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4 Efficacy of *recA* gene sequence analysis in the identification
5 and discrimination of *Lactobacillus hilgardii* strains isolated
6 from stuck wine fermentations
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Abstract

Conventional phenotypic methods sometimes lead to misidentification of some heterofermentative wine lactobacilli such as *Lactobacillus hilgardii*, *Lactobacillus buchneri*, and *Lactobacillus brevis*. We establish the specificity of 16S rDNA sequencing in the differentiation of these species and in the rejection of the *Lactobacillus vermiforme* species name. Moreover, we succeeded in differentiating these heterofermentative species by means of *recA* gene sequence comparison. Short homologous regions were amplified by PCR with degenerate consensus primers, sequenced, and 280 bp were analysed and considered for the inference of phylogenetic trees. The phylogram obtained was coherent and clearly separated the three species. The *recA* gene sequence was a reliable and useful method that allowed a good discrimination among closely related species. The validity of the *recA* gene sequence, restriction fragment length polymorphism (RFLP) analysis of the PCR-amplified 16S–23S rDNA intergenic spacer region (ISR), and random amplified polymorphic DNA (RAPD) to study the *L. hilgardii* intraspecies heterogeneity was tested in five strains isolated from stuck wine fermentations at the same winery in the same vintage. The results indicated that *L. hilgardii* is a very heterogeneous species. Since *L. hilgardii* is a malolactic species that can influence the final quality of the wine, the presence of oenological relevant genes, such as those involved in ethyl carbamate or biogenic amine production, was investigated.

Keywords: *Lactobacillus hilgardii*, *Lactobacillus vermiforme*, Wine, Heterofermentative lactobacilli, *recA* gene, typing method

1. Introduction

Winemaking involves a mixed culture of numerous microorganisms including fungal, yeast, and bacteria species. The principal bacteria present in wine are members of the lactic acid bacteria (LAB). They can transform a large variety of organic compounds present in must or wine, giving final products that affect the organoleptic characteristics of wine. LAB can be detrimental or beneficial depending on the species and the vinification moment at which they develop. Many lactobacilli have been isolated as responsible for lactic acid spoilage from sluggish fermentations and sweet fortified wines (Sponholz, 1993; Lonvaud-Funel, 1999).

Heterofermentative lactobacilli isolated from sources associated with alcohol are usually allocated to *L. brevis*, *L. hilgardii*, or related taxa such as *L. buchneri*. The taxonomy of these species however is unsatisfactory. Most of the lactobacilli species exhibit considerable phenotypic diversity and as result it is often extremely difficult to distinguish among related species (Le Jeune and Lonvaud-Funel, 1994; Sohier et al., 1999). Classically, identification of LAB is made by cell morphology, substrate assimilation and products of metabolism. However, some inconstancy arises for heterofermentative lactobacilli. *L. buchneri* is phenotypically distinguished from *L. brevis*, mainly by its ability to ferment melezitose. However, Kandler and Weiss (1986) reported that some strains identified as *L. brevis* could ferment melezitose. Strains of *L. brevis* and *L. hilgardii* are phenotypically similar and only differ in their ability to ferment arabinose. *L. brevis* ferments arabinose, whereas *L. hilgardii* cannot. Sohier et al. (1999) using molecular techniques identified three arabinose-fermenting strains as *L. hilgardii* in spite of their ability to ferment this sugar. This ambiguity was also encountered by Vescoso et al. (1979) and Pidoux et al. (1990) since strains previously

identified by their phenotype as *L. brevis* were finally classified as *L. hilgardii* according to their DNA homology.

Stratiotis and Dicks (2002) described that *L. vermiforme* was the lactobacilli species most frequently isolated from South African fortified wines. Previously, DNA hybridization studies performed by Farrow et al. (1986) on three strains identified as *L. vermiforme* indicated that they shared a high DNA homology with the type strain of *L. hilgardii*. Based on these results, the species name *L. vermiforme* was rejected. In fact, nowadays *L. vermiforme* is not included in the list of validated species. However, strains isolated from bottled South African fortified wines cluster with two strains which resemble the original description of *Betabacterium vermiforme* (later reclassified as *L. vermiforme*) and that could not be designated to any of the presently known *Lactobacillus* spp. and therefore, Stratiotis and Dicks (2002) suggested that the name *L. vermiforme* will have to be revised. Later, Du Plessis et al. (2004) reported the first *L. vermiforme* description in brandy base wines. However, these authors used as reference strain *L. vermiforme* NCDO 962 that now is provided by the NCIMB as *L. hilgardii* 700962 strain.

Since the identification of wine heterofermentative lactobacilli is often ambiguous, recently, research has focused on the application of molecular biology methods for microbial identification. The 16S rDNA approach is one of the most widely used standard technique to infer phylogenetic relationships among bacteria but is sometimes insufficient to distinguish closely related species. The *recA* gene has been proposed as a useful marker in inferring bacterial phylogeny and has been used successfully to differentiate species of some bacterial genera. LAB species included in the *L. casei* and the *L. plantarum* groups were differentiated based on their *recA* sequences (Felis et al., 2001; Torriani et al., 2001).

Premature arrest of fermentation constitutes one of the most challenging problems in wine production. The causes of stuck and sluggish fermentations are numerous, troublesome to diagnose, and difficult to rectify. To avoid this type of fermentation problem, it have been described that the compatibility properties of the malolactic bacteria and the yeast strains should be evaluated. It has been shown that a novel bacterium, *L. kunkeei*, frequently is associated to stuck fermentations regardless of the yeast strain present (Bisson, 1999).

Heterofermentative lactobacilli were isolated from several fermentations tanks suffering stuck fermentations at the same Spanish winery in the same vintage. The aims of this study were (i) to evaluate 16S rDNA sequencing to identify this wine heterofermentative lactobacilli, (ii) to compare *recA* sequencing for the discrimination of these closely related species, (iii) to interspecies discriminate the lactobacilli isolated, and (iv) to detect the presence of some enological relevant traits on them.

2. Materials and methods

2.1. Strains and growth conditions

Four *Lactobacillus* sp. strains, RM62, RM63, RM66, and RM79 (previously named BIFI-62, BIFI-63, BIFI-66 and BIFI-79) and *Lactobacillus buchneri* RM-77 were isolated from Spanish wine samples suffering stuck fermentations (Moreno-Arribas et al., 2003). Pure culture of *L. hilgardii* type strain, CECT 4786^T (ATCC 8290^T), was purchased from the Spanish Type Culture Collection (CECT).

Lactobacilli strains were grown in MRS broth (Difco, France) by incubating at 30 °C in a 5% CO₂ atmosphere.

2.2. Bacterial DNA extraction

Bacterial chromosomal DNA was isolated from overnight cultures using a protocol previously described (Vaquero et al., 2004). DNA precipitates were resuspended in an appropriate volume of TE solution (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) (Sambrook et al., 1989).

2.3. Species-specific identification techniques

2.3.1. PCR amplification and DNA sequencing of the 16S rDNA

16S rDNAs were PCR amplified using the eubacterial universal pair of primers 63f and 1387r (numbering is based on the *Escherichia coli* 16S rRNA gene) previously described (Table 1) (Marchesi et al., 1998). The 63f and 1387r primer combination generates an amplified product of 1.3 kb. PCR reaction was performed in 0.2 ml **microcentrifuge tubes in a total volume of 25 µl containing 1µl of template DNA** (aprox. 10 ng), 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM MgCl₂, **200 µM of each dNTP, primer 63f (1µM), primer 1387r (1µM) and 1 U of AmpliTaq DNA** polymerase. The reaction was performed in a GeneAmp PCR System 2400 (Perkin Elmer, USA) using the following cycling parameters: initial 5 min denaturation at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 1:30 min at 72 °C.

Amplified products were resolved on a 0.7% agarose gel. The amplifications products were purified on QIAquick spin Columns (Quiagen, Germany) for direct sequencing. DNA sequencing was carried out by using an Abi Prism 377™ DNA sequencer (Applied Biosystems, USA).

2.3.2. PCR amplification and DNA sequencing of a *recA* gene fragment

For the amplification of *recA* regions, 10 ng of chromosomal DNA was added to the 25 µl PCR mixture described above but containing RecA-up and RecA-down oligonucleotides (Table 1). These degenerate primers are based on well-conserved domains, approximately 120 amino acids apart, of the RecA proteins. The PCR program was made up of initial denaturation at 94 °C for 1 min and 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 30 sec. The expected size of the amplicon was 360 pb. Fragments of the expected sizes were purified from 2% agarose gel as described above, and sequenced.

2.4. Strain-specific typing methods used for *L. hilgardii* strains

2.4.1. RFLP of the PCR-amplified 16S-23S rDNA ISR

Restriction fragment length polymorphism (RFLP) analysis of the PCR-amplified 16S-23S rRNA gene intergenic spacer region (ISR) was performed as described previously (Zavaleta et al., 1996) by using primers 16S14F and 23S1R based on conserved regions of aligned rRNA bacterial sequences (Table 1). The amplified 16S-23S ISRs from *L. hilgardii* were digested with the restriction enzymes *NdeI*, *TaqI*,

AluI and *CfoI* (Roche, Germany). The digested products were separated in 2% agarose gels (Pronadisa, Spain).

2.4.2. Random Amplified Polymorphic DNA (RAPD)

Approximately 10 ng of DNA was subjected to PCR amplification in the reaction mixture described above, but containing only one oligonucleotide in the mixture. The reaction mixtures were subjected to amplification for 40 cycles at the following conditions 94 °C, 1 min; 36 °C, 1 min; and 72 °C, 2 min, with a final extension step at 72 °C for 7 min. Three oligonucleotides were selected for RAPD analysis, M13, OPA20 and A22 (Table 1). PCR products were electrophoresed in 0.7% agarose gels (Pronadisa, Spain).

2.5. Presence of amino acid decarboxylase genes

Chromosomal DNAs were subjected to multiplex-PCR amplification to detect simultaneously the presence of the LAB histidine, tyrosine, and ornithine decarboxylase encoding genes (Marcobal et al., 2005). Briefly, PCRs were performed in 25 µl amplification reaction mixture as described above, out of that six primers were included in the same reaction. The relative concentration of the primers was optimised previously (Marcobal et al., 2005) being 0.3 µM of primer set JV16HC-JVHC17HC for the amplification of the histidine decarboxylase encoding gene, 1 µM of primer set 3-16 for the amplification of the ornithine decarboxylase gene, and 2 µM of primer pair P1-rev-P2-for for the tyrosine decarboxylase encoding gene (Table 1). The reactions were performed by using the following cycling parameters: 1 min at 95 °C followed by 30

cycles of 30 s at 95 °C, 30 s at 52 °C, and 2 min at 72 °C, and a final extension step of 10 min at 72 °C. Amplified products were resolved on a 1.5% agarose gel (Pronadisa, Spain).

2.6. Presence of arginine deiminase pathway genes

The arginine deiminase (ADI) pathway basically includes three enzymes, arginine deiminase (ADI), ornithine transcarbamylase (OTC), and carbamate kinase (CK). Two conserved domains of each protein were selected to design synthetic primers to amplify by PCR the corresponding gene. Primer set Aup–Adown amplifies a 0.6 kb internal DNA fragment of the ADI encoding gene. Similarly, primer sets Bup–Bdown and Cup–Cdown amplify fragments of 0.3 and 0.5 kb, of the genes coding for OTC and CK, respectively (Table 1) (Arena et al., 2002). The PCR reaction mixture and the amplification conditions were described above for the amplification of the 16S rDNA.

2.7. Data analysis

Sequence similarity searches were carried out using Basic local alignment search (BLAST) (Altschul et al., 1997) on the EMBL/GenBank databases. Sequence alignments and comparison were done with the program BioEdit (<http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html>) and converted into MEGA files with MEGA version 2.1 software (<http://www.megasoftware.net>). Phylogenetic trees were constructed by the unweighted pair group method with arithmetic averages (UPGMA) with the Kimura two-parameter model. The percentage of bootstrap

confidence levels for internal branches, as defined by the MEGA program, was calculated from 10000 random resamplings.

3. Results and discussion

3.1. Molecular classification of *Lactobacillus* sp. isolated from stuck wine fermentations

A total of 78 lactic acid bacteria strains were isolated from must grape or wine of different wine-producing areas of Spain (Moreno-Arribas et al., 2003). Morphological and biochemical tests were used to taxonomically identify these strains. However, five lactobacilli strains remain without a clear species assignation. Later, a more exhaustive identification, including 16S rDNA sequencing, was done. The 16S rDNA sequence from the unclassified strains, RM62, RM63, RM66, and RM79 showed the highest identity degree to the *L. vermiforme* RTT15 partial sequence (accession no. AF375901) available on year 2001, followed by *L. hilgardii* DSM 20176^T (accession no. M58821) which possess the second sequence most similar. Therefore, we preliminary identified these strains as *L. vermiforme*. However, recently, we performed a new search on the ribosomal database project (<http://rdp.cme.msu.edu>) and then, the sequence of the *L. hilgardii* IOEB 0204 (accession no. AY241664) have become available on year 2005 and have revealed that our unclassified lactobacilli were 100% identical to this *L. hilgardii* sequence. When we compared both 16S rDNA sequences from *L. hilgardii*, IOEB 0204 and DSM 20176^T, we could observe that the type-strain sequence contains several non-determined nucleotide positions, that were surely identified in the IOEB 0204 sequence.

In order to check if *L. hilgardii* DSM 20176^T strain have a 16S rDNA sequence identical to that of IOEB 0204, we sequenced this DNA fragment from the type strain provided by the CECT, *L. hilgardii* CECT 4786^T (DSM 20176^T, ATCC 4786^T, or LMG 6895^T). This sequence confirms that both *L. hilgardii* strains possessed identical 16S rDNA sequence and it was identical to the lactobacilli strains RM62, RM63, RM66, and RM79. Therefore, we assumed that these strains belonged to the *L. hilgardii* species. Similarly, strain RM77, previously identified as *L. buchneri*, showed a 16S rDNA sequence identical to that of *L. hilgardii* strains, and therefore, was reclassified as *L. hilgardii* RM77. These data clearly showed that molecular methods are more efficient than phenotypic or biochemical studies for lactobacilli identification at the species level.

Interestingly, when we compared the 16S rDNA sequence of *L. hilgardii* CECT 4786^T to that of *L. vermiforme* RTT15, they were identical in a 492 nucleotides overlap out of two non-determined nucleotide positions present in *L. vermiforme*. In addition, the complete sequence of *L. vermiforme* ATCC 13133 (accession no. M59295), was identical to *L. hilgardii* sequence in a 1480 overlap, out of 22 non-determined nucleotide positions. Moreover, previous studies where the isolation of *L. vermiforme* strains were reported, used as control strain of this species *L. vermiforme* NCDO 962 (Plessis et al., 2004), this strain is now provided by the NCIMB (National Collection of Industrial and Marine Bacteria, Aberdeen, UK) as *L. hilgardii* NCIMB 700962. The NCIMB also offers the following *L. hilgardii* strains NCIMB 700961, NCIMB 700962 and NCIMB 1965, which were used as *L. vermiforme* NCDO strains by Farrow et al. (1986) who demonstrated that both species were synonymous, and therefore they postulated that the species name *L. vermiforme* should be rejected. Taking into account previous results, together with the results obtained on 16S rDNA sequence analysis, which were not available when Farrow's work was published, it seems that *L.*

1 *veriforme* strains are members of the *L. hilgardii* species. Consequently, the proposed
2 species *L. veriforme*, phylogenetically indistinguishable from many strains of *L.*
3 *hilgardii* should continue been rejected and not included in the list of validated species
4 names.

5 The majority of heterofermentative lactobacilli in wine belongs to *L. hilgardii*,
6 *L. buchneri* and *L. brevis* species, which are closely related. We aligned the 16S rDNA
7 sequence of the type strains representing these species. Fig. 1A shows only the
8 nucleotide position where the sequences were different among them. The highest
9 nucleotide identity (97%) was shown between *L. hilgardii* and *L. buchneri* sequences,
10 while both sequences were 94% identical to *L. brevis* sequence (Fig. 1B). These results
11 confirm the high identity showed among these wine heterofermentative lactobacilli.

12 It has been proposed the *recA* gene sequence analysis as a new method for
13 inferring relationships among very closely related species. Torriani et al. (2001)
14 indicated that the clear distinction obtained with short gene sequences validates the
15 possibility of using the *recA* gene as a phylogenetic-taxonomic marker for closely
16 related species and opens new possibilities for a rapid and reliable identification of LAB
17 of importance for food. In that sense, LAB included in the *L. casei* (Felis et al., 2001)
18 and the *L. plantarum* (Torriani et al., 2001) groups were successfully differentiated
19 based on their *recA* sequences. We sequenced a 280 bp *recA* DNA fragment, excluding
20 regions of primer annealing, from the *L. hilgardii* strains isolated from wine (RM62,
21 RM63, RM66, RM77, and RM79) and from the *L. hilgardii*, *L. buchneri* and *L. brevis*
22 type strains as representatives of these species (Fig. 2). Sequence alignments did not
23 introduce any gaps, as expected from closely related species. *L. hilgardii* strains
24 presented only five polymorphic sites, showing a 98–100% identity among them. The
25 DNA sequences differences are translationally silent, as amino acid sequences of the

RecA proteins are identical in this region. RecA is not completely free of mutation; though the RecA protein has a fundamental role in the cell and mutations must not alter its function, point mutations that do not alter gene product activity are tolerated. From the sequences showed in Fig. 2, it could be obtained that *L. hilgardii* presented an identity of 82% to *L. buchneri* and 77% to *L. brevis*. These identities are lower than those obtained from the 16S rDNA sequences, allowed then a better discrimination among closely species. Our results confirm that short *recA* gene sequences have high discriminating power for species difficult to differentiate.

Based on these partial *recA* sequences we have constructed a phylogenetic tree (Fig. 3). Fig. 3 showed that the three wine heterofermentative species were unambiguously differentiated by the comparative analysis of the short fragment of the *recA* gene. Moreover, this phylogenetic analysis permitted a clear distinction among other LAB species represented by their type strains (Fig. 3). Analysis based on the *recA* gene are in general agreement with those performed with 16S rDNA, thus validating the use of *recA* as a taxonomic marker (Felis et al. 1997). Our results confirm that the *recA* gene can be proposed as a new method for inferring relationships among very closely related species.

3.2. Molecular diversity among *L. hilgardii* strains as revealed by RFLP-ITS and RAPD analysis

Torriani et al. (2001) when compared *recA* sequences of strains from *L. plantarum* group obtained identities higher than 99% within strains from the same species. Due to the sequence diversity showed at the intra-species level, *recA* could be a useful target for simultaneous identification of species and strains. The *recA* analysis

among *L. hilgardii* strains reveals a degree of heterogeneity (0–2%) that could be also used to know the intraspecies genetic diversity in this species. Recently, multilocus sequence typing (MLST) has been shown to be a powerful technique for bacterial typing (Enright and Spratt, 1999). MLST make use of automated DNA sequencing to characterize the alleles present at different housekeeping loci. The simplest version of a MLST scheme is the analysis of only one locus. Here, by using only the *recA* locus we were able to identify four different alleles among the six *L. hilgardii* strains sequenced (Fig. 2). Only strains RM62 and RM77, and RM66 and RM79 shared identical *recA* sequence. This heterogeneity is higher than the previously reported one for other species (Torriani et al., 2001) and remarkably high since five strains were isolated from wines from different fermentation tanks suffering stuck fermentations at the same winery in the same vintage.

In order to provide more discrimination, we applied two additional methods frequently used for intraspecies bacterial typing. First, we analysed the variation in length and sequence of the 16S–23S rDNA ISRs. ISRs sequences are much more variable than that of the 16S rRNA structural gene, both in size and sequence, which makes it a suitable target for typing bacterial populations and to discriminate among strains within some species. Since ribosomal genes are present in several copies each one having a different sequence, we observed two different PCR amplification bands in the agarose gel (Fig. 4.1). However, all the *L. hilgardii* strains isolated from wine stuck fermentations showed the same PCR amplification pattern and shared the same RFLP ISR pattern, out of strain RM63 which ISR was not cut by *TaqI*. Fig. 4 shows the PCR amplification pattern and the RFLP patterns obtained. Therefore, the RFLP ISR method is not enough discriminating among the *L. hilgardii* strains analysed.

1 The intraspecies genetic diversity among these *L. hilgardii* strains was also
2 evaluated by random amplified polymorphic DNA (RAPD). Single primers
3 corresponding to arbitrary sequences are used to amplify genomic DNA sequences in
4 order to generate genomic patterns. Depending on the type of primer used and on the
5 reaction conditions, random amplification of bacterial genomes generate suitable
6 fingerprints for characterization at particular strain level. Different results were obtained
7 according to the oligonucleotide used (Fig. 5). The M13 RAPD profiles were strain
8 specific, whereas primer OPA20 gives mainly only one specific pattern. The high
9 discrimination showed by M13 RAPD is in agreement with previous work where this
10 oligonucleotide was successfully used to obtain strain-specific patterns of many LAB
11 species (Rossetti and Giraffa, 2005).

12 By the application of these typing methods we were able to differentiate all the
13 *L. hilgardii* strains analysed. Strains RM62 and RM77, and RM66 and RM79 that
14 shared identical *recA* sequence, were differentiated by RAPD analysis. Results from the
15 present study demonstrated that *L. hilgardii* is a heterogeneous species. This
16 heterogeneity is consistent with the occasional misidentification of *L. hilgardii* and *L.*
17 *brevis* species (Le Jeune and Lonavud-Funel, 1994; Sohier et al., 1999), the description
18 of the additional *L. vermiforme* species (Stratiotis and Dicks, 2002; Plessis et al., 2004),
19 and the traditional allocation of a variety of different species in *L. hilgardii* species
20 (Kandler and Weiss, 1986).

21 22 3.3. Genotypic characterization of enologically relevant safety traits 23

1 *L. hilgardii* is a heterofermentative species which can grow in wines and
2 therefore, influences the final quality of the product either by conducting malolactic
3 fermentation or by causing wine spoilage.

4 The role of malolactic wine LAB in the formation of ethyl carbamate or
5 urethane, an animal carcinogen, has been studied (Liu and Pilone, 1998). The formation
6 of ethyl carbamate is derived largely from ethanolysis of precursors compounds (e. g.,
7 urea, citrulline, and carbamyl phosphate) which are themselves derived from microbial
8 metabolism. In LAB, arginine degradation by the arginine deiminase (ADI) pathway
9 has been identified as a mechanism of ethyl carbamate precursors production. This
10 pathway basically involves three enzymes, ADI, OTC and CK. The production of ethyl
11 carbamate precursors by *L. hilgardii* in wines was investigated (Azevedo et al., 2002).
12 Recently, the genes involved in the ADI pathway in *L. hilgardii* X₁B have been
13 characterized (Arena et al., 2002). In order to detect the presence of the genes involved
14 in the ADI pathway, we amplified each of the three genes in the *L. hilgardii* strains. All
15 the strains analysed gave an amplicon of the expected size for each gene. Figure 6
16 shows the DNA fragments obtained from *L. hilgardii* RM62. These results indicated
17 that the *L. hilgardii* strains analysed possessed the ADI pathway genes and therefore
18 should be considered as potential ethyl carbamate producers. Previously, De Angelis et
19 al. (2002) demonstrated that only obligately heterofermentative lactobacilli, including *L.*
20 *hilgardii* strains, possessed the three enzymatic activities.

21 In addition to ethyl carbamate, biogenic amines are compounds present in wines
22 that have been implicated in food poisoning incidents. Biogenic amines in wine are
23 mainly formed by decarboxylation of the corresponding amino acids by
24 microorganisms. Several amino acids can be decarboxylated; as a result biogenic
25 amines are usually found, with histamine, tyramine and putrescine being the most

1 frequent. *L. hilgardii* strains from the OIEB collection and the ATCC were analysed for
2 biogenic amine production, and they were found to be strong tyramine producers
3 (Moreno-Arribas et al., 2000). In a previous study, the potential to produce biogenic
4 amines by the *L. hilgardii* strains analysed in the present work, was investigated in a
5 decarboxylase synthetic broth by reverse-phase high performance liquid
6 chromatography (Moreno-Arribas et al., 2003). None of the *L. hilgardii* strain was able
7 to produce these biogenic amines. This result was later confirmed by thin-layer
8 chromatography (data not shown). In order to correlate the non-production of biogenic
9 amines with the absence of the corresponding decarboxylase genes, we performed a
10 multiplex PCR assay for the simultaneous detection of these genes (Marcobal et al.,
11 2005). None of the *L. hilgardii* strains produced an amplicon of the expected size (data
12 not shown), so we could conclude that known decarboxylase genes were absent on
13 them. This could be an expected result since stuck fermentations are characterized by a
14 high residual sugar level, that could be easily metabolised by *L. hilgardii* strains to
15 **obtain energy. However, in “dry” fermentations, the decarboxylation of amino acids to**
16 the corresponding amines is a source to provide additional energy through electrogenic
17 transport (Konings, 2002). In relation to oenologically relevant properties, the *L.*
18 *hilgardii* strains analysed in this work seem to be potential producers of ethyl carbamate
19 precursors, however, no potential to form biogenic amines was observed.

20
21 In summary, the partial sequence of the *recA* gene was a reliable and useful
22 method to molecularly identify *L. hilgardii* species from the related species *L. buchneri*
23 and *L. brevis*. By applying different intraspecies typing methods to five strains isolated
24 at the same winery in the same vintage, we could conclude that *L. hilgardii* is a
25 heterogeneous species. Since *L. hilgardii* is a wine-isolated species, enological relevant

properties were examined. No biogenic amine production was observed, however, all the *L. hilgardii* strains examined in this study are potential producers of ethyl carbamate.

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Figure captions

Figure 1. (A) Polymorphic sites in the 16S rDNA sequences of *L. hilgardii* CECT 4786^T (ATCC 8290^T) and IOEB 0204 (accession number AY241664); *L. buchneri* CECT 4111^T (JCM 1115^T) (accession no. AB205055); and *L. brevis* CECT 4121^T (ATCC 14869^T) (accession no. M58810). Each of the sites where the sequence of one or more of the genes differs is shown (only sites that differ are shown). Numbering of the polymorphic sites (vertical format) is according to the numbering of the *E. coli* 16S rDNA sequence. (B) Pairwise comparison of 16S rDNA sequences from *L. hilgardii*, *L. buchneri*, and *L. brevis* type strains. The percentage of identical nucleotides between genes is shown. The accession numbers of the analysed sequences are also indicated.

Figure 2. Comparison of nucleotide sequences of *recA* fragments from *L. hilgardii* strains as compared to type strains of *L. buchneri* and *L. brevis* as representatives of these species. Multiple alignment was done using the program BioEdit after retrieval of *recA* partial sequences from GenBank of *L. hilgardii* CECT 4786^T (LMG 6895^T) (accession no. AJ621647) represented in the alignment as 4786; *L. buchneri* CECT 4111^T (LMG 6892^T) (accession no. AJ621626) represented as 4111; and *L. brevis* CECT 4121^T (ATCC 14869^T) (accession no. DQ080023) showed in the figure as 4121. The *L. hilgardii* strains isolated from stuck wine fermentations are represented in the alignment as RM62 (*L. hilgardii* RM62), RM63 (*L. hilgardii* RM63), RM66 (*L. hilgardii* RM66), RM77 (*L. hilgardii* RM77), and RM79 (*L. hilgardii* RM79). Asterisks indicated nucleotide identity. Nucleotide differences among *L. hilgardii* strains are typed in boldface.

Figure 3. UPGMA dendrogram showing the genetic relatedness of some lactobacilli species based on its *recA* sequences. Phylogenetic analysis was conducted with the MEGA program (version 2.1) by the UPGMA method. The *recA* partial sequences were obtained from GenBank and EMBL databases from the representative type strain of each species: *L. hilgardii* CECT 4786^T (LMG 6895^T) (accession no. AJ621647); *L. buchneri* CECT 4111^T (LMG 6892^T) (accession no. AJ621626); *L. brevis* CECT 4121^T (ATCC 14869^T) (accession no. DQ080023); *L. paraplantarum* ATCC 16673^T (accession no. AJ621661); *L. plantarum* CECT 748^T (ATCC 14917^T) (accession no. AJ621668); *L. pentosus* CECT 4023^T (LMG 10755^T) (accession no. AJ286118); *L. fermentum* subsp. *cellobiosus* DSM 20055^T (accession no. AJ579535); *L. fermentum* subsp. *fermentum* LMG 6902^T (accession no. AJ579534); *L. delbrueckii* subsp. *bulgaricus* ATCC 11842^T (accession no. AJ586864); *L. delbrueckii* subsp. *delbrueckii* ATCC 9649^T (accession no. AJ586863); and *L. delbrueckii* subsp. *lactis* ATCC 12315^T (accession no. AJ586865). The scale bar represents an estimated 5 base substitutions per 100 nucleotides positions.

Figure 4. 16S–23S rDNA ISR patterns obtained after PCR amplification (lane 1), and *A**l**u**I* (lane 2), *C**f**o**I* (lane 3), *N**d**e**I* (lane 4) and *T**a**q**I* (lane 5) digestion of the DNA fragment PCR-amplified with primers 16S14f and 23S1R of the *L. hilgardii* RM62 strain. The sizes of some fragments in the 50 pb molecular mass marker are indicated on the left.

Figure 5. RAPD patterns obtained with primers M13 (A), A22 (B), and OPA20 (C) of the *L. hilgardii* strains isolated from stuck wine fermentations, *L. hilgardii* RM62 (lane 1), RM63 (lane 2), RM66 (lane 3), RM77 (lane 4), and RM79 (lane 5). The molecular

1 sizes (in kilobases) of some standards (*Eco*RI and *Hind*III digested λ DNA) are
2 indicated on the left.

3

4 Figure 6. PCR amplification of the genes involved in the arginine deiminase pathway
5 in *L. hilgardii* RM62. The gene encoding ADI protein was amplified by using primers
6 Aup-Adown that give an amplicon of 0.6 kb (lane 1). OTC and CK genes were
7 amplified by primers Bup-Bdown and Cup-Cdown, giving amplicons of 0.3 (lane 2) and
8 0.5 kb (lane 3), respectively. A 100 bp ladder marker was included on the left, and the
9 size of some standards are indicated.

Figure 1 (MS 5821/06)

A

	000000000000000000000000000000000000
	0000000000000000000000000000000000001111111111
	1444555555566666777788888899992333344578
	9578014578901345578901236713789128934587
<i>L. hilgardii</i>	GCTCTGCATGAAGTGATTAAC TGATGTAGATCTC-CGTC A
<i>L. buchneri</i>	GCTCCCTGTGATTTAGCTGAAAGATATGGATCCTTTACTC
<i>L. brevis</i>	AAC TCCTG---AGACCGAT---CAAGAGGTCA-CACTA

L. hilgardii **AAATTAATTATGGACAGTTCGACTGGGGGCGCATCGCCGC**

L. buchneri **AACTGAACCGTAGACTACCTGGCTGGAGGCGCATCTCCGC**

L. brevis **TGAATGGTCACTATTAGACCAGTAAAACCGCAGGGTTTAT**

L. hilgardii 00001111111111111111111111111111
677900000011111122222334444
944647112312355566677564555
417447070791602323612373567
L. buchneri AGCTTCATGGGCTGCGACCTCTAGGGC
L. brevis AGTTCCATGAGTCACAACCTCTAGGAC
 GATACTTAAACC GTGGTTCTCGAAGT

B

	<i>L. hilgardii</i> (AY241664)	<i>L. brevis</i> (DQ268866)
<i>L. buchneri</i> (AB205055)	97%	94%
<i>L. brevis</i> (DQ268866)	94%	

Figure 2 (MS 5821/06)

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RM62  TTGATGATCTGTTATTATCACAAACCGGATACTGGCGAACAGGGTCTCCAAATTACCGATA 60
RM63  ***** 60
RM66  *****G 60
RM77  ***** 60
RM79  *****G 60
4786  ***** 60
4121  *****T*A**GC*G**G**G**A*****G*****A**CT*G*****CG*T***G 60
4111  ****C*****C*T**G*****A*****A**C*****G*****T**CG 60

RM62  CCTTGGTCACCAGTGGTGCAATTGATATTGTTGTAATTGATTTCAGTTGCTGCGTTGGTTC 120
RM63  ***** 120
RM66  ***** 120
RM77  ***** 120
RM79  ***** 120
4786  ***** 120
4121  *****A*TT*****G*****C*****C**GG*****C**G**A**C**A*CG* 120
4111  *A*****T*****G*****C*****C*****C*****A***** 120

RM62  CACGTGCGGAAATCGAAGGTGAAATGGGCGATACCCACGTTGGCCTTCAAGCTCGTTTGA 180
RM63  *****T***** 180
RM66  ***** 180
RM77  ***** 180
RM79  ***** 180
4786  ***** 180
4121  *****T*****T*****C*****T***G*T*****G**T**G*****C**A**** 180
4111  *C**A**T*****G*****G**CG*****T**G**G*****AC*** 180

RM62  TGTCCCAAGCGTTAAGAAAAATTGTCGGGTACGATTAGTAAAAACAAAGACAATCGCAATTT 240
RM63  *****A***** 240
RM66  *****T***** 240
RM77  ***** 240
RM79  *****T***** 240
4786  ***** 240
4121  *****A*****C**C*G**GC*A**T**G**C**C*A*****G*****GT*A* 240
4111  *****G*****CC*CC*G**C*T**T*A*****C*****G**C*****G**T**C**** 240

RM62  TTATTAATCAAATTCGTGAAAAAGTCGGTGTCATGTTTGG 280
RM63  *****G***** 280
RM66  *****G***** 280
RM77  ***** 280
RM79  *****G***** 280
4786  *****G***** 280
4121  **G*C*****G*****G*****C**G*****A 280
4111  *C*****G*****T*****G***** 280

```

Figure 3 (MS 5821/06)

Figure 4 (MS 5821/06)

Figure 5 (MS 5821/06)

Figure 6 (MS 5821/06)