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ORIGINAL ARTICLE

Evaluating *Lactobacillus* and *Pediococcus* strains for enzyme-encoding genes related to peptide and amino acid utilization in wine

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Abstract In this study, a total of 104 strains of lactic acid bacteria (LAB) were tested for the presence of genes encoding enzymes related to peptide and amino acid utilization in wine-making. Primers for PCR amplifications were designed from conserved regions of genes isolated from various LAB species belonging to *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus*. As expected, PCR assays generated single DNA fragments of the correct sizes. The PCR detection results revealed that the genes tested for were distributed across the different species of lactobacilli and pediococci investigated. However, some strains of *Pediococcus* did not possess certain enzyme-encoding genes, such as *pepO*, *pepT*, *metK* and *gshR*. In addition, *pepX* and *metB*/*metC* were not detected in any of the *Pediococcus* strains tested. The *Lactobacillus plantarum* IWBT B349 strain was selected for gene sequence verification. The results of the comparative sequence analysis demonstrated that nucleotide gene sequences of this strain are highly identical to those of other *L. plantarum* strains (WCFS1, JDM1 and ATCC 14917) published in GenBank database. Neighbour-joining trees based on the *pepC* and *pepM* gene sequences were also constructed, and these indicated that there was a similar trend of clustering of bacterial species between the two genes. Altogether, the results presented here indicate that lactobacilli and pediococci strains of wine origin have the genetic potential to degrade peptides and sulphur-containing amino acids during vinification.

Keywords *Lactobacillus* · *Pediococcus* · Peptidases · Amino acids · PCR detection · Sequencing

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Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria occurring on grapes and in wine during malolactic fermentation (MLF). The latter process, carried out mainly by *Oenococcus oeni* and *Lactobacillus plantarum*, involves the conversion of L-malic acid to L-lactic acid and CO_2. This reaction contributes to wine acidity reduction, increased microbial stability and changes in the sensory attributes of wine (Bartowsky 2005; Lerm et al. 2010). LAB are generally fastidious microorganisms with multiple amino acid requirements, thus requiring the proteolytic system necessary to degrade proteins (present in the environment in which they occur) into smaller peptides and amino acids crucial for their growth (Juillard et al. 1995). In the dairy industry, for example, the proteolytic activity of bacterial species also favours the development of texture and flavour of fermented milk products (Lynch et al. 1999; El-Soda et al. 2000; Hynes et al. 2003).

While the proteolytic system of *Lactococcus lactis*, a model microorganism used as the starter culture in the dairy industry, has been extensively investigated (Tan et al. 1993; Tynkkynen et al. 1993; Hagting et al. 1994; Foucaud et al. 1995; Kunji et al. 1996), the proteolytic enzymes of bacterial species of wine origin remain poorly understood. Nevertheless, the production of exocellular proteinases by *O. oeni* strains has been detected (Rollan et al. 1993; Manca de Nadra et al. 1997, 1999; Remize et al. 2005), albeit Davis et al. (1988) observed no protease production by several wine LAB strains of *Oenococcus*, *Lactobacillus* and *Pediococcus*. More recently, Strahinic et al. (2010) demonstrated the presence of a protease enzyme-encoding gene (*prtP*) in a natural isolate of *L. plantarum* originating from home-made cheese. This gene encodes an enzyme that participates in protein degradation to form small peptides and amino acids. The resulting peptides are subsequently translocated into the cell

via the specific transport system where they are acted upon by a variety of peptidases (Kunji et al. 1996).

Following the primary and secondary proteolysis, some peptidases are able to release sulphur-containing amino acids from peptide degradation (Kunji et al. 1996). In the dairy industry, the catabolism of sulphur amino acids by microbial enzymes plays a key role in the development of a typical cheese flavour (Dias and Weimer 1999). The LAB originating from cheese are also known to metabolize methionine into methanethiol, a volatile sulphur compound contributing to cheese flavour (Weimer et al. 1999). In wine, Pripis-Nicolau et al. (2004) showed that the LAB isolated from wine could degrade methionine to form volatile sulphur compounds. At a molecular level, Knoll et al. (2011) recently reported on the cloning and characterization of a cystathionine lyase gene from two *Oenococcus oeni* strains originating from wine. The latter authors also observed that *O. oeni* seemed to play a minor role in the production of volatile sulphur compounds during winemaking. This is the extent of our knowledge regarding the enzymes acting on sulphur-containing amino acids and, in addition, the distribution of genes encoding a variety of peptidases in wine-associated LAB strains has still not been thoroughly investigated.

The aim of this study was, therefore, to investigate the presence of genes encoding enzymes involved in the proteolytic and amino acid catabolic pathways in lactobacilli and pediococci of oenological origin. Reference strains of non-wine origin that are representative of different LAB species were also included in the study. The genes tested for included those encoding peptide transporter (DtpT), aminopeptidases (PepC, PepN, PepM), endopeptidase (PepO), tripeptidase (PepT), proline peptidase (PepX, PepI), as well as enzymes involved in the catabolism of sulphur-containing amino acids.

Materials and methods

Bacterial strains and growth conditions The bacterial strains used in this work were previously isolated from grape and wine samples obtained from five different commercial wineries situated in the Western Cape region, South Africa, during the 2001 and 2002 harvest seasons (Krieling 2003). Taxonomic identification of these isolates was based on the results of PCR analysis with species-specific primers and 16S rDNA sequence analysis. The type and reference strains included in this work are presented in Table 1. All of the strains were cultured at 30 °C in MRS medium (BIOLAB Diagnostics, Wadeville, South Africa).

Primer design To design the primers for PCR assays, we aligned nucleotide sequences of the target genes from several LAB species belonging to four LAB genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus*. Two conserved domains of each gene were then selected to design the primers for PCR amplifications. All PCR primers used in this study (Table 2) were synthesized by Integrated DNA Technologies. (Coralville, IA).

Colony PCR assays PCR amplifications were performed in a Biometra Thermocycler machine (Biometra® GmbH, Göttingen, Germany). The 25-μl PCR mixture comprised a template DNA, 0.6 μM of each primer, 1.5 mM $MgCl_2$, 250 μM of dNTP mix, 1× PCR buffer and 1.25 units of Supertherm DNA polymerase (Southern Cross Biotechnology, Cape Town, South Africa). The reaction conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 49–54 °C for 45 s and 72 °C for 1 min (specific annealing temperatures are indicated in Table 2), with a final extension step at 72 °C for 10 min. PCR fragments were checked by gel electrophoresis in 1 %w/v agarose gels containing ethidium bromide (0.5 μg ml^{-1}) and documented with Alpha Imager software (Alpha Innotech Corporation, San Leandro, CA).

Sequence verification To verify whether the PCR-generated amplicons corresponded to the target genes, the *L. plantarum* IWBT B349 strain was chosen for sequencing. DNA fragments from this strain were amplified with the same primers in a 50-μl reaction containing TaKaRa *Ex Taq*™ polymerase (Southern Cross Biotechnology) instead of Supertherm polymerase. Amplicons were cleaned with the QIAquick® PCR Purification kit (Qiagen, Southern Cross Biotechnology) and subsequently sequenced using the corresponding forward and reverse primers. The GenBank database was searched for homologous DNA sequences.

Phylogenetic analysis In an attempt to study the phylogenetic relationship between the LAB strains tested in this study and closely related taxa, we randomly selected five strains from which the *pepC* and *pepM* genes were amplified and sequenced. The obtained nucleotide gene sequence data were assembled and compared to the database sequences. The phylogenetic trees were inferred using the neighbour-joining method (Saitou and Nei 1987). The evolutionary distances were computed using Kimura 2-parameter method (Kimura 1980) from MEGA 4.1 software (Kumar et al. 2008) and are given in the units of the number of base substitutions per site. Bootstrapping analysis was performed to evaluate the reliability of topologies of the constructed phylogenetic trees using 1000 bootstrap replications (Felsenstein 1985).

Results

PCR detection of genes

A total of 104 strains of lactobacilli and pediococci were tested in this study. Using a PCR detection approach with

Table 1 Reference or type strains included in this work

Bacterial species	Strain number	Culture collection [a]
Lactobacillus spp.		
L. casei	LMG 13552	Laboratorium voor Microbiologie
L. curvatus	LMG 13553	Laboratorium voor Microbiologie
L. delbrueckii	DSM 20074	Deutsche Sammlung von Mikroorganismen und Zellkulturen
L. fermentum	DSM 20052	Deutsche Sammlung von Mikroorganismen und Zellkulturen
L. hilgardii	DSM 20176	Deutsche Sammlung von Mikroorganismen und Zellkulturen
L. malefermentans	ATCC 11305	American Type Culture Collection
L. paracasei	DSM 5622	Deutsche Sammlung von Mikroorganismen und Zellkulturen
L. paraplantarum	DSM 10667	Deutsche Sammlung von Mikroorganismen und Zellkulturen
L. pentosus	DSM 20314	Deutsche Sammlung von Mikroorganismen und Zellkulturen
L. plantarum	DSM 20174	Deutsche Sammlung von Mikroorganismen und Zellkulturen
L. reuteri	LMG 13557	Laboratorium voor Microbiologie
L. sakei	NCFB 2714	National Collection of Food Bacteria
Pediococcus sp.		
P. pentosaceus	LMG 13561	Laboratorium voor Microbiologie
	NCDO 813	National Collection of Dairy Organisms
	NCDO 1859	National Collection of Dairy Organisms

[a]Countries of culture collection: Laboratorium voor Microbiologie, University of Gent, Belgium; Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; American Type Culture Collection, Manassas, VA, USA; National Collection of Food Bacteria, Reading, UK; National Collection of Dairy Organisms, Reading, UK.

gene-specific primers, we screened these strains for the presence of genes encoding peptidases and enzymes involved in the catabolism of sulphur-containing amino acids. PCR assays generated amplicons of the expected sizes (Table 2), and the different enzyme-encoding genes detected by the PCR assays are presented in Table 3. Among the lactobacilli screened, more than 80 % possessed all of the genes tested for. It is also worth noting that the *pepC* and

Table 2 PCR primers designed in this study, annealing temperatures and amplicon sizes

Target gene	Application	Primer sequence (5′→ 3′)	Annealing temperature (°C)	Amplicon size [a] (bp)
dtpT	Di-tripeptide transporter	F-GAAATGTGGGARCGDTTYAGTTAYTAYGG R-ATCNGYYARGAACCACAKACWCATCATYTG	50	1242–1269
pepC	Cysteine aminopeptidase	F-GGNCGTTGYTGGATGTTYGCBGCHTTRAAYAC R-TTADGCHAAWGHWCCCATTGGRTCCCAHGG	54	1131–1143
pepI	Proline iminopeptidase	F-CCYGGTGGHAABCAYGARTAYTGGGAARAC R-GCRTTRTCDATCATGTGRTGRTGRCCRCCDT	53	734–743
pepN	Membrane alanine aminopeptidase	F-ATGGAAAACTGGGGNYTDGTNACHTAYCG R-ACNRCNGGRTADCCNGGYTGTTCVARCCANG	49	569
pepM	Methionine aminopeptidase	F-GGHTTTGAAGGHTAYAARTATKCNACBTGTGT R-AYCATBGGTTCRATNGTAATBGTCATVCC	49	443
pepO	Endopeptidase O	F-ATYTTVCCDGAYAMDACNTACTAYGMHGA R-CCACCARTTATBCADVTTNCCDAATTCATCRAA	49	1077–1089
pepT	Tripeptidase T	F-TTGATACDGCDGAYTTTAAYGCNGADAATG R-CCRTGCATRTTTTCDBCHCCVGCAAA	53	898–907
pepX	X-Prolyl dipeptidyl aminopeptidase	F-CTTTTATWDTNTDGNHCTNCAAYTACTNGRNTTT R-ACNGCAAANCCVCGNGMBARAAARTAATBAT	50	639–681
metK	S-Adenosylmethionine synthase	F-GAAMGMCAYTTATTTACDTCDGA R-AATBCCAGCWGGBCGYAARTCAAA	54	1080
metB/metC	Cystathionine γ-lyase/β-lyase	F-ATGAAATTYRAWACMMAAYTWATTCAYGGYGG R-ACCVACHGAKARRCGRATYAGYTCGTCTT	49	1080–1083
gshR	Glutathione reductase	F-ATGGCGGAACAGTACGATG R-TTAATACAAATATTGTAAGTCACTAGCCG	49	1332

[a] Theoretical amplicon sizes based on the nucleotide gene sequences of several LAB species used as templates for primer design.

Table 3 Results of PCR detection of genes with enzyme-specific primers

Species	*dtpT*	*pepC*	*pepI*	*pepN*	*pepM*	*pepO*	*pepT*	*pepX*	*metK*	*metB/C*	*gshR*
Lactobacillus spp.	94[a] (88[b])	94 (94)	94 (88)	94 (89)	94 (94)	94 (85)	94 (88)	94 (81)	94 (89)	94 (84)	94 (79)
L. casei	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)
L. curvatus	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)
L. delbrueckii	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)
L. fermentum	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (02)	03 (03)	03 (02)	03 (03)	03 (03)	03 (03)
L. hilgardii	02 (02)	02 (02)	02 (02)	02 (02)	02 (02)	02 (02)	02 (02)	02 (01)	02 (01)	02 (02)	02 (01)
L. malefermentans	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (00)	01 (01)	01 (01)	01 (01)	01 (00)	01 (00)
L. paracasei	05 (05)	05 (05)	05 (05)	05 (05)	05 (05)	05 (05)	05 (05)	05 (04)	05 (05)	05 (05)	05 (03)
L. paraplantarum	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (00)	03 (02)	03 (03)	03 (03)
L. pentosus	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (02)	03 (02)	03 (03)	03 (02)
L. plantarum	71 (66)	71 (71)	71 (66)	71 (67)	71 (71)	71 (66)	71 (65)	71 (66)	71 (69)	71 (64)	71 (63)
L. reuteri	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)
L. sakei	02 (01)	02 (02)	02 (01)	02 (01)	02 (02)	02 (00)	02 (02)	02 (01)	02 (02)	02 (00)	02 (00)
Pediococcus spp.	10 (10)	10 (10)	10 (10)	10 (10)	10 (10)	10 (07)	10 (04)	10 (00)	10 (07)	10 (00)	10 (03)
P. acidilactici	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (00)	01 (00)	01 (00)	01 (00)
P. parvulus	06 (06)	06 (06)	06 (06)	06 (06)	06 (06)	06 (05)	06 (00)	06 (00)	06 (04)	06 (00)	06 (00)
P. pentosaceus	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (01)	03 (03)	03 (00)	03 (03)	03 (00)	03 (03)

[a] Total number of strains tested

[b] Number of positive strains

pepM genes were present in all of the *Lactobacillus* species tested and that although *L. plantarum* strains seemed to possess different combinations of the tested genes, other *Lactobacillus* species, including *Lactobacillus casei*, *L. curvatus*, *L. delbrueckii* and *L. reuteri*, possessed all of the genes tested for. Among the *L. fermentum strains* tested, only the reference strain (DSM 20052) did not possess the *pepO* and *pepX* genes. All ten strains of pediococci possessed the *dtpT*, *pepC*, *pepI*, *pepN* and *pepM* genes. In contrast, the *pepX* and *metB*/*metC* genes were not detected in any of the *Pediococcus* strains tested.

Sequence verification

PCR amplifications of the IWBT B349 strain of *L. plantarum* were verified by sequencing using the same sets of primers used for the PCR amplifications. The newly determined sequence data were first assembled and aligned, and then a comparative analysis of the DNA sequences was performed. A homology search was performed by comparing these sequences to other DNA sequences published in GenBank database. The BLAST results confirmed that the sequenced fragments corresponded to the genes tested. The highest homology of nucleotide sequences was recorded with three *L. plantarum* strains (JDM1, WCFS1 and ATCC 14917) published in the NCBI databank (Table 4). The comparative analysis of sequences revealed that IWBT B349 showed a higher sequence identity to the JDM1 strain than WCFS1 and ATCC 14917.

Phylogenetic analysis

The phylogenetic relationship between bacterial strains was also studied by constructing neighbour-joining trees based on nucleotide sequences of the *pepC* and *pepM* genes as

Table 4 Highest identities found between DNA sequences of the *Lactobacillus plantarum* IWBT B349 isolate and other *L. plantarum* strains published in the NCBI database[a]

Target gene	Percentage identity of *L. plantarum* IWBT B349 gene sequences with those of:		
	L. plantarum WCFS1[b]	*L. plantarum* JDM1	*L. plantarum* ATCC 14917
pepC	99.8[c] (NP_784371[d])	100 (YP_003062126)	99.7 (ZP_04014238)
pepI	100 (NP_784587)	100 (YP_003062296)	99.9 (ZP_04014422)
pepN	99.0 (NP_784655)	100 (YP_003062364)	99.6 (ZP_04014501)
pepM	99.7 (NP_784075)	100 (YP_003061815)	100 (ZP_04015434)
pepO	99.1 (NP_786662)	100 (YP_003064328)	99.1 (ZP_04015003)
pepT	99.9 (NP_785446)	100 (YP_003063184)	99.7 (ZP_04012774)
pepX	99.7 (NP_784590)	100 (YP_003062299)	99.7 (ZP_04014425)
metK	99.5 (NP_784949)	100 (YP_003062685)	99.7 (ZP_04013270)
metB/metC	99.7 (NP_784073)	100 (YP_003061813)	99.9 (ZP_04015432)
gshR	98.9 (NP_784176)	99.8 (YP_003061917)	99.5 (ZP_04015540)

[a] NCBI database: www.ncbi.nlm.nih.gov/BLAST

[b] GenBank accession numbers: *L. plantarum* WCFS1 (AL935263); *L. plantarum* JDM1 (CP001617); *L. plantarum* ATCC 14917 (ACGZ00000000)

[b] Percentage identity

[c] GenBank accession number

examples. Five strains belonging to *L. plantarum*, *Lactobacillus paracasei*, *Lactobacillus paraplantarum*, *Pediococcus parvulus* and *Pediococcus pentosaceus* were randomly selected, from which the *pepC* and *pepM* genes were PCR-amplified and subsequently sequenced. Based on the phylogenies inferred with nucleotide sequences of the two selected genes (Fig. 1), there was a similar trend of clustering between bacterial species—a conspicuous feature observed for both phylogenies derived from *pepC* and *pepM* genes. For example, *L. plantarum* IWBT B349 clustered with other *L. plantarum* strains (WCFS1 and JDM1) whose nucleotide gene sequences were retrieved from GenBank database. Similarly, *P. pentosaceus* LMG 13561 also clustered with ATCC 25745 strain of the same species.

Discussion

The proteolytic system has been studied extensively in lactococci. This system consists of protein breakdown, peptide transport and hydrolysis (Kunji et al. 1996). The degradation of proteins is initiated by an extracellular cell-wall-

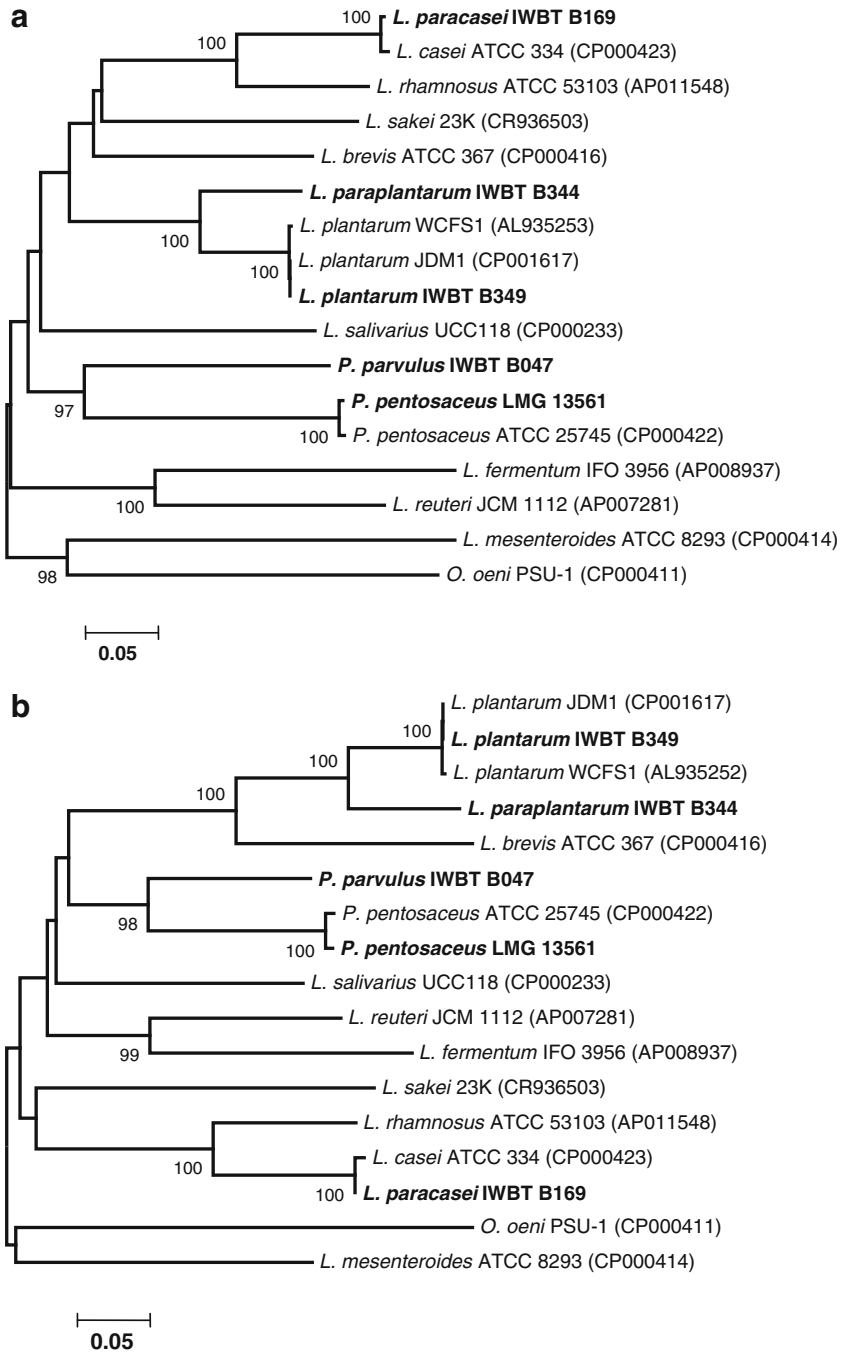

Fig. 1 Phylogenetic tree showing the relationships between various lactic acid bacteria strains tested in this study (*in bold*) and closely related species (GenBank accession numbers *in parentheses*) based on partial *pepC* (**a**) and *pepM* (**b**) gene sequences. The tree was constructed by the neighbour-joining method with Kimura's two-parameter correction model. Bootstrap values of >80 % are shown at branching points. The *horizontal scale bar* represents the number of base substitutions per site

associated proteinase (PrtP) enzyme that releases small peptides and amino acids essential for bacterial growth. The degradation products of the proteins are then transported across the cell membrane into the cell via the peptide transporters (e.g. DtpT, DtpP, Opp). Once inside the cell, these peptides are further hydrolyzed into amino acids by a variety of intracellular peptidases (Kunji et al. 1996; Christensen et al. 1999).

Strahinic et al. (2010) reported on the presence of *prtP* proteinase gene in a natural strain of *L. plantarum* isolated from home-made cheese. In our study, we screened different oenological species of lactobacilli and pediococci for the presence of genes encoding various peptidases. These peptidases can be classified into aminopeptidases (PepC, PepN, PepM, PepA), endopeptidases (PepO, PepF), dipeptidases (PepD, PepV), tripeptidase (PepT) and proline-specific peptidases (PepX, PepI, PepR, PepP, PepQ) (Liu et al. 2010). Among the bacterial strains tested in this study, it was shown that peptidase genes are present in the majority of different species. The presence of the *dtpT* gene encoding a di-/tripeptide transporter was also detected in all pediococci strains as well as in the majority of lactobacilli (>90 %).

In order to complement the amino acid auxotrophy, LAB require a range of proteolytic and peptidolytic enzymes to enable them to garner amino acids from the proteins and peptides. As such, wine also contains proteins which can ultimately be degraded by protein-utilizing LAB to produce smaller peptides and amino acids. Other studies have indicated that wine peptides act as carbon and nitrogen substrates for bacterial growth (Aredes Fernandez et al. 2004). The presence of peptidolytic enzyme-encoding genes in wine lactobacilli and pediococci strains tested in this study has implications on the genetic capability of these strains to release amino acids from peptides. This can also favour the development of wine flavour, as has been reported for dairy LAB (El-Soda et al. 2000; Hynes et al. 2003).

Following the breakdown of peptides by various peptidases, sulphur-containing amino acids can also be released (Kunji et al. 1996). These sulphurous amino acids have been shown to enhance flavour formation in various dairy products, such as cheese (Dias and Weimer 1999). In our study, we also tested the LAB strains for the presence of enzyme-encoding genes involved in the catabolism of sulphur-containing amino acids. Different *Lactobacillus* species displayed the presence of these genes (i.e. *metK*, *gshR* and *metB*/*metC*), albeit not all the strains tested possessed the genes. Some strains of pediococci only possessed the *metK* and *gshR* genes, whereas the *metB*/*metC* gene was not detected in any of the *Pediococcus* strains tested. These findings suggest that *Pediococcus* strains differ with regard to their amino acid utilization and that the presence of genes in some strains is rather strain-dependent. On the other hand, the genomes of *O. oeni* oenological strains PSU-1 (accession no. CP000411), ATCC BAA-1163 (accession no. AAUV00000000) and AWRI B429 (accession no. ACSE00000000) possess *metK*, *gshR*, *metB* and *metC* genes, suggesting that these strains have the genetic potential to metabolize sulphur-containing amino acids. This was also evidenced in a recent study (Knoll et al. 2011) in which two oenological strains of *O. oeni* were shown to contribute to the formation of volatile sulphur compounds, although the role played by these strains during vinification was insignificant.

Conclusion

To conclude, the results presented in this study provide an indication that *Lactobacillus* and *Pediococcus* strains, particularly those of wine origin, possess the genetic potential to degrade peptides and amino acids during winemaking. It should also be noted that certain genes were not present in some strains, suggesting that the presence of the tested enzyme-encoding genes is rather strain-dependent. Nevertheless, it remains to be further confirmed if the tested enzyme-encoding genes are active under oenological conditions and that they are not repressed by winemaking parameters such as pH, temperature and ethanol.

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