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Lactobacillus uvarum sp. nov. – A new lactic acid bacterium isolated from Spanish Bobal grape must ☆

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Abstract

Five strains isolated from grape musts in Spain in 1997, have been characterized by several molecular techniques, and three of them have been identified as pertaining to a new species. All strains are Gram-positive rods, aerotolerant and homofermentative bacteria that do not exhibit catalase activity. Phylogenetic analysis based on 16S rRNA gene sequences placed these strains within the genus *Lactobacillus*, closely related to *Lactobacillus mali*. DNA–DNA hybridization experiments confirmed that strain 71 belongs to the lately described species *L. satsumensis*, strain 88 belongs to *L. mali* and the other three isolates have an independent status at species level. Restriction analysis of the amplified 16S rRNA gene (16S-ARDRA), internal spacer region (ISR) analysis, random amplified polymorphism DNA (RAPD) and ribotyping were performed in order to establish genotypic similarities and differences between the new species and their closest species. The three isolates can be genetically differentiated from their closest relatives by RAPD analysis and ribotyping. Phenotypically, they can be distinguished by several traits such as their ability to grow at pH 3.3 and NaCl 5% (w/v) and by certain carbohydrate fermentations. The name *L. uvarum* sp. nov. is proposed. The type strain is 8^T (= DSM 19971^T = colección española de cultivos tipo (CECT) 7335^T).
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Keywords: *Lactobacillus uvarum* sp. nov.; *Lactobacillus*; Must; Winemaking; 16S rRNA; ARDRA; ISR; RAPD; Ribotyping

Abbreviations: CECT, colección española de cultivos Tipo; DSMZ, deutsche Sammlung von Mikroorganismen und Zellkulturen; ISR, internal spacer region; *L.*, *Lactobacillus*; LAB, lactic acid bacteria; RAPD, random amplified polymorphism DNA; 16S-ARDRA, restriction analysis of the amplified 16S rRNA gene

Introduction

The genus *Lactobacillus* is one of the most important taxa related to foods and many species included in it play an important role in food preservation, pro duction and alteration [1,2]. *Lactobacillus* species occur naturally on grapes and their ability to grow in grape juice and wine is well documented [4,6,8, 18,19,24,25,27,30,37]. Some new lactobacilli species as

☆The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 8^T, 24 and 68 are AY681126, EU345000 and AY681128, respectively.

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Lactobacillus kunkeei [9], *L. nagelii* [10], *L. bobalius* [23] and *L. vini* [31] have been isolated from this habitat in recent years. Their role in winemaking could be beneficial, as they could perform malolactic fermentation which decrease excessive acidity of wine, but also detrimental. In fact, some wine lactobacilli have been described as spoilage microorganisms because of their production of acetic acid, off-flavours, ropiness and biogenic amines [7,13,20–22,28,33,36,37].

In an earlier study, Rodas et al. [30] carried out an extensive polyphasic analysis to characterize 178 wine lactobacilli isolated from different moments of the winemaking process. They found five strains (strains 8, 24, 68, 71 and 88) that did not cluster with any of the reference species of *Lactobacillus*. The 16S rRNA gene of four of these strains was sequenced, and their phylogenetic relationship with the rest of published lactobacilli sequences established. The results revealed that the closest relative of strains 8, 68, 71 and 88 was *L. mali*, with 96.8, 97.8, 96.7 and 100% sequence similarity. In the same year, a new *Lactobacillus* species, *L. satsumensis* was described [11] and then, *L. vini* [31], that are besides *L. nagelii*, the closest species to *L. mali* at that moment.

The relationships of strains 8, 24, 68, 71 and 88 between them and their nearest relatives are studied in depth in our work, by applying different analysis: phylogenetic, genotypic and phenotypic. On the basis of these results, a new species of *Lactobacillus*, *L. uvarum* sp. nov. is proposed comprising three wine strains: 8, 24 and 68, with strain 8^T (= colección española de cultivos tipo (CECT) 7335^T = DSM 19971^T) as the type strain.

Materials and methods

Bacterial strains, origin, growth and storage conditions

Strains 8, 24, 68, 71 and 88 were isolated by Rodas et al. [30] from Bobal must in different wineries from Utiel–Requena Origin Denomination of Spain. Strain 8 was isolated from an Utiel winery, strains 24 and 68, from the Requena experimental winery of Viticulture and Enology School, strain 88 from a Camporrobles winery, and strain 71 from a Cuevas de Utiel winery. Reference strains used in this work were *L. mali* CECT 4149, *L. mali* Lb44, *L. mali* Lb206, *L. satsumensis* DSM 16230^T and *L. satsumensis* ENOLAB 4555.

All strains used in this study were grown in MRS broth (Scharlab) supplemented with $0.5\,g\,l^{-1}$ L-cysteine hydrochloride (mMRS) under the conditions described by Rodas et al. [29]. Cultures were preserved frozen at $-20\,^{\circ}C$ in 20% glycerol, and lyophilised.

Phylogenetic analysis

Sequences of 16S rRNA gene of strains 8, 68, 71 and 88 are accessible from GenBank with accession numbers AY681126, AY681128, AY681129 and AY681130, respectively. Sequence corresponding to 16S rRNA gene of strain 24 was obtained in this work by applying the methods described by Rodas et al. [30].

Almost complete 16S rRNA gene sequences of wine isolates 8, 24, 68, 71 and 88 and their nearest relatives were subjected to phylogenetic analysis. Several reconstruction methods (neighbour-joining, maximum parsimony and maximum likelihood) were applied in the BioNumerics V2.5 software package to infer the phylogeny of those strains. Pairwise distances were calculated with the Mega 3.1 software.

DNA–DNA hybridization

Genomic DNA was extracted from pure cultures following the method described by Sambrook et al. [32] but adding CTAB extraction buffer as reported Gardes et al. [15]. DNA was spectrophotometrically quantified by a DU 800 spectrophotometer (Beckman Coulter) at 260 nm and adjusted to a final concentration of $300\,ng\,\mu l^{-1}$ in Mili U water.

A set of DNA–DNA hybridization experiments was performed between wine and reference strains in order to know if they belonged to the same or different species (Table 1). Hybridizations were performed at $63.2\,^{\circ}C$ and experiments were done in duplicate as described by Ziemke et al. [38].

Genotypical characterization

Amplification and *Mse*I and *Bfa*I restrictions of 16S rRNA gene were done as described by Rodas et al. [29]. Amplification and *Hae*III restriction of internal spacer region (ISR) were done as described Chenoll et al. [5]. random amplified polymorphism DNA (RAPD) analysis with COC and 17R primers and ribotyping with *Eco*RI enzyme were performed as reported by Rodas et al. [30]. All these techniques were applied to genotipically characterize wine strains.

Digitalized gel images were analyzed by the BioNumerics V2.5 software package and a dendrogram was obtained for each technique using Pearson's product moment correlation coefficient for RAPD and ribotyping, and Dice similarity coefficient for the rest of experiments. A dendrogram derived from comparison of all combined techniques was constructed using UPGMA clustering method maintaining the same similarity coefficients used for single pattern analysis. Similarity values between genotypic patterns were calculated.

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