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The arginine deiminase pathway in the wine lactic acid bacterium *Lactobacillus hilgardii* X_1B: structural and functional study of the *arcABC* genes

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Abstract

The genes implicated in the catabolism of the amino acid arginine by *Lactobacillus hilgardii* X_1B were investigated to assess the potential for formation of ethyl carbamate precursors in wine. *L. hilgardii* X_1B can use arginine via the arginine deiminase pathway. The complete nucleotide sequence of the *arc* genes involved in this pathway has been determined. They are clustered in an operon-like structure in the order *arcABC*. No evidence was found for the presence of a homologue of the *arcD* gene, coding for the arginine/ornithine antiporter. The *arc* genes have been expressed in *Escherichia coli* resulting in arginine deiminase (ArcA), ornithine carbamoyltransferase (ArcB) and carbamate kinase (ArcC) activities. The results indicate the need for caution in the selection of lactic acid bacteria for conducting malolactic fermentation in wine since arginine degradation could result in high amounts of ethyl carbamate.

Keywords: Ethyl carbamate; Urethane; Arginine deiminase pathway; Wine; Arginine

1. Introduction

Most fermented foods and beverages, including wine, contain trace amounts of ethyl carbamate (also known as urethane) (Ough, 1976),an animal carcinogen (Zimmerli and Schlatter, 1991). In recent years, considerable efforts have been done to investigate the possible origin of this substance in wine (Monteiro et al., 1989; Monteiro and Bisson, 1991; Sponholz, 1992; Tegmo-Larsson et al., 1989). The formation of ethyl carbamate is a spontaneous chemical reaction involving ethanol and a compound that contains a carbamyl group such as urea, citrulline, and carbamyl phosphate (Ough et al., 1988). The role of malolactic wine lactic acid bacteria (LAB) in ethyl carbamate formation has been studied (Arena et al., 1999a; Liu et al., 1994; Sponholz, 1992; Tegmo-Larsson et al., 1989). Some wine LAB are known to degrade L-arginine, one of the major amino acids found in grape juice and wine, with the formation of ornithine and ammonia (Arena et al., 1999a; Arena and Manca de Nadra, 2001; Granchi et al., 1998; Mira de Orduña et al., 2001; Tonon et al., 2001). It has been demonstrated that arginine catabolism in wine LAB involves the arginine deiminase (ADI) pathway. This pathway basically includes three enzymes, ADI (EC 3.5.3.6), ornithine transcarbamylase (EC 2.1.3.3) (OTC), and carbamate kinase (EC 2.7.2.2) (CK), which catalyze the following reactions:

$$\text{L-arginine} + H_2O \xrightarrow{\text{ADI}} \text{L-citrulline} + NH_3$$

$$\text{L-citrulline} + P_i \overset{\text{OTC}}{\leftrightarrow} \text{L-ornithine} + \text{carbamyl-P}$$

$$\text{carbamyl-P} + \text{ADP} \overset{\text{CK}}{\leftrightarrow} \text{ATP} + CO_2 + NH_3$$

The energy (ATP) formed during arginine catabolism can be coupled to bacterial growth. There are large variations in the distribution of the arginine-catabolizing enzymes among wine LAB, with some strains possessing only part of the enzyme system. Arginine is apparently

Abbreviations: LAB, lactic acid bacteria; ADI, arginine deiminase; CK, carbamate kinase; OTC, ornithine carbamoyltransferase; ORF, open reading frame; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside.

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transported mainly via the arginine-ornithine antiporter system after the initiation of arginine uptake in the presence of a fermentable sugar. The metabolism of arginine in wine LAB has practical significance in terms of taxonomic utility, biological significance and oenological implications (Liu and Pilone, 1998).

Lactobacillus hilgardii is a facultative heterofermentative LAB commonly isolated from grape juice and wine. In previous studies Arena et al. (1999a) demonstrated the utilization of arginine and citrulline via the ADI pathway by *L. hilgardii* X_1B. This observation could be considered the result of a strain adaptation for an enhanced viability during the stationary phase when cells are grown under anaerobiosis as have been demonstrated for *Lactobacillus sakei* (Champomier et al., 1999). However, the citrulline excreted to the medium during arginine catabolism, could be a source of ethyl carbamate formation in wine.

Interestingly, the ADI pathway only has been thoroughly studied in a few LAB at the molecular level. The genetics of the arginine deiminase pathway has been well studied in *L. sakei* (formerly *L. sake*) (Zúñiga et al., 1998). In this organism, the genes encoding the enzymes of the ADI pathway are clustered. The complete sequence data of the ADI genes in *Lactococcus lactis* ssp. *cremoris* (EMBL/GenBank/DDBJ accession no. AJ250129) and *L. lactis* ssp. *lactis* (Bolotin et al., 2001; accession no. AF282249) are also available. Recently, the ADI pathway genes of *Oenococcus oeni*, the main species that induces malolactic fermentation in wine, have been cloned (Tonon et al., 2001).

To gain deeper insight into the arginine catabolism of *L. hilgardii* X_1B, we reported here the characterization of the genes involved in the ADI pathway. The proteins implicated in this pathway have been overproduced in *Escherichia coli* and biochemically characterized.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

L. hilgardii X_1B has been described previously (Manca de Nadra and Strasser de Saad, 1987). This strain was isolated from Argentinean wines. The *E. coli* strain DH5α (Sambrook et al., 1989) was used as host for recombinant plasmids. Plasmid pGEM-T (Promega) was used for cloning polymerase chain reaction (PCR) fragments. The construction of the recombinant plasmids pRM5 and pRM6 is described in the text. Plasmid pT7-7 is an expression vector that allows the hyperexpression of the desired protein upon induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in an *E. coli* strain (*E. coli* JM109 (DE3) [*endA1 recA1 gyrA96 hsdR17 supE44 relA1 thi*Δ (*lac-pro*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15) λ*c*I857 *ind1 Sam/nin5 lacUV5*-T7 gene 1; Promega]).

L. hilgardii was routinely grown in MRS medium (purchases from Difco, Detroit, MI) at 30°C without shaking. *E. coli* cells were incubated in Luria-Bertani medium (Sambrook et al., 1989) at 37°C with shaking. When required, ampicillin was added to the medium at 100 μg ml^{-1}. Chromosomal DNA, plasmid purification and transformation of *E. coli* were carried out as described elsewhere (Muñoz et al., 1998).

2.2. DNA manipulations and hybridization

Restriction endonucleases, T4 DNA ligase and the Klenow fragment of DNA polymerase were obtained commercially and used according to the recommendations of the suppliers. Gel electrophoresis of plasmids, restriction fragments, and PCR products were carried out in agarose gels as described (Sambrook et al., 1989). DNA was digoxigenin-labeled and chemiluminescently detected by using the DIG High Prime DNA labeling and detection Starter Kit (Roche) according to the manufacturer's instructions.

PCR amplifications were performed as previously described (Muñoz et al., 1998) but using *Pfu* DNA polymerase (Stratagene). Conditions for amplifications were chosen according to the G plus C content of the corresponding oligonucleotides. The oligonucleotides primers mentioned in the text were: Aup (5′-GAYCCNATGCCNAAYYTRTAYTTYAC), coding for DPMPNLYFT; Adown (5′-GTRTTRSRNCCRTCRTTCCAYTGYTC), coding EQWNDGSNT; Bup (5′-TGGCAYCCNACNCARATGMTNGCNGA), coding for WHPTQMLAD; Bdown (5′-TCNCCCATKSWNACCCANACRTCNGT), coding for TDVWVSMGE; Cup (5′-TCNCAYGGNAAYGGNCCNCARGTNGG), coding for SHGNGPQVG, and Cdown (5′-GCNGTNATHGAYAARGAYTTYGCNTC), coding for AVIDKDDFAS (Y = C or T; N = A, C, G or T; R = A or G; S = C or G; M = A or T; K = G or T; W = A or T; H = A, C or T). For inverse PCR we used: primer 21 (5′-GACGAGCAACGAATGTCATATGG) and primer 22 (5′-GAAATTTATCGAGAACGGGGATC) for the *Pvu*II fragment, and primer 23 (5′-AATTTCAGTTGGTGGTGGCGG) and primer 25 (5′-CTGGTGTAATTGTCCAAGTATC) for the *Bcl*I fragment.

DNA sequencing was carried out by using an Abi Prism 377™ DNA sequencer (Applied Biosystems, Inc.). Unless otherwise stated, DNA and protein sequence analysis were preformed using software form the Wisconsin Genetics Computer Group (Madison, WI, USA). Sequence similarity searches were carried out using the EMBL/GenBank, SWISS-PROT, and PIR databases.

2.3. Heterologous expression of arcA, arcB and arcC in E. coli

To clone *arcB* and *arcC* in the absence of its own promoter, these genes were first PCR amplified form *L. hilgardii* X_1B DNA by using *Pfu* DNA polymerase and

oligonucleotides based in the nucleotide sequence previously determined. To amplify *arcB* we used primer 19 [(2066) 5′-GGAGAATTCCATATGACAAAAG], and 20 [(3135/c) 5′-TTATTCATCGATTATTGAGCTTG); and primer 27 [(3223) 5′-GGAGGGAAA*CATATG*GGACGCGTAAAATCG], and 28 [(4234/c) 5′-GCCTTACACTCCGATCGATAACCGC] for *arcC* (the underlined sequences indicate restriction sites for *Nde* I in primers 20 and 27, and *Cla* I in 21 and 28). The numbers indicate the position of the first nucleotide of the primer in the sequence reported here, and c/ indicates that the sequence corresponds to the complementary strand of that included in the database. The amplified DNAs were digested with *Nde* I and *Cla* I and ligated to the expression vector pT7-7 (Tabor, 1990) previously digested with the same enzymes.

Analysis of the nucleotide sequence showed the presence of an internal *Nde* I restriction site in the *arcA* gene. We eliminated this site using a two-step mutagenesis strategy based on PCR. In the first step, we divided the gene in two overlapping fragments including the *Nde* I site in one end. We designed oligonucleotides destroying the *Nde* I site but keeping the His-181 residue in the ArcA protein. We used primers 33 [(716) 5′-TTCAAGGGACATATGGAGGTTA CAC], and 34 [(1284/c) 5′-GCAACGAATGTCAT**G**TGGT TAATAC] to amplify the 3′ fragment, and primers 35 [(1260) 5′-GTATTAACCA**C**ATGACATTCGTTGC] and 36 [(2022/c) 5′-GGACTGCATCGATAAGTAATACTGC] for the 5′ one (the underlined sequences indicate restriction sites for *Nde* I in primer 33 and *Cla* I in 36; the changed nucleotide that destroy the internal *Nde* I restriction site is shown in boldface). We used *Pfu* DNA polymerase to amplify both fragments. The resulting PCR products were purified and used as template for a second PCR amplification with primers 33 and 36. The 1.2-kb amplified DNA was digested with *Nde* I and *Cla* I and ligated to pT7-7 as described above.

All the ligation mixtures were introduced by transformation into the expression strain *E. coli* JM109 (DE3).

2.4. Enzyme assays

Cell extracts for enzyme assays were obtained from induced cultures. Briefly, *E. coli* JM109 (DE3) cells harboring the recombinant plasmids were grown in LB broth supplemented with ampicillin (100 μg ml^{-1}) at 37°C to an optical density at 600 nm of 0.6, the cultures were shifted to 30°C, and expression of the corresponding genes was induced by adding IPTG to 0.5 mM (final concentration). After 3 h of induction, samples of the cultures were harvested by centrifugation (10,000 × *g*, 5 min) and the pelleted bacteria were resuspended in 10 mM phosphate buffer (pH 7.5) and disrupted by sonication. The insoluble fractions were separated by centrifugation (15,000 × *g*, 15 min), and the supernatants were used for enzyme assays. Protein concentration was determined with the Coomassie protein assay reagent (Pierce).

The standard assay to determine ADI activity was performed as described by Zúñiga et al. (1998) but with 10 mM arginine (final concentration) in the reaction. Citrulline concentration was analyzed as previously described (Arena et al., 1999a). One enzyme unit was the amount of enzyme needed to produce 1 μmol of citrulline per h per mg of protein. OTC activity was assayed according to the method described previously (Zúñiga et al., 1998) in cell extracts containing 100 μg of protein. OTC activity was determined by measuring the formation of citrulline from ornithine and carbamyl phosphate. CK activity was measured by an indirect assay described by Ruepp and Soppa (1996). Briefly, in a first step, cell extract of *E. coli* JM109 (DE3) harboring the *arcC* gene cloned into plasmid pT7-7 and cell extract with pT7-7 as control were incubated for different times at 37°C in the presence of 40 mM ornithine and 10 mM carbamyl phosphate. In the second step, the carbamyl phosphate, which was not degraded by CK, was subsequently converted into citrulline by the addition of ornithine and OTC from a culture of *E. coli* JM109 (DE3) harboring the cloned *arcB* gene. The citrulline was detected by a colorimetric assay, as described above.

3. Results and discussion

3.1. Amplification of the L. hilgardii arc cluster

To locate the genes involved in the ADI pathway we aligned amino acid sequences of known ADI (ArcA), OTC (ArcB) and CK (ArcC) proteins from *L. sakei* (Zúñiga et al., 1998), *Bacillus licheniformis* (Maghnouj et al., 1998) and *Clostridium perfringens* (Ohtani et al., 1997). Two conserved domains of each protein were selected to design synthetic primers to amplify by PCR the corresponding gene. We obtained different fragments when using this set of primers: Aup + Adown (0.6-kb), Bup + Bdown (0.3-kb) and Cup + Cdown (0.5-kb), whose sequences shared high similarity at the amino acid level with other ADI, OTC and CK sequences, respectively. We also obtained two overlapping fragments, Aup + Bdown (1.6-kb) and Bup + Cdown (1.4-kb) (Fig. 1) confirming the gene order as *arcA*, *arcB* and *arcC*. A total of 2.7-kb DNA fragment was sequenced containing the complete sequence of *arcB* and incomplete sequences for *arcA* and *arcC*. To sequence the 5′ end of *arcA* and the 3′ end of *arcC* we synthesized two specific probes. The 0.6 and 0.5-kb PCR fragments were ligated to pGEM-T (Promega), and the ligation mixture was used to transform *E. coli* DH5α. The recombinant plasmids were named pRM5 (*arcA*) and pRM6 (*arcC*) (Fig. 1). Genomic DNA from *L. hilgardii* X_1B was treated with restriction endonucleases, and the resultant fragments were run on agarose gels (Sambrook et al., 1989). Restriction fragments were blotted and hybridized with pRM5 and pRM6 probes. Southern blot hybridizations (not shown) indicated that the 0.6-kb fragment of *arcA* was

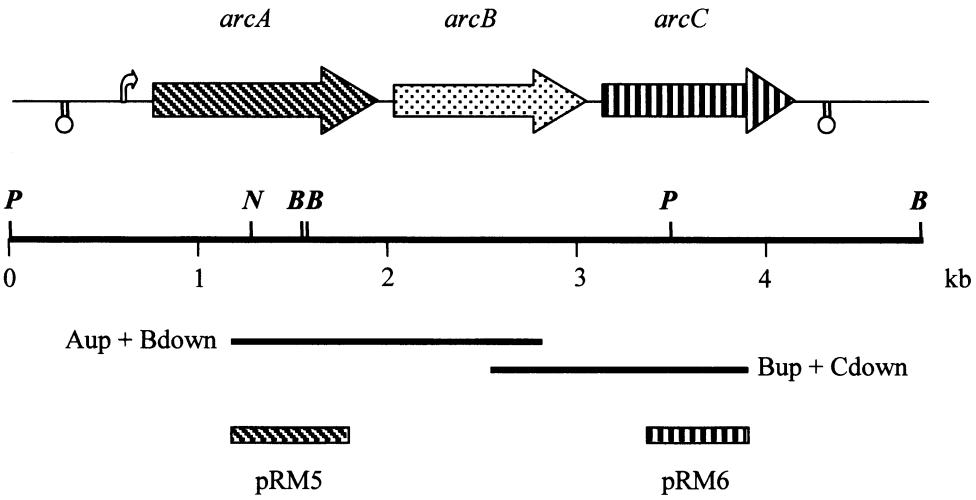

Fig. 1. Genetic organization of *L. hilgardii* X_1B containing the *arc* cluster. Arrows represent complete ORFs. Some of the plasmids and PCR fragments used in this study are indicated, as are relevant restriction sites (*P*, *Pvu* II; *N*, *Nde* I; and *B*, *Bcl* I). The location of the putative promoter (vertical bent arrow) and transcription terminator regions (ball and stick) are also indicated. The partial restriction map of the region is also shown.

included in a 3.4-kb *Pvu* II fragment and the 0.5-kb of *arcC* in a 3.2-kb *Bcl* I fragment (Fig. 1). Chromosomal *L. hilgardii* X_1B DNA was then digested with these enzymes. Fragments with appropriate sizes were religated and the ligation mixtures were used as templates for inverse PCR. To amplify the 3.4-kb *Pvu* II fragment we used primer 21, sequence close to the 3′ end of the 2.7-kb fragment, and primer 22, located near the unique internal *Pvu* II restriction site. Similarly, we amplified the 3.2-kb *Bcl* I fragment using primer 23, based on the sequence close to the 3′ end of the 2.7-kb fragment and primer 25, near one of the internal *Bcl* I sites. The PCR fragments were purified and sequenced. A total of 4756 bp DNA fragment from *L. hilgardii* was PCR amplified and sequenced.

3.2. Structure of the ADI cluster of L. hilgardii

Homology searches enabled us to locate the *arcA*, *arcB* and *arcC* genes on the 4756 bp fragment (Fig. 1). The analysis of the nucleotide sequence reported in this work suggested that the *arcABC* genes are organized as a single operon. In fact, only one putative promoter was detectable upstream of the first open reading frame (ORF). Computer promoter predictions carried out at the Internet site http://www.fruitfly.org/cgi-bin/seq_tools/promoter.pl showed the sequence (nucleotide 651) TcGACA-19 bp-TAcgAT (681) that could function as a promoter and also predicted the putative transcription start site at 11 nucleotides after the −10 consensus sequence. Sequence analysis revealed three possible open reading frames all transcribed in the same direction and separated by short intergenic spaces (Fig. 1). A typical ribosome binding site was recognized upstream of each gene. Putative transcription terminators followed the TAA stop codon of the third ORF.

The first complete open reading frame (1254 bp) was assigned to the *arcA* gene. It encodes a 418-amino acid residue, 47.2-kDa protein, 70% identical to the putative *Oenococcus oeni* arginine deiminase (accession no. AF124851) and 66% identical to that of *L. sakei* (Zúñiga et al., 1998). The 1029 bp open reading frame immediately downstream of *arcA* encodes a 38.3-kDa (343-amino acid residue) protein. The high similarity (77% identity) observed between this protein and the ornithine transcarbamoylase of *O. oeni* suggests that this open reading frame corresponds to the *arcB* gene. The third complete open reading frame (954 bp) was identified as the *arcC* gene. It encodes a 318-amino acid residue, 33.6-kDa protein, showing 68 and 64% identity with the putative carbamate kinase of *O. oeni* and *L. sakei*, respectively. Interestingly, the nucleotide sequence of the *arcABC* genes of *L. hilgardii* were more similar to the sequence of the *O. oeni* genes, which is also found in wines, than those of a bacteria evolutionary more related as *L. sakei*. The genes encoding the enzymes involved in the arginine deiminase pathway of *L. hilgardii* are clustered and are arranged in the same order as those of *L. sakei* and *O. oeni*.

Homology searches were also carried out with the remaining sequences of the 728 bp located upstream of *arcA* and the 565 bp between the TAA stop condon of *arcC* from the downstream *Bcl* I site. No additional ORFs were identified. No evidence was found for the presence of a homologue of the *arcD* gene, coding for the arginine/ornithine antiporter. Gene organization is thus different from that of the other *arc* clusters described so far except from that of *O. oeni*, another wine LAB (Tonon et al., 2001). However, in *O. oeni* upstream of *arcA*, an open reading frame called *orf229* encodes a protein which shares common features with proteins involved in transcription activation. In several *arc* gene clusters have been described different putative regulatory genes, i.e. *arcR* in *Halobacterium salinarum* (Ruepp and Soppa, 1996) and *ahrC* in *Clostridium prefringens* (Ohtani et al., 1997) and in *L. lactis*

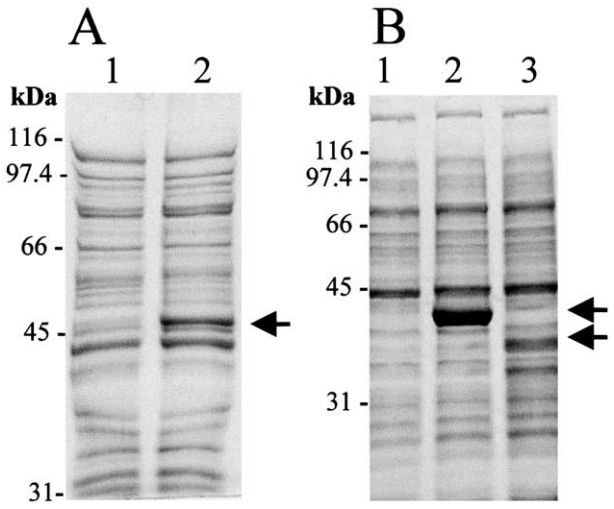

Fig. 2. sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of cells extracts of IPTG-induced cultures of *E. coli* JM109 (DE3) bearing recombinant plasmids for identification of protein production. (A) ADI production (10% gel). Lane 1, *E. coli* JM109 (DE3) (pT7-7); lane 2, sample of *E. coli* JM109 (DE3) (pRM9). (B) OTC and CK production (12.5% gel). Lane 1, *E. coli* JM109 (DE3) (pT7-7), lane 2, *E. coli* JM109 (DE3) (pRM7), lane 3, *E. coli* JM109 (DE3) (pRM8). The arrows indicated the overproduced protein. The gel was stained with Coomassie blue. Molecular mass markers are indicated at the left (SDS-PAGE Standards, Bio-Rad).

(Bolotin et al. 2001); in contrast, regulatory genes have not been described in the *L. sakei arc* operon (Zúñiga et al., 1998). Moreover, an extra gene (*arcT*) has been found in *L. sakei* and in *L. lactis*. This gene may encode a transaminase of class I. In *L. sakei*, the *arc* genes followed the order *arcA*, *arcB*, *arcC*, *arcT* and *arcD*. The *arc* cluster in *L. lactis* showed another special feature, the presence of two copies of the *arcC* gene coding for different carbamate kinases. The gene order in *L. lactis* is $arcABDC_1C_2T$.

3.3. Enzymatic analysis of the arc genes

The *arcA*, *arcB* and *arcC* genes of *L. hilgardii* were expressed in *E. coli* following the strategy described in Section 2.3 consisting in amplifying the genes by PCR and cloning the products under the control of the T7 RNA polymerase-inducible ϕ 10 promoter. All the ligation mixtures were introduced by transformation into the expression strain *E. coli* JM109 (DE3). The correct sequence and insertion of *arcA*, *arcB* and *arcC* into recombinant plasmids pRM9, pRM7 and pRM8, respectively, were verified by restriction analysis and DNA sequencing.

Cell extracts were prepared from *E. coli* JM109 (DE3) cells harboring the recombinant plasmids as described in Section 2.4. The extracts were used to detect the presence of hyperproduced proteins. Control cells containing the pT7-7 vector plasmid alone did not show expression over the 3-h time course analyzed, whereas expression of additional 47, 38 and 33-kDa proteins were apparent with JM109 (DE3) cells harboring pRM9, pRM7 and pRM8, respectively (Fig. 2). These molecular masses are in good agreement with the M_r deduced from the nucleotide sequence of the *arcA, arcB* and *arcC* genes.

As reported above (see Section 3.2), sequence similarities had suggested that ArcA might be an ADI, ArcB an OTC and ArcC a CK. Supernatants of sonicated cell lysates prepared from *E. coli* JM109 (DE3) harboring pRM9, pRM7 and pRM8 as described were assayed for ADI, OTC and CK activities, respectively, by following modified protocols based on methods previously described. We tested enzymatic activities in cell extracts of *E. coli* JM109 (DE3) harboring pT7-7 and pRM9. The levels of activity were 1 and 5×10^3 U/mg protein, respectively. OTC activity was determined by measuring the formation of citrulline from ornithine and carbamyl phosphate. Under the experimental conditions used, the enzyme is able to perform this reaction, which is the reverse of the in vivo reaction. The amount of citrulline formed was determined as described in Section 2.4. Cell extracts from *E. coli* JM109 (DE3) harboring the recombinant plasmid pRM7 showed OTC activity (7×10^3 U/mg protein), whereas extracts prepared from control cells containing the vector plasmid alone did not. CK activity was measured by an indirect assay described by Ruepp and Soppa (1996). In cell extracts of *E. coli* JM109 (DE3) harboring pRM8, CK activity, which was absent in control cultures, could be measured. Thus, we could prove experimentally that the *arcC* gene encodes a CK.

These biochemical analyses of the enzyme activities demonstrated that *arcA*, *arcB* and *arcC* codes for a ADI, OTC and CK, respectively, involved in the arginine deiminase pathway and represents the first example of cloning and heterologous expression of a *L. hilgardii* gene.

3.4. Conclusions

We have sequenced a gene cluster of *L. hilgardii* encoding the enzymes involved in the ADI pathway and demonstrated that this cluster encodes functional enzymes of this pathway. From these findings and previous results (Arena et al., 1999a) we can conclude that *L. hilgardii* X_1B utilizes arginine and citrulline via the ADI system. It has been shown for several LAB of non-wine origin that they increased their acid tolerance and their viability during the stationary phase by degrading arginine (Arena et al., 1999b; Champomier et al., 1999; Manca de Nadra et al., 1982, 1986, 1988; Marquis et al., 1987; Stuart et al., 1999). However the citrulline excreted to the medium during arginine catabolism is a source of ethyl carbamate formation in wine (Liu et al., 1994). The characterization of the ADI pathway described in this work would be fundamental in order to achieve a better knowledge of the ethyl carbamate production by *L. hilgardii*. A further analysis of the regulation of this pathway and the influence of different wine constituents on the degradation of arginine will allow to minimize the formation of ethyl carbamate by LAB during vinification.

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