

Multi-Loci Sequence Typing (MLST) for Two Lacto-Acid Bacteria (LAB) Species: *Pediococcus parvulus* and *P. damnosus*

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Abstract The control of wine microbial population during and beyond fermentation is of huge importance for wine quality. Lactic acid bacteria (LAB) in wine are responsible for malolactic fermentation (MLF) which can be desired in some cases and undesirable in others. Some LAB do not perform MLF and their uncontrolled growth could contribute to severe wine spoilage such as undesired flavours. Their identification and detection is considered crucial for numerous biotechnological applications in food fermentations, where, through acidification and secretion of bacteriocins, they contribute to reduce food spoilage and growth of pathogenic microorganisms. LAB have traditionally been classified using morphological or biochemical features. Primary isolation, biochemical identification and phenotypic analysis are laborious, time consuming and inaccurate and often lead to misidentification within some genera such as *Pediococcus*. Molecular identification based on suitable marker genes could be an attractive alternative to conventional morphological and biochemical methods. We assessed here the applicability of four housekeeping genes *recA*, *rplB*, *pyrG* and *leuS* in combination with the *mle* gene in multi-loci sequence typing (MLST) of *Pediococcus parvulus* and *Pediococcus damnosus*. Sequencing and comparative analysis of sequence data were performed on 19 strains collected during wine fermentation. A combination of these five marker genes allowed for a clear differentiation of the strains analysed, indicating their applicability in molecular typing. Analysis of the observed

nucleotide polymorphisms allowed designing highly discriminative primers for a multi-loci sequence typing (MLST) method that proved successful in detecting a particular isolate or sequence type of *P. parvulus* when using either conventional PCR or Real Time PCR.

Keywords Lacto-acid bacteria · Multi-loci sequence typing · *Pediococcus parvulus* · *Pediococcus damnosus* · PCR · Real time PCR

Abbreviations

API	Appareillage et procédé d'identification
LAB	Lactic acid bacteria
MLF	Malolactic fermentation
MLST	Multi-loci sequence typing
PCR	Polymerase chain reaction

Introduction

Lactic acid bacteria (LAB) are among the principal bacteria present during the process of wine making [1, 2]. They are believed to be essential to the quality of many fermented beverages [3]. During wine fermentation, LAB may play dual roles as the main effectors of the malolactic fermentation (MLF) [4] or as wine spoilage agents under certain conditions. *Oenococcus oeni* is considered as the main LAB species during MLF [5]. LAB such as *Pediococcus* spp. might lead to different alterations of wine quality through production of exopolysaccharides [6], acetic acid [7] and biogenic amine [8]. The control of wine population during and beyond fermentation is therefore of huge importance for wine quality, and the identification and detection of LAB is considered crucial for wine

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fermentation as well as for many food fermentations processes involving LAB [9–11].

Established methodologies, routinely used for assessing the evolution of LAB populations, rely on plate counting, morphological descriptors and phenotypic methods based on biochemical systems such as API® (API, appareillage et procédé d'identification) by Biomérieux (Lyon, France) and phenotypic arrays (Biolog, Hayward, CA, USA) [12, 13]. These methods are strongly dependent of the physiological state of the bacteria, are time consuming and, due to the very slow growth of these LAB bacteria on synthetic media (up to 20 days), yield delayed results. As a consequence, for instance, misidentification within the genus *Pediococcus* is common [13]. Alternative molecular LAB typing methods are being developed in several laboratories [9, 10, 14–16], aiming for higher sensitivity and specificity and better reproducibility. Multi-loci sequence typing (MLST) is one of the preferred methods in molecular typing of LAB and has been applied successfully to *Lactococcus casei*, *Oenococcus oeni*, *Lactobacillus plantarum* and *Pediococcus acidilactici* [17–20]. The existing MLST schemes were based on housekeeping genes.

Phylogenetic relationships of LAB bacteria are most commonly based on the variability in their ribosomal RNA genes [21, 22]. However, for the classification of closely related species and subspecies, rRNA genes are too conserved and alternative marker genes exhibiting greater differences between species are required [9, 23]. As an alternative, protein encoding gene sequences have been applied for the classification of LAB bacteria [9, 10]. The *mle* gene, encoding the malolactic enzyme, has been shown to reflect the phylogenetic position of LAB cocci [23], with a better phylogenetic resolution than with the 16S rRNA gene.

The objective of the present study was to perform a comparative sequence analysis of relevant marker genes and to exploit the obtained information for developing a DNA-based assay for isolate identification, which could replace traditional morphological identification of the species *Pediococcus parvulus* and *Pediococcus damnosus*. The desired method to develop would be reproducible, quick, cheap, relatively free of personnel error and would generate results not only fully compatible with traditional identification schemes but offering a possibility of between strains identification. A multi-loci sequence typing method (including the malolactic fermentation gene) was developed as a modification of the MLST method of Maiden et al. [24] and tested against 19 *P. parvulus* and *P. damnosus* strains collected during a batch fermentation experiment. In a second step a set of highly discriminative primer pairs was designed for discriminating two *P. parvulus* strains Bpe 149 and Bpe 184 and proved to be unambiguously effective in detecting a single sequence type (ST) of *P. parvulus*.

Materials and Methods

Bacterial Strains and Culture Method

Pediococcus strains used in this study are listed in Table 1. *Pediococcus* strains were obtained from DSMZ (German Resource Centre for Biological Material) or isolated from wine during fermentation at the School of Engineering of Changins. Strains were grown aerobically in MRS vegitone medium (Sigma, Basel, Switzerland) according to manufacturer recommendations at 28°C. All the strains listed in Table 1 have been checked morphologically and by 16S ribosomal DNA sequencing (see below).

Genomic DNA Extraction

Cells from 5–7 days old cultures were harvested and chromosomal DNA for the polymerase chain reaction (PCR) was prepared by using the UltraClean™ Microbial DNA Isolation kit (MO BIO Laboratories, Inc., St Quentin-Yvelines, France) according to the manufacturer's instructions. Quality was checked by visualization under UV light following electrophoresis in 2% agarose. Concentrations were determined with a NanoDrop ND-1000 UV spectrophotometer (Witec AG, Lucerne, Switzerland).

Table 1 Origin and allelic profiles of the 19 *P. parvulus* and *P. damnosus* strains analysed

Species	Strain	ST	Allele					Source
			leuS	pyrG	recA	rplB	mle	
<i>P. parvulus</i>	Bpe 165	1	1	1	1	1	1	EIC
	Bpe 207	1	1	1	1	1	1	EIC
	Bpe 290	1	1	1	1	1	1	EIC
	Bpe 149	2	1	1	2	1	1	EIC
	Bpe 184	3	1	2	3	1	1	EIC
	Bpe 301	3	1	2	3	1	1	EIC
	Bpe 299	4	1	3	4	2	2	EIC
	CBS 20332	4	1	3	4	2	2	DSMZ
<i>P. damnosus</i>	Bpe 202	1	1	1	1	1	1	EIC
	Bpe 230	1	1	1	1	1	1	EIC
	Bpe 238	1	1	1	1	1	1	EIC
	Bpe 283	1	1	1	1	1	1	EIC
	Bpe 243	1	1	1	1	1	1	EIC
	Bpe 260	1	1	1	1	1	1	EIC
	Bpe 226	2	2	1	1	1	1	EIC
	Bpe 233	2	2	1	1	1	1	EIC
	Bpe 263	2	2	1	1	1	1	EIC
	Bpe 294	3	3	1	1	2	2	EIC
	CBS 20331	3	3	1	1	2	2	DSMZ

16S rDNA PCR Amplification

16S rDNA amplification of bacterial samples was carried out using the previously described primers fd1 and rp2 [25]. The reaction mixture contained 1× PCR buffer (75 mM Tris–HCl (pH 9.0), 50 mM KCl, 20 mM (NH₄)₂SO₄), 0.1 mM dNTPs, 0.25 μM of each primer, 1.5 mM MgCl₂, 1 U of Taq polymerase (Biotools, Spain) and 1 ng of bacterial DNA in a total volume of 50 μl. PCR conditions included an initial denaturation step of 95°C for 2 min followed by 35 cycles of 95°C (20 s), 55°C (30 s) and 72°C (2 min) terminated with a final 20 min chain elongation step.

Amplification with Universal Primers for Other Genes

PCR amplifications of the 16S–23S spacer gene (Jensen et al. 1993), the RNA polymerase *rpoB* gene and the *fusA*, *gyrB*, *ileS*, *lepA*, *pyrG*, *recA*, *recG* were carried out with the respective primers [9] and degenerated primers [26]. Not all the strains amplified with these so-called universal primers, which implied redesigning primers specific of the *Pediococcus* genus. This was achieved on the basis of obtained sequences for some of these genes and of GenBank deposited sequences (NCBI, Bethesda, USA).

Design of *Pediococcus* spp. Specific Primers and PCR Amplification

In particular, specific primers were designed for the *recA*, *rplB*, *pyrG*, *leuS* genes with the constraint to display the same annealing temperature range. PCR amplification of *recA*, *rplB*, *pyrG*, *leuS* and *mle* from chromosomal DNA

was performed by using the respective PCR primers (Table 2). For each target, PCR reactions of 50 μl volume each, consisting of 1× PCR buffer (75 mM Tris–HCl (pH 9.0), 50 mM KCl, 20 mM (NH₄)₂SO₄), 0.1 mM dNTPs, 0.25 mM of both primers, 1.5 mM MgCl₂, 1 U of Taq Polymerase (Biotools, Madrid, Spain), 1 ng genomic DNA as template and 1 U of Taq polymerase (Invitrogen, Switzerland), were performed in a Master Gradient thermocycler (Eppendorf, Hamburg, Germany) according to the following amplification programme: an initial denaturation step of 95°C for 5 min followed by 39 cycles including denaturation for 60 s at 94°C, annealing for 60 s at 55°C and extension for 1 min at 72°C. Amplification was terminated by a final extension step of 10 min at 72°C. Amplicons were checked by gel electrophoresis as previously described.

DNA Sequencing

For DNA sequencing, PCR products of 16S rDNA, *recA*, *rplB*, *pyrG*, *leuS* and *mle* genes were purified using the MiniElute PCR Purification Kit (Qiagen, Basel, Switzerland), according to manufacturer's specifications. Quantity and quality were checked as described above for DNA extraction and used as templates in sequencing reactions. Bidirectional sequencing of *recA*, *rplB*, *pyrG*, *leuS* and *mle* genes was performed at Microsynth AG (Balgach, Switzerland) by using the respective primer pair used for PCR amplification (Table 2) as sense and antisense sequencing primers. All bacterial DNA samples were sequenced twice and a consensus sequence was created from the duplicates. All DNA sequences have been deposited in GenBank under

Table 2 PCR primers used in this study

Primer	Sense	Species	Sequence (5' → 3')	Gene location
RecApp-F	Forward	<i>P. parvulus</i> and	CAAATTGCAACGATGCCTACTGGATCC	Recombinase A
RecApp-R	Reverse	<i>P. damnosus</i>	GGTGCCATCCTTAATCTGTTCGCCCG	
RplBoo-F	Forward	<i>P. parvulus</i> and	GGTCGTATGACTGTTCCGCCATCATGGTGGTGG	Ribosomal protein L2
RplBoo-R	Reverse	<i>P. damnosus</i>	GGGTTTCATAACAGAACCACGAACATGTGGA	
PyrGppento-F	Forward	<i>P. parvulus</i> and	GGAATGAATCATTCCCTCAATCCCACG	CTP synthase
PyrBppento-R	Reverse	<i>P. damnosus</i>	AAATATATTTTTGTTACAGGTGGGGTTGTTT	
LeuSoo-F	Forward	<i>P. parvulus</i> and	GCAGGTTAAATCACTTGGTTTTTCTTATGACTGG	Leucyl-tRNA synthase
LeuSoo-R	Reverse	<i>P. damnosus</i>	TCACGCGTATCATGAGCTGGAACAGCCAT	
Mlepar-F	Forward	<i>P. parvulus</i>	CGATCGCCGACACCATCGAAAATTACAGTG	Malolactic enzyme
Mlepar-R	Reverse		TGTGGTGATTTGGTCTTATACTTATTCAAGATATTAGC	
Mledam-F	Forward	<i>P. damnosus</i>	CCATTGCCGACACAATTGAAAACCTATAGTGA	Malolactic enzyme
Mledam-R	Reverse		TGTCGTGATTTTGTCTTTGTACGTATTTAAGATATTAGC	
RecA-ST3-F	Forward	<i>P. parvulus</i> ST3	GGATCCTTAGCACTAGATGAC	Recombinase A
RecA-ST3-R	Reverse		AATGTTAACCCTAAGTGTTC	
RecA-ST2-F	Forward	<i>P. parvulus</i> ST2	GGATCCTTAGCACTAGATGAT	Recombinase A
RecA-ST2-R	Reverse		AATGTTAACCCTAGGTGTTC	

the accession numbers EU331244–EU331291 (*P. parvulus*) and EU331292–EU331357 (*P. damnosus*).

DNA Sequence Analysis

The resulting sequences, augmented of the GenBank accession CP000422, were imported into the Seaview software package [27] and manually aligned according to the alignment of amino acid sequences originating from translated DNA sequences. Alignments were trimmed to a uniform length (corresponding to nucleotide positions 334–1010 for *leuS*, 80–1022 for *pyrG*, 111–694 for *recA*, 148–671 for *rplB* and 291–760 for *mle* of the genome of *Pediococcus pentosaceus* ATCC 25745 (Acc. No. CP000422)). The trimmed alignments were also used to construct a concatenated alignment. Trees based on the neighbour-joining method were built from the individual alignments as well as from the concatenated alignment. Reliability of the tree structure was assessed via bootstrap analysis, using the PhyML program [28] according to Belbahri et al. [29].

Primer Design and Real Time PCR Analysis

Primers specific to the strains Bpe 149 and Bpe 184 were developed through visual comparison of the alignment of all sequences used for the phylogenetic analysis of the *recA* gene (Table 2). Primers were specifically designed to discriminate not only between both strains but also among closely related lab Cocci such as *P. pentosaceus*, *P. damnosus* and *P. dextrinicus* and other wine bacteria, particularly those widely represented such as *Oenococcus oeni*. The specificity of potential primers was further tested by BLAST [30] searching GenBank for compatible sequences. Following the identification of suitable target regions in the allelic sequences of the *recA* gene of *P. parvulus* Bpe 149 and Bpe 184 strains, primers were tested against putative cross-hybridizing species listed above. Amplifications were carried out in capped capillary tubes in a Lightcycler 2.0 Real Time PCR systems (Roche Diagnostics, Rotkreuz, Switzerland) using an initial denaturation at 95°C for 10 min, followed by 60 cycles of 95°C for 10 s, 68°C for 10 s and 72°C for 7 s. Amplification mixtures (20 µl final volume) were built with the Light Cycler Fast Start DNA Master SYBR Green I kit added of 2 ng of template DNA and primers at a final concentration of 0.5 µM.

Results

16S rDNA Strains Identification

As shown by 16S rDNA amplification and sequencing, seven strains belonged to *P. damnosus* and 10 to *P. parvulus*.

Another strain (not shown here) belonged to *Oenococcus oeni* and was thus discarded.

Selection and Characteristics of MLST Marker Genes

General criteria for MLST marker gene selection included (i) the genes have to be preferably interspaced across the entire genome based on LAB bacteria complete genome sequences available [31], (ii) the functions of the encoded proteins, preferably conserved and well characterized and (iii) presence of the selected genes in all the strains as a single copy of a minimum size of 1 kb allowing PCR primer design. The *mle* gene was selected based on the results of previous studies on *Oenococcus oeni* and other LAB bacteria [18, 23]. Selection of the remaining loci (*recA*, *rplB*, *pyrG* and *leuS*) was based on a preliminary analysis of several housekeeping genes (*gyrB*, *recA*, *rpoB*, *rplB*, *recG*, *pyrG*, *leuS* and *mle* and the 16S rRNA) with degenerated universal primers (data not shown). This preliminary study showed that *recA*, *rplB*, *pyrG* and *leuS* displayed sufficient sequence diversity among *P. parvulus* and *P. damnosus* strains investigated, providing the best phylogenetic resolution amongst the genes tested.

Comparative Sequence Analyses

Pediococcus parvulus sequences were registered under the following GenBank accessions: EU331244–EU331251 for *rplB*, EU331252–EU331259 for 16S rRNA gene, EU331260–EU331267 for *leuS*, EU331268–EU331275 for *mle*, EU331276–EU331283 for *pyrG*, EU331284–EU331291 for *recA*. *Pediococcus damnosus* sequences were registered under the following GenBank accessions: EU331292–EU331302 for *rplB*, EU331303–EU331313 for 16S rRNA gene, EU331314–EU331324 for *leuS*, EU331325–EU331335 for *mle*, EU331336–EU331346 for *pyrG*, EU331347–EU331357 for *recA*.

The characteristics of the marker genes are summarized in Table 3. Individual marker genes allowed for the discrimination of up to three distinct allelic types, differentiated by at least one nucleotide difference (Fig. 1). Sequence variation was low and ranged, for instance, from 0 to 2.67%, for *P. parvulus* with the *rplB* gene. For *P. damnosus* all the genes analysed displayed very little sequence variability (Table 3). The genes *rplB*, *mle*, *recA* and *pyrG* proved to be suitable markers for *P. parvulus* strain level differentiation with 2, 2, 3 and 3 alleles, respectively (Fig. 1), in the collection of the nine strains analysed (Table 3). The 11 *P. damnosus* strains displayed a variability reduced to a unique polymorphic site within the *rplB*, *leuS* and *mle* genes, with 2, 2 and 2 alleles, respectively (Fig. 1). The gene *leuS* displayed no variability in all

Table 3 Characteristics of the marker genes used in this study

Species	Gene	Size for analysis (bp)	G + C (%)	No. of alleles	No. of sequences analysed	No. of polymorphic sites
<i>P. parvulus</i>	recA	584	43	3	8	4
	rplB	524	40.6	2	8	14
	pyrG	943	38.5	3	8	2
	leuS	677	43	1	8	0
	mle	470	40.2	2	8	8
<i>P. damnosus</i>	recA	584	42.8	1	11	0
	rplB	524	42.1	2	11	1
	pyrG	943	38.3	1	11	0
	leuS	677	41.1	2	11	1
	mle	470	40.2	2	11	1

Fig. 1 Polymorphic nucleotide sites in *P. parvulus* and *P. damnosus* MLST genes. Only the variable sites are shown.

The nucleotide at each site is shown for a putative consensus sequence; only those that differ from the nucleotide in the consensus sequence are shown for the alleles. Nucleotide sites are numbered in a vertical format from the first nucleotide position of the considered gene. The number of strains harbouring the allele is indicated in brackets

		<i>P. parvulus</i>																												
		2	7	1	2	3	3	2	2	3	3	3	3	4	4	4	4	6	6	4	5	5	5	5	6	6				
		6	6	4	0	0	1	0	4	8	2	2	5	9	9	1	4	5	6	0	1	0	1	1	2	3	4	6	9	
		7	1	4	4	9	6	4	0	8	4	7	4	0	7	7	7	3	5	7	8	8	0	9	2	1	0	3	1	
	Consensus	G	C	T	C	A	C	G	T	C	G	C	C	G	A	C	T	C	C	A	T	C	T	C	C	T	A	G	C	
	ST1 (3)
	ST2 (1)	G
	ST3 (2)	A	.	C	T	T	T
	ST4 (2)	.	T	A	C	T	A	A	T	A	G	T	C	T	T	G	C	T	C	T	T	A	C	T	T	
		pyrG			recA			rplB						mle																
		<i>P. damnosus</i>																												
		6	3	5																										
		6	9	5																										
		6	0	1																										
	Consensus	C	A	T																										
	ST1 (6)	.	.	.																										
	ST2 (3)	A	.	.																										
	ST3 (2)	.	G	C																										
		leuS			rplB			mle																						

strains *P. parvulus* while *recA* and *pyrG* displayed no variability in all strains of *P. damnosus* (Fig. 1, Table 3).

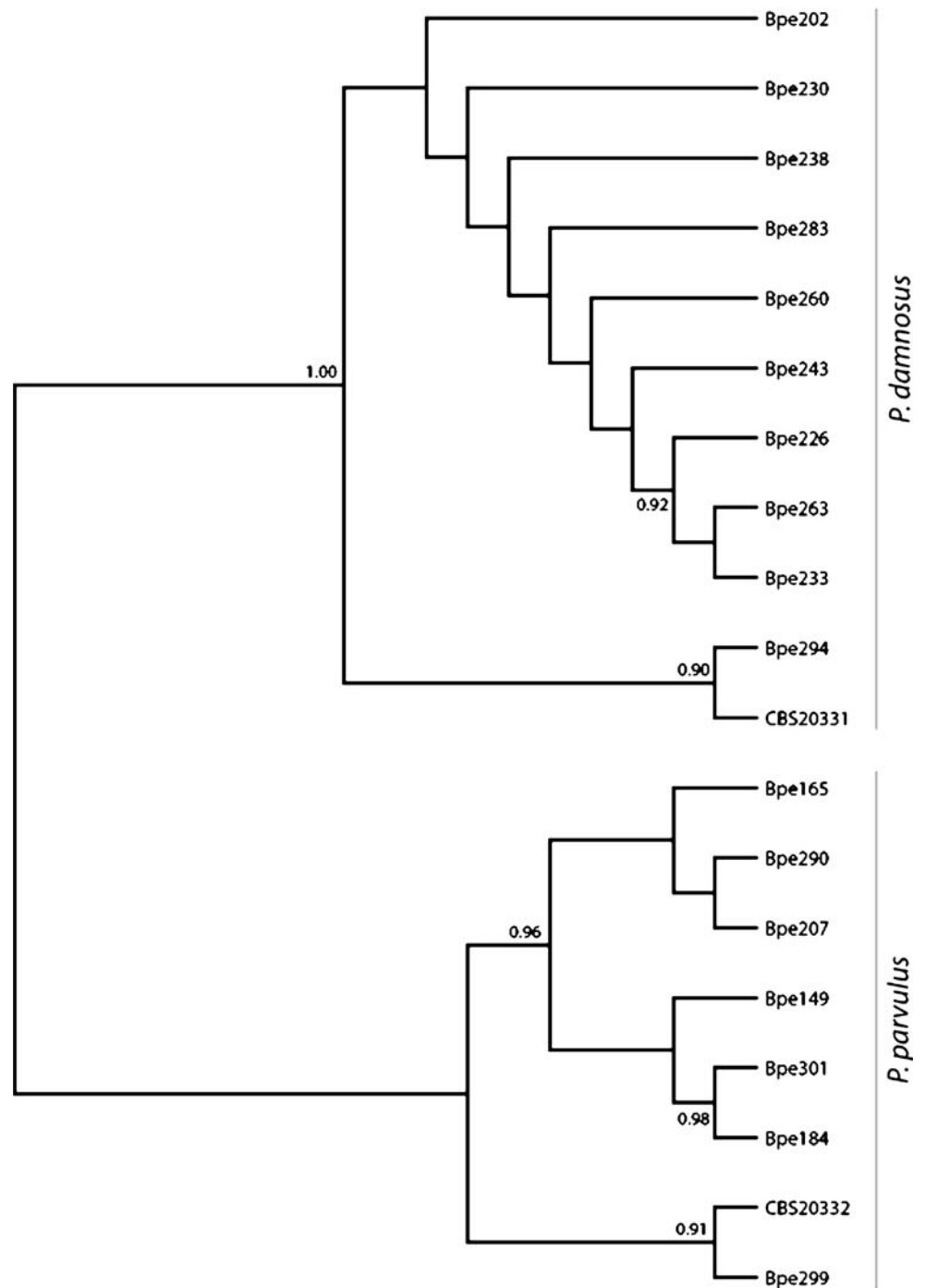
MLST Differentiation of *Pediococcus* Strains

Based on the *recA*, *rplB*, *pyrG*, *leuS* and *mle*, 4 and 3 sequence types (ST) could be identified amongst the 8 *P. parvulus* and 11 *P. damnosus* isolates respectively (Table 1, Fig. 1), corresponding to a discriminatory ability of 1 ST for 2 isolates of *P. parvulus* and 1 ST for 3.6 isolates. While none of the individual marker genes allowed for the identification of at least a majority of the isolates analysed, their combination provided a resolution well beyond that level (Fig. 2). *P. parvulus* strain Bpe 299 matched the strain CBS 20332. Similarly *P. damnosus* strain Bpe 294 matched the reference strain CBS 20331 (Fig. 2).

Primer Design and Real Time PCR Analysis

The PCR primer pair (RecA-149-F, RecA-149-R) produced a single band approximately 207 bp in size for *P. parvulus* ST2 (Bpe 149). At the highest temperature where amplification was still possible (66°C) no bands were produced when the primers were tested against genomic DNA from all closely related STS such as ST3 (illustrated by strain Bpe 301 in Fig. 3) and species i.e. *P. damnosus*, *P. dextrinicus* and *Oenococcus mesenteroides*. The primer pair (RecA-184-F, RecA-184-R) produced a single band approximately 207 bp in size for *P. parvulus* ST3 (illustrated by strain Bpe 301 in Fig. 4). At the highest annealing temperature allowing amplification (66°C), no cross amplification was observed with ST2 (strain Bpe 149, Fig. 4) as well as closely related species *P. damnosus*,

Fig. 2 Neighbour-joining dendrogramme based on concatenated nucleotide sequences of the *recA*, *rplB*, *pyrG*, *leuS* and *mle* genes. The positions considered to generate the tree are shown in Fig. 1. Bootstrap values higher than 50% from 100 replicates are shown. The bar represents 1% evolutionary sequence divergence



P. dextrinicus and *Oenococcus mesenteroides* (Fig. 4). In Real Time PCR similar results have been obtained except that some cross amplification was observed when using primer pair (RecA-149-F, RecA-149-R) against ST3 (illustrated by strain Bpe 301 in Fig. 3). However melting curve analysis could easily be used to rule out misunderstanding of the amplification observed, and under gel electrophoresis analysis the amplicon seems to correspond to a non-specific product of higher size (Fig. 3).

Discussion

Identification of strains belonging to the same species is still difficult to achieve in LAB such as the genus *Pediococcus*. This is mainly due to difficulties in morphological identification based on phenotypic features and biochemical traits highly dependent on physiological state of cells. Suitable and highly precise *Pediococcus* typing techniques are not available. A reproducible,

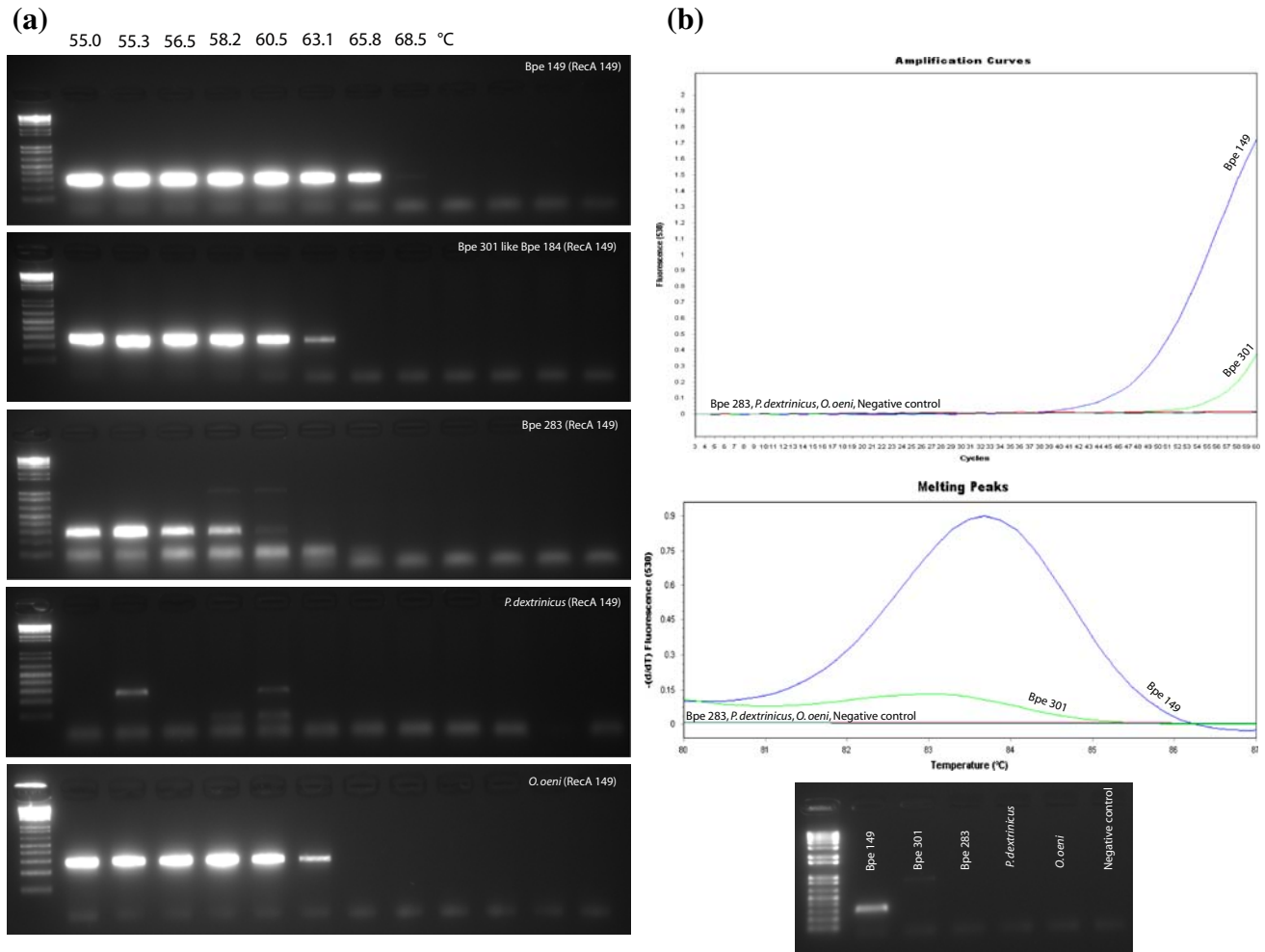


Fig. 3 Reaction of strains Bpe 149, Bpe 301, Bpe 283, *P. dextrinicus* and *Oenococcus oeni* to the PCR primer pairs RecA-149-F and RecA-149-R. **(a)** Specificity of the primer pairs RecA-149-F and RecA-149-R at different annealing temperatures with DNA from respective strains listed above. Annealing temperatures ranging between 55 and

75°C are indicated in the figure. **(b)** Specificity of the primer pairs RecA-149-F and RecA-149-R in real time experiments. Amplification curves, Melting peaks analysis and agarose gel electrophoresis of amplification products are shown

cheap, fast and error-free method with a higher resolution than conventional identification schemes would be a priority for the biotechnological applications of these bacteria.

Multi-loci sequence typing (MLST) tends to be the preferred method in molecular typing of LAB bacteria and has been applied successfully to *Lactococcus casei*, *Oenococcus oeni*, *Lactobacillus plantarum* and *Pediococcus acidilactici* [17–20]. The existing MLST schemes were based on housekeeping genes. In this study the combination of *recA*, *rplB*, *leuS*, *pyr* housekeeping genes and the malolactic enzyme gene *mle* conveniently grouped *P. parvulus* and *P. damnosus* strains into groups corresponding to recognised species (Fig. 1). Besides that, the most widely used phylogenetic marker, the 16S rDNA gene displayed very little sequence variability within the

two *Pediococcus* species investigated. The number of alleles of the 4 housekeeping and the *mle* genes ranged from 1 to 3 (Table 1). Sequence variation ranged from 0 to 2.67%, for *P. parvulus* with the *rplB* gene. The obtained variability was low, in comparison to those observed in other studies using MLST on related LAB (0.3–6.6% for *Oenococcus oeni* [18], 1–7.7% in *Lactobacillus plantarum* [19] and 1.4–7.8% [17], lab cocci [17, 18]. This could be explained by the fact that strains of the present study were collected from the same fermentation experiment in the same location. Surprisingly a strain of *P. parvulus*, Bpe299 was similar to the CBS20332 strain and they shared the ST4 type which was the rarest type among these isolates. This ST4 type diverged fairly from the consensus sequence obtained from these local strains. These strains were however identified without any

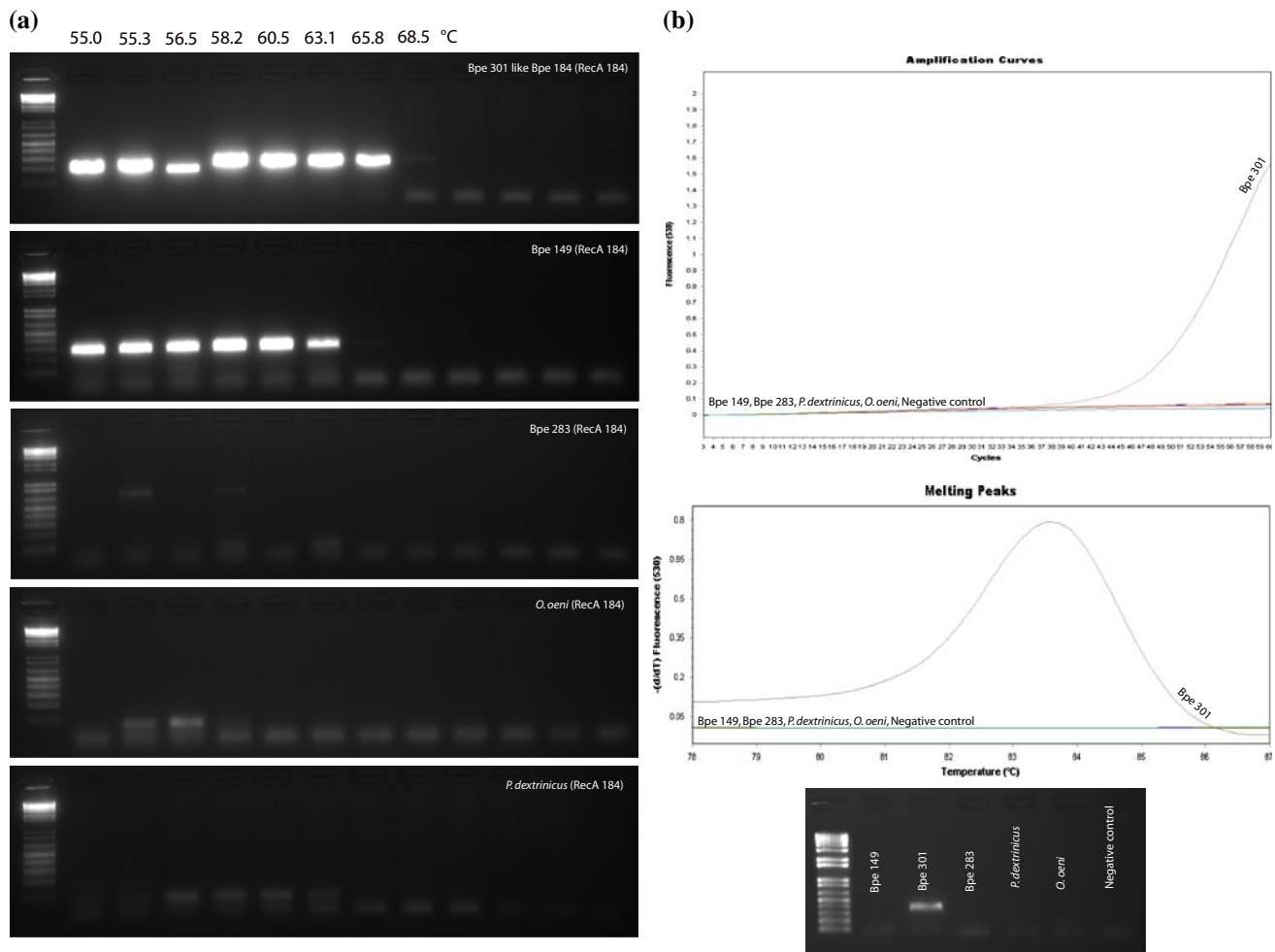


Fig. 4 Reaction of strains Bpe 301, Bpe 149, Bpe 283, *P. dextrinicus* and *Oenococcus oeni* to the PCR primer pairs RecA-184-F and RecA-184-R. **(a)** Specificity of the primer pairs RecA-184-F and RecA-184-R at different annealing temperatures with DNA from respective strains listed above. Annealing temperatures ranging between 55 and

75°C are indicated in the figure. **(b)** Specificity of the primer pairs RecA-184-F and RecA-184-R in real time experiments. Amplification curves, melting peaks analysis and agarose gel electrophoresis of amplification products are shown

ambiguity as *P. parvulus* by the 16S rDNA sequence; consequently this variation could originate from recombination events or horizontal gene transfer between compatible bacteria species. A comprehensive sequence database would be required in order to maximize the full potential of molecular methods for *P. parvulus* and *P. damnosus* typing. The *recA*, *rplB*, *pyrG*, *leuS* and *mle* sequences obtained from 8 *P. parvulus* and 11 *P. damnosus* isolates showed substantial intra-species phylogenetic variation, indicating a strong typing potential. While the selected isolates could be considered as local isolates, sequence data from other countries, from other geographical regions of Switzerland and neighbour countries, would most likely further broaden the known diversity. Establishing such a comprehensive sequence database would also shed light onto the evolution of LAB cocci *P. parvulus* and *P. damnosus* and allow for their

large-scale characterization. The combination of all five marker genes would be required for a reliable typing of all the isolates, taking into consideration potential minor sequence variations.

Meanwhile our study showed unambiguously that MLST could provide with a resolution at the infra-specific level. The existing variability was sufficient for designing primers that allowed for the detection of a specific ST of the species *P. parvulus* in PCR and Real Time PCR experiments (Figs. 3 and 4). This type of studies is highly suited in numerous biotechnological applications such as identification and monitoring of specific wine spoilage bacteria during fermentation experiments, for which these protocols are being validated with LAB mixes (data not shown). Other applications could include strain characterization prior to patent and release of a valuable LAB species for commercial applications [19].

Conclusions

The combination of housekeeping genes *recA*, *rplB*, *pyrG*, *leuS* and the malolactic enzyme encoding gene *mle* showed good discrimination amongst the *P. parvulus* and the *P. damnosus* strains investigated. These results indicate that the approach enabled efficient molecular typing for the closely related *P. parvulus* and *P. damnosus* species as well as an intra-species resolution. Taking advantage of the sequence differences, we could design isolate specific PCR primers that proved effective in detecting without ambiguity a specific strain of *P. parvulus* and *P. damnosus*. The primers proved also effective in Real Time experiments allowing fast and reliable identification of these wine spoilage bacteria.

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