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Characterization of wine *Lactobacillus plantarum* by PCR-DGGE and RAPD-PCR analysis and identification of *Lactobacillus plantarum* strains able to degrade arginine

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Summary

Screening of strains isolated from red wine undergoing malolactic fermentation allowed the identification of lactic acid bacteria able to degrade arginine. A denaturing gradient gel electrophoresis approach, using the *rpoB* gene as the molecular target, was developed in order to characterize the isolated strains. Several strains were identified as *Lactobacillus plantarum* and were typed by RAPD-PCR with several randomly designed primers. Almost all of the *L. plantarum* strains identified were able to produce citrulline and ammonia, suggesting that the ability of *L. plantarum* to degrade arginine is a common feature in wine. During the characterization of the newly identified *L. plantarum* strains, the presence of genes coding for the arginine deiminase (ADI) pathway was observed in the strains able to produce citrulline, while the lack of this genes was observed in strain unable to produce citrulline. These results suggest that the degradation of arginine in *L. plantarum* is probably strain-dependent.

Introduction

Wine lactic acid bacteria (LAB) are responsible for the malolactic fermentation (MLF) which usually follows the alcoholic fermentation performed by yeast (Liu 2002). During malolactic fermentation, lactic acid bacteria, mainly belonging to the *Lactobacillus*, *Oenococcus oeni* and *Leuconostoc* spp. cause a biological deacidification of wine, due to the conversion of the dicarboxylic malic acid into monocarboxylic lactic acid and a broad range of secondary compositional modifications of great importance for taste and flavour improvement of wine (Lonvaud-Funel 1999; Liu 2002).

However, with regard to wine quality, undesirable substances may be released by LAB during and after winemaking. Ethyl carbamate (or urethane), a well known animal carcinogen (Zimmerli & Schlatter 1991) found in many fermented foods, including wine (Canas *et al*. 1994; Kodama *et al*. 1994), may be produced from precursors such as urea which are produced by yeasts, or citrulline and carbamoyl phosphate mainly produced by LAB through the arginine deiminase (ADI) pathway (Liu & Pilone 1998; Mira de Ordùna *et al*. 2000). This pathway consists of the following three enzymes: ADI, ornithine carbamoyltransferase (OTC), and carbamate kinase. Arginine is first converted into citrulline, which is subsequently transformed into ornithine and carbamoyl phosphate.

The organization of the genes encoding the ADI pathway has been reported in several bacteria (Zuniga *et al*. 1998; Arena *et al*. 2002; Tonon *et al*. 2001) including homofermentative LAB isolated from red wine (Spano *et al*. 2004). More recently, a gene cluster encoding the enzymes of the ADI pathway of *Oenococcus oeni*, the main species that induces malolactic fermentation in wine, has been cloned and sequenced (Tonon *et al*. 2001). The genes (named *arcA*, *arcB* and *arcC*) are clustered in the same order as in *Lactobacillus sakei* and *Lactobacillus hilgardii* (Arena *et al*. 2002), the other LABs for which the corresponding loci have been sequenced. Moreover, their expression is induced by arginine although they are differently regulated in different bacterial species (Zuniga *et al*. 1998; Tonon *et al*. 2001).

Additionally, there are reports concerning the characterization of a fourth gene encoding a transport protein, catalysing an electroneutral exchange between arginine and ornithine (Divol *et al*. 2003). We have previously reported the presence of the ADI pathway in wine *L. plantarum* (Spano *et al*. 2004). The high identities among ADI, OTC and CK protein sequences between *O. oeni* and *L. plantarum* and the induction of *arc* genes by arginine suggested that the cloned genes control arginine catabolism in *L. plantarum*.

The aim of this paper was to develop suitable molecular methods to identify *L. plantarum* in wine and

to analyse its ability to degrade arginine in red wine. From our studies we suggest that strains of *L. plantarum* unable to degrade arginine lack the *arc* genes.

Materials and methods

Bacterial strains, plasmids and growth conditions

Lactobacillus plantarum strains (Lp24, Lp65, Lp12, Lp61, Lp60, Lp34, Lp11 and Lp77) were isolated from wine undergoing MLF during harvesting in 2002.

In addition, previously identified *L. plantarum* strain Lp90 (Spano *et al*. 2004) able to produce ammonia and citrulline was used in this study.

PCR amplified fragments previously cloned in a pGEM-T easy vector (Promega) were delivered into *Escherichia coli* JM109 High Efficiency Competent Cells (Promega). Plasmid DNA was purified with Wizard *Plus* SV Minipreps (Promega) and DNA sequencing was performed on both strands with universal primers (T7 and SP6) by MWG Biotech (Germany).

Lactobacillus plantarum ATCC 14917^T (American Type Culture Collection) unable to produce citrulline was used as a negative control.

For PCR-DGGE analysis, the reference strains *L. plantarum* ATCC 14917^T, *Lactobacillus brevis* DSMZ 20054, *Lactobacillus hilgardii* DSMZ 20176, *Lactobacillus sakei* subs. *sakei* DSMZ 20017, and the strain of *L. plantarum* previously identified (Spano *et al*. 2004) were used.

Production of citrulline and ammonia from arginine degradation

For arginine degradation by the ADI pathway, Niven medium (per liter 2 g of K_2HP_4, 3 g of L-arginine, 0.1% (v/v) Tween 80, 50 mg of $MnSO_4$; pH adjusted to 5 with 2% (w/v) glucose) was used. Strains were inoculated in MRS broth for 24 h and then transferred to Niven medium. The cultures were centrifuged, and 1 ml of distilled water 1 ml of H_2SO_4/H_3PO_4 (1:3) and 0.13 ml of 3% (w/v) diacetylmonoxime were added to 1 ml of culture supernatant (Curk *et al*. 1996). The samples were then boiled for 10 min and the development of orange colour was monitored, allowing the detection of citrulline, the arginine degradation product. Ammonia was detected with Nessler's reagent (Pilone *et al*. 1991).

Two replicate tests were carried out for each strain analysed.

DNA manipulation, amplification and sequencing of the rpoB gene

Genomic DNA of *L. plantarum* ATCC 14917^T, *Lactobacillus brevis* DSMZ 20054, *Lactobacillus hilgardii* DSMZ 20176, *Lactobacillus sakei* subs. *sakei* DSMZ 20017 and strains Lp24, Lp65, Lp12, Lp61, Lp60, Lp11, Lp34, Lp77, Lp90, was extracted using the AquaPure Genomic DNA Kit (BIO-RAD) as recommended by the manufacturer.

Primers *rpoB*1698f and *rpoB*2014r (Dahllöf *et al*. 2000) were used to amplify a region of approximately 350 bp of the *rpoB* gene. For the PCR experiment, about 100 ng of genomic DNA were added to a 50 (l PCR mix and the reaction mix was cycled through the following temperature profile: 94 °C 5 min; 94°C 1 min; 60 °C 1 min; 72 °C 1 min for the first 15 cycles, then 15 cycles at 52 °C as the annealing temperature. When PCR products were to be used for sequencing analysis (MWG Biotech, Germany), an unclamped forward primer was used for generation of the amplicons (Dahllöf *et al*. 2000).

Analyses of DNA sequences was carried out using a set of programs accessible at the sites DEAMBULUM (www.infobiogen.fr/services/deambulum/fr) and NCBI (www.ncbi.nlm.nih.gov).

Identification of Lactobacillus plantarum strains by PCR-DGGE and PCR amplification of random DNA fragments

The Dcode Universal Mutation Detection System^{TM} (BioRad, Hercules, CA, USA) was used for specific separation of the PCR products. Electrophoresis and gel staining was performed as previously reported (Rantsiou *et al*. 2004).

RAPD-PCR amplification was performed using 12 different primers of arbitrary nucleotide sequence with GC contents $> 50\%$.

The similarities among RAPD patterns were calculated by the Dice similarity index (Brosch *et al*. 1994) using the Taxotron software Restrictotyper module (Taxolab software, Institute Pasteur, France).

Total RNA isolation and RT-PCR analysis

For total RNA isolation, induction was performed at pH 3.6 adding 5 g arginine l^{-1} to a culture (in MRS medium) of *L. plantarum* strains Lp90 and Lp34, previously grown to an absorbance at 600 nm (A_{600}) of 0.5. RNA preparation was carried out using the NucleoSpin RNA II (Macherey-Nagel, Duren, Germany) as recommended by the manufacturer. Synthesis of cDNA was carried out with the ProStar ^{TM} Single-Tube RT-PCR System (Stratagene) as recommended by the manufacturer. The primers correspond to the internal portion of the 23S rRNA and the *arcABC* genes isolated from the *L. plantarum* strain Lp90 as already published (Spano *et al*. 2004). The reaction mix was cycled through the following temperature profile: 45 °C 15 min (reverse transcriptase reaction); 95 °C 1 min; 95 °C, 30 s (denaturation), 60 °C 1 min (annealing), 68 °C, 30 s: 40 cycles. PCR negative controls with water instead of cDNA and chromosomal DNA resulted in no amplification. Genomic DNA of the strain Lp90 was used as a positive control. The *L. plantarum* 23S rRNA gene was used as a constitutive expression control, since it is was not induced by arginine (Tonon *et al*. 2001).

Results and discussion

Identification of new Lactobacillus plantarum strains able to produce citrulline

In this study, an alternative approach to 16S rDNA-target identification of LAB populations was assessed, involving exploitation of sequence variation in the *rpoB* gene encoding the beta subunit of RNA polymerase. The *rpoB* gene has already been used as a target for the identification of lactic acid bacterial populations during food fermentation (Rantsiou *et al*. 2004). Using the primer set *rpoB*1698f and *rpoB*2014r, a PCR product of approximately 350 bp was identified for the strains analysed (data not shown). Following separation of *rpoB* amplicons, species-specific bands were obtained by DGGE (Figure 1). No differences between the *rpoB* amplicons corresponding to *L. plantarum* ATCC 14917^T, *L. plantarum* strain Lp90 and the new strains isolated were observed. Therefore, the newly identified strains represent *L. plantarum* species. However, because *L. plantarum* and *L. helveticus* have been reported to share an identical DGGE pattern (Rantsiou *et al*. 2004), three amplified fragments (corresponding to strains Lp34, Lp11 and Lp61) were excized, reamplified and sent for sequencing to a commercial facility (MWG Biotech, Germany). The DNA sequence was analysed and compared with the GenEMBL databases, using the FASTA program or the BLAST network service (NCBI). The resultant fragments corresponded to the *rpoB* gene isolated from *L. plantarum* strain WCFS1 (Kleerebezem *et al*. 2003).

RAPD-PCR patterns of each strain were obtained with several randomly designed primers in separate reactions (Figure 2). The RAPD profile was different for strains Lp12, Lp34, Lp60 and Lp61, while it was identical for strains Lp24 and Lp65 or Lp77, Lp11 and Lp90. The isolates Lp24 and Lp65 clustered together and shared the same clonal origin. The isolates Lp77 and Lp11 were similar to strain Lp90. Strains Lp11 and Lp90 had identical RAPD patterns, while the third, Lp77, differing by only one band, can be considered closely related to them. In contrast, the other four RAPD patterns (Lp12, Lp60, Lp34 and Lp61) were clearly unrelated since they showed $>50\%$ difference according to the Dice similarity index (Figure 2B).

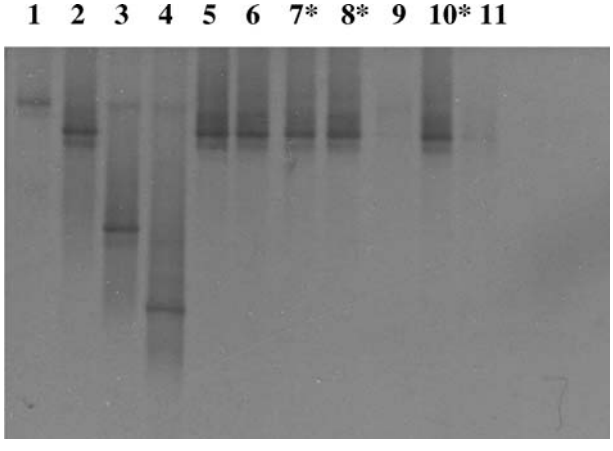

Figure 1. Example of DGGE profiles of the *rpoB* amplicons obtained from strains isolated from wine undergoing malolactic fermentation during harvesting in 2002. Bands indicated by asterisks were excized and, after reamplification, subjected to sequencing. A denaturing gradient from 30 to 60% was used. Lane 1, *Lactobacillus brevis* DSMZ 20054; lane 2, *L. plantarum* ATCC 14917T; lane 3, *Lactobacillus sakei* subs. *sakei* DSMZ 20017; lane 4, *Lactobacillus hilgardii* DSMZ 20176; lane 5, *L. plantarum* strain Lp90; lanes 6, 7, 8, 9 10 and 11, strains Lp24, Lp65, Lp61, Lp12, Lp34, Lp60, respectively.

All of the strains analysed except strain Lp34 were able to form citrulline and ammonia from arginine. However, the ability to use arginine and the presence of the ADI pathway has been proven to be strain-related in *O. oeni* (Tonon *et al*. 2001; Divol *et al*. 2003). In order to verify if the same dependence exists in wine *L. plantarum*, a strain unable to use arginine (strain Lp34 isolated in this work) and a strain able to degrade it (strain Lp90) were used to carry out RT-PCR experiments specific to each *arcABC* genes (Figure 3). The 23S RNA gene was used as an internal control. In the strains tested we were able to amplify the 23S RNA gene, suggesting that the DNA was present in all the PCR samples; however, amplification of the *arcABC* genes was positive only when cells of strain Lp90 were examined (Figure 3A), whereas no fragments were observed with cells of strain Lp34 (Figure 3B). The experiment was repeated twice on DNA extracted after independent isolation procedures, with the same results.

Conclusions

Wine malolactic bacteria (MLB) vary in their ability to degrade arginine. Homofermentative wine lactobacilli were found to be unable to degrade arginine (Edwards *et al*. 1993; Liu *et al*. 1995) although arginine is degradable by a number of homofermentative LAB (including *L. plantarum*) isolated from other sources such as fish (Jonsson *et al*. 1983) and orange juice (Arena *et al*. 1999). In contrast, all heterofermentative wine LAB examined, including strains of *L. buchneri* and *L. brevis* (Edwards *et al*. 1993; Liu *et al*. 1994; Liu 1996) and the heterofermentative species *Oenococcus oeni* (Liu & Pilone 1998), hydrolyzed arginine. The organization of the genes encodes the ADI pathway in wine LAB has been reported in *Oenococcus oeni*, the main species which induces malolactic fermentation in wine (Tonon *et al*. 2001; Divol *et al*. 2003). Furthermore, the presence of putative *arcABC* genes in wine *L. plantarum* has been reported (Spano *et al*. 2004).

In this study, new strains of *L. plantarum* able to produce citrulline were isolated from wine undergoing MLF in 2002. The strains were classified as belong to *L. plantarum* species using the *rpoB* gene as the molecular target. During the characterization of the newly identified *L. plantarum* strains, the presence of all the *arcABC* genes was observed in the strains able to

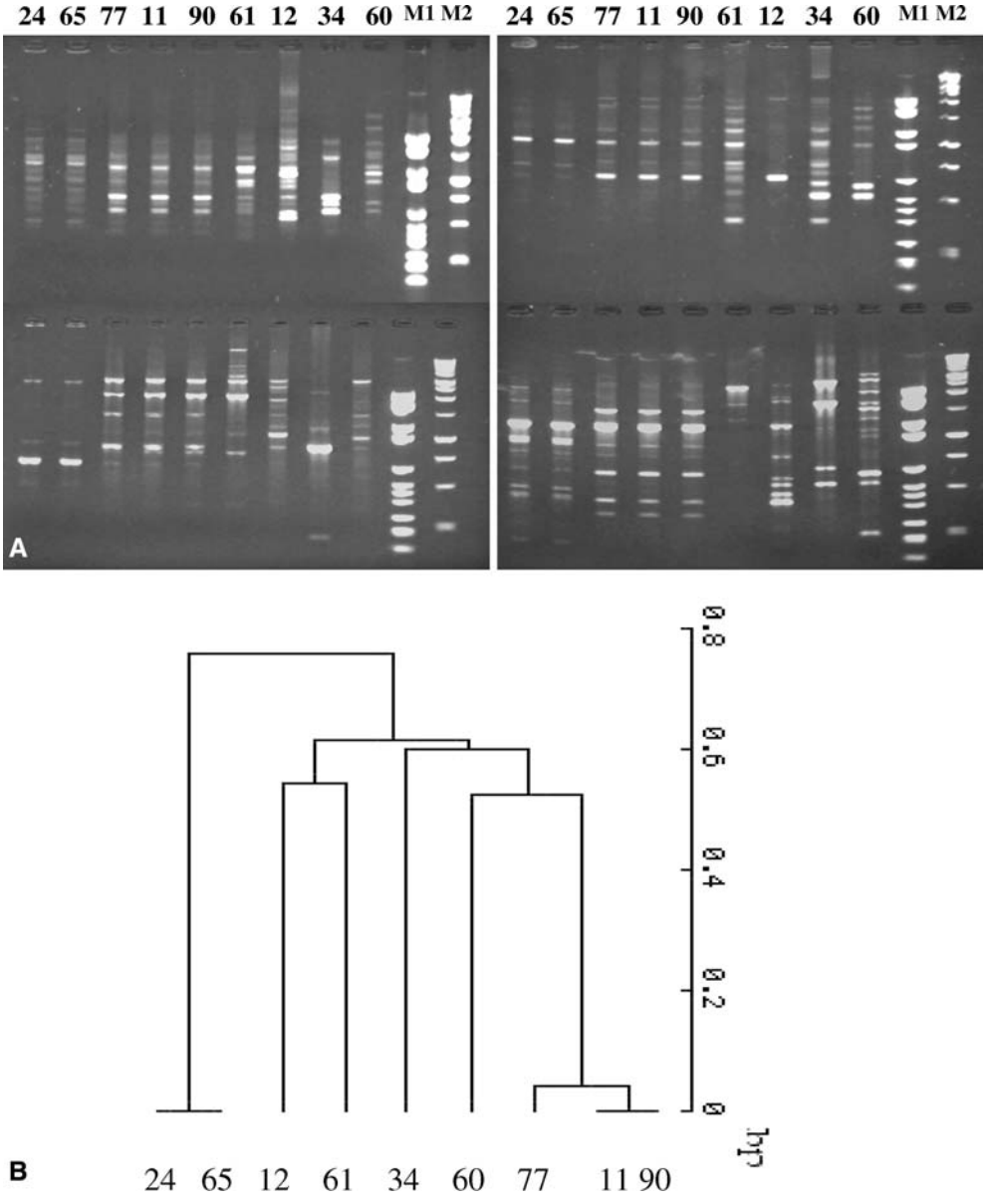

Figure 2. (A) Examples of RAPD-PCR profiles obtained from DNA extracted from *Lactobacillus plantarum* strains able to produce citrulline (numbers as Lp24 → Lp60) and amplified with four different primers. (B) UPGMA dendrogram based on the RAPD analysis reported above. M1 ed M2, molecular markers.

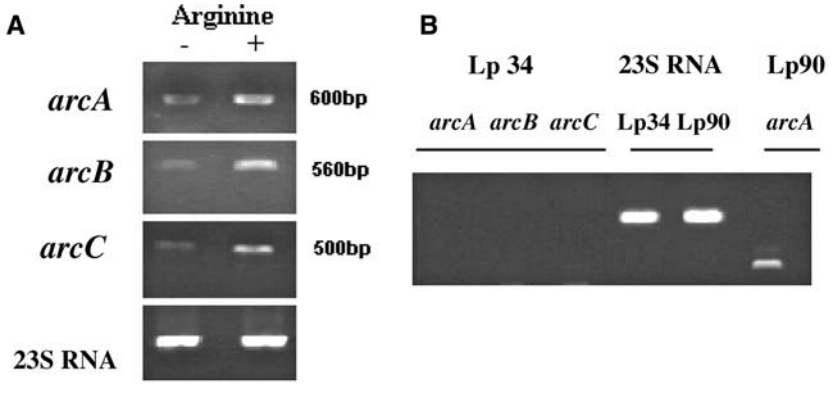

Figure 3. RT-PCR analysis of *arcABC* gene expression from *L. plantarum* Lp90 strain. (A) Left panel, RT-PCR obtained with cDNA made from *L. plantarum* Lp90 strain extracted after 20 h of induction with 5 g l^{-1} arginine (+) or without arginine (−) at pH 3.6. (B) Genomic DNA from strain Lp34 was amplified with primers specific to the *arcABC* genes. The genomic DNA from strain Lp90 was used as positive control and was amplified with primers specific to the *arcA* gene. Amplification with the 23S RNA gene confirmed that the same amount of DNA was added in the PCR reaction.

produce citrulline, while the lack of *arcABC* genes was observed in strain Lp34 unable to produce citrulline. These results suggest that the degradation of arginine in *L. plantarum* is probably strain-dependent as already reported for some strains of *O. oeni* (Tonon *et al.* 2001; Divol *et al.* 2003).

The production of citrulline in wine its appears to be strictly dependent from the availability of both arginine and LAB strains able to degrade it. Therefore, wine makers must known the characteristics of indigenous LAB in order to keep as low as possible the level of those strains able to produce citrulline a precursor in the formation of ethyl carbamate.

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