Lactobacillus vini sp. nov., a wine lactic acid bacterium homofermentative for pentoses

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Six strains with more than 99.5 % 16S rRNA gene sequence similarity, identical internal spacer region profiles and restriction analysis of the amplified 16S rRNA gene patterns were isolated from fermenting grape musts during independent studies carried out in France and Spain many years apart. Strains are Gram-positive, motile, facultatively anaerobic rods that do not exhibit catalase activity and have the ability to utilize pentose sugars (ribose and/or L-arabinose), although they are homofermentative bacteria. Strains ferment pentoses exclusively yielding lactic acid as the end product. A broad set of molecular techniques has been applied to characterize these strains and the results show a high degree of genotypical congruence, sharing identical profiles with 16S rRNA-based techniques. Phylogenetic analysis based on 16S rRNA gene sequences placed these strains within the genus *Lactobacillus*, closely related to *Lactobacillus mali*, *Lactobacillus nagelii* and *Lactobacillus satsumensis* (with approximately 95 % sequence similarity). DNA–DNA hybridization experiments confirmed the independent status at the species level of these fermenting grape-musts strains. Phenotypically they can be distinguished from the closest relatives by several traits such as growth temperatures and fermentation of carbohydrates. The name *Lactobacillus vini* sp. nov. is proposed, with strain Mont 4^T (= DSM 20605^T = CECT 5924^T) as the type strain.

Several species of lactic acid bacteria have been described by different authors as being associated with the winemaking process (Davis *et al.*, 1985; Wibowo *et al.*, 1985; Fleet, 1993; Rodas *et al.*, 2005). Some of them are beneficial for wine quality, whereas others have been described as detrimental (Sponholz, 1993; Edwards *et al.*, 1998).

In 1978, P. Barre isolated one wine lactobacillus from high-temperature fermenting grape must (Barre, 1978), named as

Mont 4, that was deposited at the Deutsche Sammlung von Micro-organismen und Zellkulturen GmbH (DSMZ) as *Lactobacillus* sp. DSM 20605. Afterwards, this bacterium was incorporated in the Spanish Type Culture Collection (CECT) from DSMZ as *Lactobacillus* sp. CECT 5924. Chenoll *et al.* (2003) included it in a molecular taxonomic study in order to establish an rRNA-based identification scheme for *Carnobacterium*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* species. In this study, the authors reported that strain CECT 5924 (= Mont 4 = DSM 20605) had singular rRNA-RFLP, internal spacer region (ISR)-PCR and ISR-RFLP profiles that allowed its differentiation from the rest of the analysed members of the genus *Lactobacillus*. In addition, and based on 16S rRNA gene sequence analysis, it was suggested that this strain might represent a novel species. Later on, Rodas *et al.* (2005), following an extensive polyphasic study of wine lactobacillus strains, found a cluster of wine lactobacilli that showed 100 % 16S rRNA gene sequence similarity value with strain Mont 4 (= CECT 5924). The closest phylogenetic neighbours of strain CECT 5924 are *Lactobacillus mali*, *Lactobacillus nagelii* and the recently described novel species *Lactobacillus satsumensis*, with 95.1, 95.3 and 95.6 % sequence similarity, respectively.

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Abbreviations: 16S-ARDRA, amplified 16S rDNA restriction analysis; ISR, internal spacer region; RAPD, random amplified polymorphic DNA.

A table detailing phenotypic traits of *Lactobacillus vini* and its closest phylogenetic neighbours *L. mali*, *L. nagelii* and *L. satsumensis*, and figures showing phylogenetic trees constructed using the maximum-parsimony and maximum-likelihood methods and a dendrogram derived from the comparison of combined molecular techniques' patterns obtained from wine lactic acid bacteria and reference strains are available as supplementary material in IJSEM Online.

This study presents the phenotypic and genotypic characterization of wine and reference strains, including DNA–DNA hybridization experiments. On the basis of these results, a novel species of the genus *Lactobacillus*, *Lactobacillus vini* sp. nov. is proposed, with strain Mont 4^T (=DSM 20605^T=CECT 5924^T) as the type strain.

In addition to strain Mont 4^T (=DSM 20605^T=CECT 5924^T), wine strains 116 (CECT 7072), 119 and 209P were isolated from fermenting red grape Bobal must, whereas strains 154 and 155 came from fermenting red grape Tempranillo must (Rodas *et al.*, 2005), both musts were used for winemaking in the Utiel-Requena Origin Denomination in Spain. These strains and the reference strains *L. mali* CECT 4149, *L. nagelii* CECT 5983^T and *L. satsumensis* DSM 16230^T were grown in modified MRS (mMRS) broth that consisted of MRS (Scharlab) supplemented with L-cysteine hydrochloride (0.5 g l⁻¹) as described by Rodas *et al.* (2003). They were incubated at 28 °C in sealed plastic bags, and stored in 20 % (v/v) glycerol at -80 °C or lyophilized. Phenotypic characterization of strains was done following standardized methods.

Colonies grown on mMRS agar plates at 28 °C for 4 days are 0.7–1.5 mm in diameter, white, smooth, circular and convex with entire edges. Cells are rod-shaped, 0.49–0.82 × 1.36–2.8 µm in size, a few cells are even longer, and the mean value is 0.66 × 2.14 µm in size. Cells are motile, non-spore-forming and occur mainly in pairs but also in short chains of up to five cells. Although a few cells grown in mMRS are motile when observed on wet mounts, no flagella are observed under the Heimbrosk's staining method (Heimbrosk *et al.*, 1989). All strains are Gram-positive, catalase-negative, facultative anaerobes. They form DL-lactate from glucose, as determined by the L-lactic kit (Roche) and HPLC (Frayne, 1986), but not gas and they do not ferment gluconate, thus being considered as homo-fermentative. However, all of them are able to ferment L-arabinose and Mont 4^T ferments D-ribose as well, exclusively yielding DL-lactate as the end product. The molar ratios are 1.57:1.70 (lactate:pentose) for all strains and pentoses, near to the theoretical 1.67 molar ratio lactate:pentose value. These results are in agreement with those obtained by Barre (1978), and verified by Kandler (1983) and Picataggio *et al.* (1998) for strain Mont 4^T. Moreover, Picataggio *et al.* (1998) demonstrated that Mont 4^T had transaldolase and transketolase activities, allowing it to use pentose sugars via an inducible pentose phosphate pathway. This pathway, which exclusively yields lactate as a final product, is different from the 6-phosphogluconate pathway used by hetero-fermentative facultative lactobacilli.

Strains 116, 119, 154, 155, 209P and Mont 4^T grow at 25, 37 and 45 °C but not at 5 or 15 °C. They grow at pH 3.7, 4.5 and 8.0 and in medium with 5 % (w/v) NaCl added but not with 10 % (w/v) NaCl. None of them produce exopolysaccharides from sucrose and only strains 116 and 119 are able to produce ammonia from arginine.

Fermentation profiles of the wine and reference strains were tested in API 50 CHL galleries (bioMérieux) according to the manufacturer's instructions. Strains were incubated up to 5 days at 28 °C. They are shown together with other phenotypic traits in Supplementary Table S1 available in IJSEM Online. Strains 116 and 155 do not ferment cellobiose when tested in the API 50 CHL gallery, but they do when assayed in MRS fermentation broth supplemented with 0.5 % (w/v) cellobiose (Rodas *et al.*, 2005), and therefore we consider all strains positive for this test. Strains present different fermentative abilities for the following carbohydrates: ribose is only fermented by strain Mont 4^T; galactose and D-tagatose are fermented by strains 116, 119, 155 and 209P; strain 154 ferments rhamnose and splits arbutin; and methyl α-D-mannoside is fermented by all strains with the exception of Mont 4^T. API 50 CHL profiles were analysed with the BioNumerics 2.5 (Applied Maths) software using the Simple Matching similarity coefficient and the un-weighted pair group method using arithmetic averages (UPGMA). Strains 116, 119, 155 and 209P share an identical fermentation profile, being slightly different from the other Spanish strain, 154 (data not shown). Therefore, strain 116 was chosen as a representative of these four strains and compared with strains 154 and Mont 4^T in further analysis.

As stated in the online catalogue of DSMZ (<http://www.dsmz.de>), the cell-wall peptidoglycan of strain Mont 4^T (=DSM 20605^T=CECT 5924^T) is L-Lys-D-Asp.

The G+C content of the type strain was determined by HPLC at DSMZ, following the procedure of Mesbah *et al.* (1989). Strain Mont 4^T has a G+C content of 39.4 mol%, a value within the range (32–53 mol%) established for the genus *Lactobacillus*.

GenBank accession numbers of the 16S rRNA gene sequences of about 1500 bp corresponding to strain *Lactobacillus* sp. CECT 5924^T (AJ576009) and wine strains 116 (AY681131) and 154 (AY681132) were published in previous studies (Chenoll *et al.*, 2003; Rodas *et al.*, 2005). Subsequent sequence analysis was conducted using the ARB program package (Ludwig *et al.*, 2004). Sequence similarity values were calculated by comparing nucleotides at the corresponding positions, and, following the recommendations of Ludwig *et al.* (1998), three alternative treeing methods (neighbour-joining, maximum-parsimony and maximum-likelihood) and data subsets were employed using the corresponding ARB tools. The phylogenetic analysis established that the wine strains 116 and 154 showed 99.5 % 16S rRNA gene sequence similarity to that of strain Mont 4^T. The three sequences formed a tight clade in the vicinity of *L. mali*, *L. nagelii* and *L. satsumensis* sequences. The tree shown in Fig. 1 was constructed using the neighbour-joining method. When other tree reconstruction methods were applied, the relative position of some branches varied slightly (see Supplementary Fig. S1 available in IJSEM Online); however, there is no doubt about the independent phylogenetic position of the group formed by strains Mont 4^T, 116 and 154. Sequence similarities with all members of the

Fig. 1. Phylogenetic tree constructed using the neighbour-joining method based on rRNA gene sequences. *Lactobacillus vini* strains Mont 4^T, 116 and 154 grouped together within the *Lactobacillus casei* cluster of micro-organisms (see Collins *et al.*, 1991) and close to strains *L. nagelii* CECT 5983^T and *L. mali* CECT 4149. Bootstrap values appear on each node and were calculated from 1000 replications. Values $\geq 90\%$ are considered statistically significant. Bar, 10% nucleotide substitution.

genus *Lactobacillus* were between 81.7 and 95.6%, their closest neighbours being *L. satsumensis* (95.6%), *L. nagelii* (95.3%) and *L. mali* (95.1%). In all three cases, the similarity in terms of the 16S rRNA gene is below the species level according to Stackebrandt & Goebel (1994).

DNA–DNA hybridization experiments were performed by the chemiluminescent-HA method as described by Ziemke *et al.* (1998) using genomic DNA of strain Mont 4^T, two representative wine strains (116 and 154), and *L. mali* CECT 4149 and *L. nagelii* CECT 5983^T. The reference DNAs were those of strains Mont 4^T and 116 and the results are expressed as mean percentage values based on three independent hybridization experiments. Intraspecific hybridization assays, performed with strains Mont 4^T, 116 and 154, gave values over 77%, thus including these strains in the same genomic species (Stackebrandt & Goebel, 1994). The relative binding ratio values of strains 116 and 154 to strain Mont 4^T were always 100% or slightly higher. Reciprocal hybridization experiments using genomic DNA of strain 116 (=CECT 7072) as template rendered values around 77% with strain Mont 4^T and 97% with strain 154. The interspecific hybridization values of strain Mont 4^T with *L. mali* CECT 4149 and *L. nagelii* CECT 5983^T were 49 and 52%, respectively. When reference DNA was that of strain 116 the values were slightly lower (31 and 38%, respectively). All these results confirm the separate status of the wine strains at the species level.

Genotypic characterization by rRNA-based techniques (Chenoll *et al.*, 2003; Rodas *et al.*, 2005) showed that wine strains and reference strain Mont 4^T had identical ISR profiles and amplified 16S rDNA restriction analysis (16S-ARDRA) patterns with any of the four restriction enzymes utilized (Supplementary Fig. S2 available in IJSEM Online), but they are peculiar profiles that allow the differentiation of this group of strains from other *Lactobacillus* species (Chenoll *et al.*, 2003; Rodas *et al.*, 2005). In addition,

intraspecific differentiation by random amplified polymorphic DNA (RAPD) and RFLP-PFGE revealed the existence of three patterns, one corresponding to strain Mont 4^T, another shown exclusively by strain 154 and the third one shared by the remaining wine strains (116, 119, 155 and 209P). This result is consistent even when the restriction enzyme *Sma*I, which yielded more than 35 fragments per digestion, was used. The overall data of each strain obtained from each technique were combined on the BioNumerics software maintaining the same coefficients used for single pattern analysis (Chenoll *et al.*, 2003; Rodas *et al.*, 2005), and clustered using the UPGMA method. At 74% similarity all wine strains clustered together with strain Mont 4^T and apart from *L. mali* and *L. nagelii* (Supplementary Fig. S2 available in IJSEM Online). Strains 116, 119, 155 and 209P grouped together at 98% similarity and strains Mont 4^T and 154 at 76% similarity. The calculated global cophenetic correlation value for the global analysis was 1.00, indicating an excellent level of reliability.

All data reported here confirm that strain Mont 4^T and the wine strains constitute a novel taxon in the genus *Lactobacillus* at the species level. It is well isolated in terms of genomic similarity from its closest phylogenetic neighbours, and readily recognizable by their phenotypic characteristics, in agreement with the criteria of Wayne *et al.* (1987). The novel species can be phenotypically differentiated from the closest species of *Lactobacillus* by the properties shown in Table 1 and genotypically by ISR, 16S-ARDRA and RAPD profiles. The most typical physiological feature of this group of strains is the homofermentative utilization of pentoses with lactate as the sole end product, which represents a unique inducible metabolic pathway, unknown among other lactobacilli.

We propose the name *Lactobacillus vini* sp. nov. with strain Mont 4^T (=DSM 20605^T=CECT 5924^T), isolated from grape must by Barre in 1978, as the type strain.

Table 1. Phenotypic traits that differ between *Lactobacillus vini* sp. nov. and its closest phylogenetic neighbours *L. mali*, *L. nagelii* and *L. satsumensis*

Species: 1, *L. vini*; 2, *L. mali*; 3, *L. nagelii*; 4, *L. satsumensis*. Data obtained in this study unless otherwise stated. +, All strains positive; –, all strains negative; DAP, diaminopimelic acid.

Characteristic	1	2	3	4
Growth in MRS at 15 °C	–	+	+	+
Growth with 5 % NaCl	+	–	+	+
Fermentation of:				
L-Arabinose	+	–	–	–
L-Sorbose	–	–	+	+
L-Rhamnose	–	–	+	+
D-Mannitol	–	+	+	+
D-Sorbitol	–	+	+	–
Methyl α-D-glucoside	–	–	+	+
Salicin	+	–	+	+
D-Cellobiose	+	+	+	–
D-Maltose	+	–	+	+
β-Gentiobiose	+	+	–	+
D-Turanose	–	–	–	+
Lactic acid isomer	DL*	L†	DL‡	L§
Formation of exopolysaccharides from sucrose	–	+†	+‡	+§
Peptidoglycan type	L-Lys–D-Asp	DAP	DAP	DAP

*Racemic mixture.
†Carr & Davies (1970).
‡Edwards *et al.* (2000).
§Endo & Okada (2005).

Description of *Lactobacillus vini* sp. nov.

Lactobacillus vini (vi'ni. L. gen. n. *vini* of wine).

Gram-positive, motile, non-spore-forming rods, 0·49–0·82 µm wide by 1·36–2·8 µm long. Cells are found singly, in pairs and in short chains. Facultatively anaerobic. Colonies on MRS agar after 4 days incubation at 28 °C are 0·7–1·5 mm in diameter, with entire edges, smooth, glistening and white. Catalase-negative. Growth occurs from 25 to 45 °C, but not at 15 °C or less. Homofermentative; no gas is produced from glucose. Cells contain L-Lys–D-Asp in their peptidoglycan. DL-Lactate is exclusively produced as the end product from hexoses and pentoses. Ammonia production from arginine is variable since only strains 116 and 119 are positive. Mannitol is not produced from fructose. Exopolysaccharide is not produced from sucrose. Citric and malic acids are utilized. All strains tested ferment L-arabinose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, amygdalin, salicin, cellobiose, maltose, sucrose, trehalose and β-gentiobiose. Aesculin is hydrolysed. None of the analysed strains ferment glycerol, erythritol, D-arabinose, D-xylose, L-xylose, D-adonitol, methyl β-D-xyloside, L-sorbose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-glucoside, lactose, melibiose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate.

The type strain, Mont 4^T (=DSM 20605^T=CECT 5924^T), was isolated from fermenting grape must in 1978 by P. Barre. Reference strains are 116 (=CECT 7072) and 154 (=CECT 7073), both isolated from fermenting Spanish grape musts (Rodas *et al.*, 2005). Strains 119, 155 and 209P are also strains of this species. The type strain shows the following additional traits: it does not produce ammonia from arginine, it ferments D-ribose, it does not ferment D-galactose, methyl α-D-mannoside or D-tagatose, it is unable to hydrolyse arginine and it does not split arbutin. The G + C content is 39·4 mol%.

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