985). In recent years, three of them have been described as novel species, namely *Lactobacillus kunkeei* (Edwards *et al.*, 1998), *Lactobacillus nagelii* (Edwards *et al.*, 2000) and *Lactobacillus vini* (Rodas *et al.*, 2006). Wine lactobacilli are sometimes considered as spoilage organisms since they can produce acetic acid and/or off-flavours (Davis *et al.*, 1985; Wibowo *et al.*, 1985; Fleet, 1993; Sponholz, 1993) and biogenic amines (Lonvaud Funel, 2001; Ribéreau-Gayon *et al.*, 2000a; Landete *et al.*, 2005).

Abbreviations: ARDRA, amplified rDNA restriction analysis; ISR, intergenic spacer region; RAPD, random amplification of polymorphic DNA.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 203^T is AY681134.

Neighbour-joining and maximum-parsimony phylogenetic trees for strain 203^T and members of the genus *Lactobacillus* are available as supplementary material with the online version of this paper.

In an earlier study, Rodas *et al.* (2005) carried out an extensive polyphasic analysis using genotypic and phenotypic methods to characterize 170 lactobacilli isolated from musts and wines. The dendrogram built by comparing the results obtained from the different techniques showed that strain 203^T did not cluster with any of the reference species of the genus *Lactobacillus*. An analysis of 16S rRNA gene sequence similarity showed that strain 203^T was most closely related to the type strains of *Lactobacillus kimchii* (99.3%), *Lactobacillus paralimentarius* (97.8%) and *Lactobacillus alimentarius* (98.2%). However, the study results did not confirm whether strain 203^T represented a novel species or not.

The phylogenetic, genotypic and phenotypic characterization of isolate 203^T and its closest phylogenetic relatives are studied in depth in our work, these issues having not been analysed by Rodas *et al.* (2005). On the basis of our results, it is proposed that strain 203^T represents a novel species of the genus *Lactobacillus*.

Strain 203^T was isolated from a Bobal wine from the Spanish Utiel-Requena Denominación de Origen (D.O.) by Rodas *et al.* (2005). In the current work, the following reference strains were used: *L. alimentarius* CECT 570^T, *L.*

kimchii DSM 13961^T, *Lactobacillus mali* CECT 4149 (the type strain of *Lactobacillus yamanishiensis*), *L. nagelii* CECT 5983^T, *L. paralimentarius* DSM 13238^T, *Lactobacillus satsumensis* DSM 16230^T and *L. vini* CECT 5924^T. All of them, as well as wine isolate 203^T, 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).

An almost-complete 16S rRNA gene sequence of isolate 203^T was compared with those of its closest relatives. In the comparison, three species not previously described by Rodas *et al.* (2005) were included, namely *Lactobacillus tucetii*, *Lactobacillus nantensis* and *Lactobacillus crustorum* (Chenoll *et al.*, 2006; Valcheva *et al.*, 2006; Scheirlinck *et al.*, 2007). Phylogenetic trees were constructed using neighbour-joining, maximum-parsimony and maximum-likelihood methods in the BioNumerics software package version 2.5 to infer the phylogeny of strain 203^T. The tree shown in Fig. 1 was constructed using the maximum-likelihood method. When other treeing methods were applied, the topology of the branches was the same (see Supplementary Fig. S1, available in IJSEM Online). The 16S rRNA gene of strain 203^T showed the following sequence similarities with respect to members of the genus *Lactobacillus*: 99.4 % for *L. kimchii* MT-1077^T, 99.3 % for *L. paralimentarius* DSM 13238^T, 98.2 % for *L. alimentarius* DSM 20249^T, 97.8 % for *L. mindensis* TMW 1.80^T and *L. crustorum* LMG 23699^T, 97.7 % for *L. farciminis* ATCC 29644^T, 96.8 % for *L. nantensis* LP33^T, 94.6 % for *L. versmoldensis* KU-3^T and 94 % for *L. tucetii* CECT 5920^T. These similarity values are similar to those obtained by Rodas *et al.* (2005) in the case of *L. kimchii* and *L. alimentarius*; however, the value obtained with *L. paralimentarius* DSM 13238^T was slightly different, because the sequence used by Rodas *et al.* (2005) (GenBank accession no. AB018528) was not the same as the one used in our study (AJ417500). We prefer to use the sequence AJ417500 to build trees as it is longer and seems more accurate than the one used previously. Following the phylogenetic analysis, we observed that strain 203^T fell within the phylogenetic group *L. alimentarius*–*L. farciminis*, recently described by Felis & Dellaglio (2007). The aforementioned

work demonstrated how the phylogenetic structure of the genus *Lactobacillus* can change when novel species are introduced in the comparison, as is the case with *L. tucetii* and *L. nantensis*.

Although the 16S rRNA gene sequence similarities between strain 203^T and the type strains of *L. kimchii*, *L. paralimentarius*, *L. alimentarius*, *L. mindensis*, *L. crustorum* and *L. farciminis* were greater than 97 % (the level generally considered as the threshold for defining bacterial species; Stackebrandt & Goebel, 1994), there was some evidence indicating that strain 203^T differed from these species. Moreover, several *Lactobacillus* strains exhibiting more than 99 % similarity with regard to their 16S rRNA gene sequences represent distinct species (Bringle *et al.*, 2001).

DNA–DNA hybridization experiments were performed with strain 203^T and *L. paralimentarius* DSM 13238^T, *L. kimchii* DSM 13961^T and *L. alimentarius* CECT 570^T, as described by Ziemke *et al.* (1998). The results, expressed as mean percentages based on three independent hybridization experiments, were 64.2, 62.5 and 42.6 %, respectively. Reciprocal hybridization experiments using genomic DNA from strain *L. kimchii* DSM 13961^T as the template rendered a value of 52 % with strain 203^T. As these values were below 70 %, strain 203^T can be classified as representing a novel species (Stackebrandt & Goebel, 1994).

Amplified rDNA restriction analysis (16S-ARDRA), analysis of the 16S–23S rDNA intergenic spacer region (ISR), random amplification of polymorphic DNA (RAPD) profiling and ribotyping were applied to characterize these strains genotypically, as described previously (Rodas *et al.*, 2003, 2005; Chenoll *et al.*, 2006). Strain 203^T could be differentiated from the reference strains by using RAPD and ribotyping profiles. RAPD enabled differentiation of the four species because it is a technique directed at total DNA and is therefore a more powerful means of obtaining evidence of differences among species or strains. Ribotyping rendered different profiles, revealing different targets for *EcoRI* in the 23S rRNA gene or flanking regions and enabling species discrimination. ISR-*DdeI* showed small differences between *L. alimentarius* and the other species compared, while ISR-*EcoRI* gave two profiles (one shared by *L. kimchii* and strain

Fig. 1. Phylogenetic tree constructed using the maximum-likelihood method. Strain 203^T joins with the type strains of *L. alimentarius*, *L. kimchii* and *L. paralimentarius* and is close to those of *L. mindensis*, *L. nantensis*, *L. farciminis* and *L. crustorum*. Bootstrap percentages (based on 1000 replications) are shown at nodes; only values ≥ 50 % are shown. *Oenococcus oeni* DSM 20252^T was used as an outgroup. Bar, 10 % nucleotide substitution. Neighbour-joining and maximum-parsimony trees are available in Supplementary Fig. S1.

203^T and the other shared by *L. alimentarius* and *L. paralimentarius*). As described recently by Ferchichi *et al.* (2008), analysis of the 16S–23S rRNA ISR is more suitable for differentiating between *L. paralimentarius* and related species that cannot be distinguished by analysing their 16S rRNA gene sequences. Ferchichi *et al.* (2008) reported slight differences in ISR sequences that allowed differentiation between *L. alimentarius* and *L. paralimentarius*, which we have demonstrated using ISR-*Dde*I profiles. However, they did not give data for *L. kimchii*. In the current study, restriction analysis of ISR sequences did not produce fingerprint differences that could be used to differentiate between *L. kimchii* and strain 203^T (Fig. 2). 16S-ARDRA was not useful for distinguishing species, because of the high levels of similarity in their 16S rRNA gene sequences. As can be deduced from the composite dendrogram constructed using all of the fingerprinting analyses, four clusters showing >58 % similarity could be delineated, each of which comprised a single species (Fig. 2).

The G+C content was determined by hydrolysing DNA enzymically and quantifying the nucleosides by using HPLC as reported previously (Tamaoka & Komagata, 1984; Ziemke *et al.*, 1998). Strain 203^T has a DNA G+C content of 34.03 ± 0.77 mol%, a value that is within the range (32–53 mol%) established for the genus *Lactobacillus*.

The peptidoglycan types for strain 203^T and *L. paralimentarius* DSM 13238^T were determined as described by Schleifer (1985) and Schleifer & Kandler (1972), with the modification that TLC on cellulose was used instead of paper chromatography. Quantitative analysis of the peptidoglycan amino acids was performed using GC as described by MacKenzie (1987). From these results, it was concluded that strain 203^T and *L. paralimentarius* DSM 13238^T both possess peptidoglycan of the A4 α L-Lys–D-Asp type, just like their phylogenetic neighbours *L. kimchii*, *L. alimentarius*, *L. mindensis* and *L. farciminis* (Ehrmann *et al.*, 2003). Dinitrophenylation (Schleifer, 1985) revealed that both strains contained aspartic acid as the N-terminal amino acid of the interpeptide bridge.

Strain 203^T is a Gram-positive, catalase-negative, micro-aerophilic lactobacillus. It forms DL-lactate from glucose, no gas is released and it ferments gluconate and ribose and thus can be considered as facultatively heterofermentative.

Colonies are 1.8–2.0 mm in diameter, white, smooth and circular to slightly irregular when grown on mMRS agar at 28 °C for 4 days. Cells are rod-shaped, 0.71–1.03 μ m wide and 1.65–3.41 μ m long (mean, 0.92×2.42 μ m). Cells are non-motile, non-spore-forming and generally occur in pairs (but can also form short chains comprising up to five cells).

Strain 203^T grows at 15, 25, 37 and 45 °C but not at 5 °C. It grows at pH 3.3, 4.5 and 8.0 and in the presence of 5 % (but not 10 %) (w/v) NaCl. It produces neither exopolysaccharides from sucrose nor ammonia from arginine.

The ability of strain 203^T and reference species to ferment carbohydrates was tested with API 50 CHL galleries

Fig. 2. Dendrogram derived from comparison of combined RAPD (COC, 16R and 17R primers), ribotyping, ISR-ARDRA (*Hae*III, *Dde*I and *Eco*RI) and 16S-ARDRA (*Eco*RI and *Dde*I) analyses for strain 203^T and reference strains. Levels of similarity between patterns were calculated by using the similarity coefficient of each technique; the clustering is based on the UPGMA method. The vertical dotted line indicates the 58 % similarity value for delineating clusters.

Table 1. Differential traits for strain 203^T with respect to its closest phylogenetic neighbours, *L. alimentarius*, *L. kimchii* and *L. paralimentarius*

Strains: 1, strain 203^T; 2, *L. alimentarius* CECT 570^T; 3, *L. kimchii* DSM 13961^T; 4, *L. paralimentarius* DSM 13238^T. Data were obtained in this study unless otherwise indicated. All of the strains are positive for the following: growth with 5% NaCl and fermentation of D-ribose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, amygdalin, arbutin, aesculin ferric citrate, salicin, cellobiose, maltose, sucrose, trehalose and gentiobiose. All are negative for the following: production of CO₂ from glucose, ammonia from arginine, mannitol from fructose and dextran from sucrose and fermentation of glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-xyloside, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, lactose, melibiose, inulin, raffinose, starch, glycogen, xylitol, turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol and 5-ketogluconate. +, Positive; −, negative; w, weakly positive; ND, no data available.

Characteristic	1	2	3	4
Growth in MRS with 10 % NaCl	−	+ ^a	− ^b	− ^c
Growth in MRS at pH 3.3	+	−	− ^b	− ^c
Fermentation of:				
L-Arabinose	−	+	+	−
D-Xylose	−	−	+	−
Galactose	−	+	w	−
Melezitose	+	−	+	−
Gluconate	+	+	+	−
2-Ketogluconate	−	w	−	−
Gas production from gluconate	w	−	+	− ^a
Lactic acid isomer	DL	L(D) ^a	DL ^b	ND
DNA G + C content (mol%)	33.26–34.80	36–37 ^a	35 ^b	37.2–38 ^c

*Data from other studies as follows: a, Reuter (1983); b, Yoon *et al.* (2000); c, Cai *et al.* (1999).

(bioMérieux) according to the manufacturer’s instructions. Strain 203^T ferments ribose, glucose, fructose, mannose, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose, trehalose, melezitose, β-gentiobiose and gluconate and hydrolyses aesculin. It does not ferment glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl β-xyloside, galactose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, lactose, melibiose, inulin, raffinose, starch, glycogen, xylitol, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate or 5-ketogluconate (Table 1). Strain 203^T differs from *L. paralimentarius* in its ability to grow at pH 3.3 and to ferment melezitose and gluconate (Cai *et al.*, 1999). Unlike *L. alimentarius*, strain 203^T is unable to grow with 10% NaCl, is able to grow at pH 3.3 and can ferment melezitose but not L-arabinose (Reuter, 1983). Strain 203^T

differs from *L. kimchii* in its ability to grow at pH 3.3 and in its inability to ferment L-arabinose and D-xylose (Yoon *et al.*, 2000) (see Table 1).

All of these data serve to confirm the separate status of strain 203^T at the species level.

Description of *Lactobacillus bobalius* sp. nov.

Lactobacillus bobalius (bo.ba’li.us. N.L. masc. adj. *bobalius* pertaining to the grape variety Bobal).

Cells are Gram-positive, non-motile, non-spore-forming rods, 0.71–1.03 μm wide and 1.65–3.41 μm long. Cells are found singly, in pairs and in short chains. Microaerophilic. Colonies on MRS agar after 4 days incubation at 28 °C are 1.8–2.0 mm in diameter, smooth, circular to slightly irregular and white. Catalase-negative. Grows at 15–45 °C, but not at 5 °C. Facultatively heterofermentative; no gas is produced from glucose. DL-Lactate is produced as an end product from hexoses and pentoses. Ammonia is not produced from arginine and mannitol is not produced from fructose. Dextran is not produced from sucrose. Citric and malic acids are used. Ferments ribose, glucose, fructose, mannose, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose, trehalose, melezitose, β-gentiobiose and gluconate and hydrolyses aesculin. Does not ferment glycerol, erythritol, D- or L-arabinose, D- or L-xylose, adonitol, methyl β-xyloside, galactose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, lactose, melibiose, inulin, raffinose, starch, glycogen, xylitol, turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol or 2- or 5-ketogluconate. The DNA G + C content of the type strain is 34.03 ± 0.77 mol%. The cell wall contains peptidoglycan of the A4α L-Lys–D-Asp type.

The type strain, 203^T (=CECT 7310^T =DSM 19674^T), was isolated in 1997 by one of the authors (A.M.R.) from a Bobal grape must.

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