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Detection of *arc* Genes Related with the Ethyl Carbamate Precursors in Wine Lactic Acid Bacteria

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Trace amounts of the carcinogen ethyl carbamate can appear in wine by the reaction of ethanol with compounds such as citrulline and carbamyl phosphate, which are produced from arginine degradation by some wine lactic acid bacteria (LAB). In this work, the presence of *arc* genes for the arginine−deiminase pathway was studied in several strains of different species of LAB. Their ability to degrade arginine was also studied. To detect the presence of *arc* genes, degenerate primers were designed from the alignment of protein sequences in already sequenced LAB. The usefulness of these degenerate primers has been proven by sequencing some of the amplified PCR fragments and searching for homologies with published sequences of the same species and related ones. Correlation was found between the presence of genes and the ability to degrade arginine. Degrading strains included all heterofermentative lactobacilli, *Oenococcus oeni*, *Pediococcus pentosaceus*, and some strains of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*.

KEYWORDS: ***arc*** **genes; arginine deiminase; ethyl carbamate; lactic acid bacteria; wine**

INTRODUCTION

Two groups of undesirable substances for the health of the wine consumer may be released by lactic acid bacteria (LAB) during and after winemaking: biogenic amines and ethyl carbamate precursors (*1*). Wine, like most fermented foods and beverages (*2*), contains trace amounts of ethyl carbamate (EC) (*3*). EC, also referred to as urethane, is a genotoxic compound in vitro and in vivo; it binds covalently to DNA and is an animal carcinogen (*4*, *5*). EC is formed by reaction between ethanol and *N*-carbamyl compounds, such as urea, citrulline, and carbamyl phosphate, at acid pH levels, and its formation is dependent on reactant concentration (*6*, *7*). This reaction is favored by increasing temperature and acid pH (*8*). The content of EC is therefore higher in wines that have been stored for a long time and where temperature has not been well controlled (*9*).

Even though the urea produced by yeast is the main potential EC precursor in wine, LAB can contribute to the formation of EC due to the production of citrulline and carbamyl-P from arginine (*10*−*12*), and significant levels of EC found in some wines have been correlated with the former presence of LAB (*9*). Some wine LAB are known to degrade L-arginine, which is one of the main amino acids found in grapes and wine (*13*). It has been demonstrated that arginine catabolism by these LAB involves the arginine deiminase (ADI) pathway (*14*−*16*). This pathway is the most widespread anaerobic route for arginine degradation (*17*, *18*), and its presence has been reported in bacteria, Archaea, and some anaerobic eukaryotes (*19*).

This pathway 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:

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

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

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

This pathway contributes positively to growth and viability of LAB due to ATP formation and the acidity decrease caused by ammonium production (*20*). However, the degradation of arginine yields citrulline and carbamyl phosphate, which can react with ethanol to form EC.

Moreover, the ADI pathway is sometimes indirectly related with biogenic amine production, specifically putrescine, because this amine can be produced from ornithine by LAB such as *Oenococcus oeni* (*21*).

Wine lactobacilli vary in their ability to degrade arginine. All heterofermentative lactobacilli are found to be degradative (*22*). Facultative heterofermentative lactobacilli from wine were believed not to degrade arginine (*14*, *23*), but recently some *Lactobacillus plantarum* have shown to be arginine-consuming

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by the ADI pathway (*24*). Likewise, no homofermentative pediococci isolated from wine seem to degrade arginine, but some of the isolated ones from other fermented food (cheese, sausages) can do it (*22*).

As reported for other bacteria (*25*−*27*), genes encoding the three enzymes of this pathway in LAB are clustered in an operon-like structure: *arcA* (ADI), *arcB* (OTC), and *arcC* (CK). These genes have been characterized in different LAB such as *Lactobacillus sakei* (*28*), *Lactobacillus hilgardii* (*29*), *Lb. plantarum* (*24*), and *O. oeni* (*30*).

Related to this pathway, other proteins, such as transcriptional regulators (ArcR, ArgR/AhrC) and arginine/ornithine transporters (ArcD1, ArcD2), have been characterized for some LAB species (*31*−*34*). Genes coding for other enzymes not related to the ADI pathway have also been described as part of an *arc* operon, such as *arcT* (a putative transaminase) and *argS* (arginyl-tRNA synthetase), both in *Lactococcus lactis* (*33*, *35*), and *argS2* (a putative arginyl-tRNA synthetase) in *O. oeni* (*36*).

Due to the relevance of *O. oeni* in winemaking, there are several molecular studies of their ADI pathway genes (*30*, *34*, *39*). Although *O. oeni* is the main species responsible for malolactic fermentation (MLF) (*37*, *38*), other LAB may proliferate during the early stages of MLF (*1*) or later if the conditions are propitious, such as low acidity (*1*). Nevertheless, little information is available about the potential of these LAB species to degrade arginine under wine conditions. The presence of the *arc* genes has been analyzed only in *Lb. hilgardii* and *Lb. plantarum*. However, there are no studies regarding the other wine LAB species.

In this work, we studied the presence of *arc* genes in LAB frequently found in wine. We analyzed a wide range of different strains to establish the relationship between their ability to degrade arginine and the presence of the ADI pathway genes.

MATERIALS AND METHODS

Microorganisms and Growth Conditions. We used 39 strains of different species of *Lactobacillus*, *Oenococcus*, *Leuconostoc*, and *Pediococcus*, most of which were isolated from wine (**Table 1**). These strains were grown anaerobically at 27 °C in MRS (*40*) supplemented with 4 g/L DL-malic acid and 5 g/L D-(−)-fructose.

Arginine Degradation. Cells grown as above detailed were inoculated (1% v/v) in 5 mL of a modified MRS medium adjusted at pH 4.5, following the method of Tonon et al. (*29*), supplemented with 5 g/L of arginine, and incubated anaerobically at 27 °C. Samples were taken at the end of the logarithmic phase. Arginine in supernatants was quantified according to the colorimetric method of Sakaguchi (*41*). To confirm the production of citrulline by some strains, it was quantified with HPLC, following the method of Gómez-Alonso et al. (*42*).

Genomic DNA Extraction. DNA was prepared by extraction with chloroform and previous incubation with lysozyme (2.5 mg/mL) in TE buffer, following the method of Persing et al. (*43*) with some modifications, as published by Reguant and Bordons (*44*).

Detection of arc Genes in Different Species of LAB. Degenerate primers were designed (**Table 2**) from multiple alignment of conserved regions of protein sequences of the three enzymes of the ADI pathway (ADI, OTC, and CK). These sequences are known in other already sequenced LAB: *Lactococcus lactis* ssp. *lactis* ML3 (GenBank data library accession number AF282249), *O. oeni* ATCC23279^T (AF124851), *Lb. sakei* BL13 (AJ1330), and *Lb. hilgardii* X1B (AJ421514). General recommendations on primer design were followed (*45*, *46*) and parameters (%GC, T_m, and specificity) were optimized using Primer Express v1.5 from Applied Biosystems (Foster City, CA) and Amplify 1.2 (*47*). Likewise, two specific primers were used to amplify the region downstream of *arcA* in *Leuc. mesenteroides* strains: LM-F, 5′-GAAGGTTATACGGTCATCCCTATC-3′; and LM-R, 5′-GCAACCGTCCACGACGATCA-3′.

Amplification was performed using the described primers synthesized by Ecogen (Barcelona, Spain). The PCR volume was 20 μL and contained 0.02 μg of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 unit of Taq DNA polymerase, and 2 μM of each primer in 10× PCR buffer. The amplification profile was 94 °C for 2 min, 55 °C for 2 min, and 72 °C for 2 min, and this was repeated for 30 cycles. Also included were a preincubation step of 94 °C for 2 min and a final extension of 72 °C for 10 min. The amplification products were resolved by electrophoresis in 1% (w/v) agarose TBE gels.

Sequencing of Amplified DNA Fragments. Amplicons were purified from the gel bands in minicolumns Wizard SV Gel and PCR Clean-Up System (Promega Biotech Ibérica SL, Barcelona, Spain) and sequenced by Biopremier Inc. (Lisbon, Portugal). The homology of their sequences was compared with all published sequences using the BLAST program at NCBI and the ClustalW software from the European Bioinformatics Institute (http://www.ebi.ac.uk/).

Nucleotide Accession Numbers. The nucleotide sequenced of these amplified fragments were deposited in the GenBank database under the accession numbers shown in **Table 4**.

RESULTS

Arginine Degradation by Wine LAB Strains. As can be seen in **Table 1**, most of the tested strains of heterofermentative lactobacilli (*Lb. hilgardii*, *Lb. brevis*, and *Lb. buchneri*) degraded over 50% of 5 g/L arginine. Most of them degraded it quickly, in <2 days, before ending the log phase. Regarding the facultative heterofermentative *Lb. plantarum*, only the strain isolated from wine showed the ability to degrade arginine. All of the tested *O. oeni* strains resulted in arginine degrading, but a great variability in arginine consumption rate was found, depending on the strain. This variability was not related with different growth of strains, because samples were taken at the end of logarithmic phase, with values of OD around 0.9−1.4 for all strains, and arginine consumption was not higher in the strains with higher growth, nor vice versa. With regard to the *Pediococcus* genus, none of the tested strains of *P. parvulus* degraded arginine, whereas all *P. pentosaceus* representatives did so. In the case of *Leuc. mesenteroides*, two strains isolated from wine were found to degrade arginine. Nevertheless, another two strains of this species, one isolated from wine and the other from olives, did not. The contents of citrulline found in the supernatants of the arginine-degrading strains of *Leuc. mesenteroides* were significantly higher than in assays with all other species (**Table 3**). The molar ratio of citrulline produced versus arginine consumed was around 30%, whereas for other arginine-degrading species, such as *Lb. hilgardii*, it was <10%.

Detection of arc Genes. The degenerate primers, designed in this work, were used to detect the genes *arcA*, *arcB*, and *arcC* in 39 strains of 8 different species of LAB (**Table 1**). The three genes were detected in all of the tested strains of *Lb. hilgardii*, *Lb. brevis*, *Lb. buchneri*, *O. oeni*, and *P. pentosaceus*. The corresponding amplification products were not obtained with *P. parvulus*. In all of these cases, the presence or absence of the *arcA* gene agreed with their ability to degrade arginine. Results of the electrophoretic gels, showing the amplified bands of one strain from each of these species, are shown in **Figure 1**.

To verify the identity of the amplicons obtained from the species *Lb. brevis*, *Lb. buchneri*, and *P. pentosaceus* (not used in the primer design), the purified fragments of a representative strain of each were sequenced and compared to published sequences. **Table 4** shows the highest homologies found between the sequences obtained and published sequences. The three fragments of *arcA*, *arcB*, and *arcC* obtained from *Lb. brevis* 4246 presented homologies of >90% with the corresponding sequences published for strain 367 from the same

Table 1. Arginine Degradation and Presence of *arc* Genes in Strains of Different Lactic Acid Bacteria

strain	source[a]	origin	degradation of arginine[b] (%)	presence of *arc* genes		
				arcA	*arcB*	*arcC*
			Lactobacillus hilgardii			
4786^T	CECT	wine	88	+	+	+
4681	CECT	wine	88	+	+	+
4682	CECT	wine	88	+	+	+
			Lactobacillus brevis			
4121^T	CECT	fecal	78	+	+	+
216	CECT	beer	97	+	+	+
3824	UV	wine	99	+	+	+
4246	UV	wine	98	+	+	+
4300	UV	wine	98	+	+	+
C12	CM	wine	92	+	+	+
JM11	CM	wine	93	+	+	+
J12	CM	wine	33	+	+	+
			Lactobacillus buchneri			
4111^T	CECT	tomato	99	+	+	+
4674	CECT	beer	61	+	+	+
			Lactobacillus plantarum			
CB21	CM	wine	93	+	+	+
220	CECT	silage	0	−	+	−
			Oenococcus oeni			
217^T	CECT	wine	89	+	+	+
4727	CECT	wine	99	+	+	+
4733	CECT	wine	45	+	+	+
4772	CECT	wine	13	+	+	+
Mf15	URV	wine	23	+	+	+
E2201	URV	wine	83	+	+	+
O43	CM	wine	90	+	+	+
F41	CM	wine	97	+	+	+
			Pediococcus pentosaceus			
923	CECT	milk	88	+	+	+
4695^T	CECT	beer	82	+	+	+
4208	UV	wine	100	+	+	+
4214	UV	wine	100	+	+	+
E2137	URV	wine	93	+	+	+
E2203	URV	wine	87	+	+	+
E1804	URV	wine	94	+	+	+
E1203	URV	wine	82	+	+	+
			Pediococcus parvulus			
813^T	CECT	silage	0	−	−	−
3908	UV	wine	0	−	−	−
3909	UV	wine	0	−	−	−
3911	UV	wine	0	−	−	−
			Leuconostoc mesenteroides			
219^T	CECT	olives	0	−	+	−
BIFI-54	IFI	wine	0	−	+	−
V41	CM	wine	99	+	−	−
L17	CM	wine	99	+	−	−

[a] CECT, Colección Española de Cultivos Tipo, Valencia, Spain; UV, S. Ferrer, Enolab, Fac. Biologia, Universitat de València, Spain; CM, M. C. Masqué, Incavi, Reus, Spain; URV, authors' collection, Rovira i Virgili University, Tarragona, Spain; IFI, R. Muñoz, Instituto de Fermentaciones Industriales, CSIC, Madrid. [b] Degradation of 5 g/L (29 mmol/L) arginine in MRS medium at pH 4.5. Values are the averages of two cultures for each strain.

Table 2. Degenerate Primers Used for the Detection of *arc* Genes in Strains of Different Lactic Acid Bacteria.

primer name	sequence (5′ to 3′)[a]	fragment amplified (bp)
ADI deg-F1	CAYGCNATGATGCAYYTNGAYACNGT(26)	266
ADI deg-R1	GTRTTNSWNCCRTCRTTCCAYTGYTC(26)	
OTC deg-F3	ATGCAYTGYYTNCCNGCNTTYCAYGA(26)	181
OTC deg-R3	CCNARNGTNGCNGCCATDATNGCYTT(26)	
CK deg-F1	CAYGGNAAYGGNCCNCARGTNGGNAA(26)	343
CK deg-R1	CKNCKNYANCCNCKNCCNGCRTCYTC(26)	

[a] Nomenclature for incompletely specified bases in nucleic acid sequences, following the recommendations of the Nomeclature Committee of the International Union of Biochemistry (http://www.chem.qmul.ac.uk/iubmb/misc/naseq.html): Y: T or C; N: A, T, C or G; R: A or G; S: G or C; W: A or T; D: G, A or T; K: G or T.

Table 3. Molar Ratio of Citrulline Produced versus Arginine Consumed by Some Strains of *Leuconostoc mesenteroides* and a Reference Arginine-Degrading Strain of *Lactobacillus hilgardii*

strain	arginine consumed[a] (mM)	citrulline produced[a] (mM)	citrulline produced/ arginine consumed (%)
Leuc. mesenteroides 219^T	0	0	
Leuc. mesenteroides V41	1.02	0.29	28.4
Leuc. mesenteroides L17	0.91	0.30	33.0
Lb. hilgardii 4786^T	0.99	0.09	9.1

[a] These values are the differences from initial and 24 h after inoculation of MRS medium at pH 4.5, with initial 2.9 mM arginine. Values are the averages of two cultures for each strain.

species. With regard to *Lb. buchneri* 4674, for the moment, there are no available sequences of the ADI pathway genes for this species other than those reported in this work. The greatest homologies for this strain were found with *Lb. brevis* in the case of *arcA* and with *L. hilgardii* in the case of *arcB* and *arcC*.

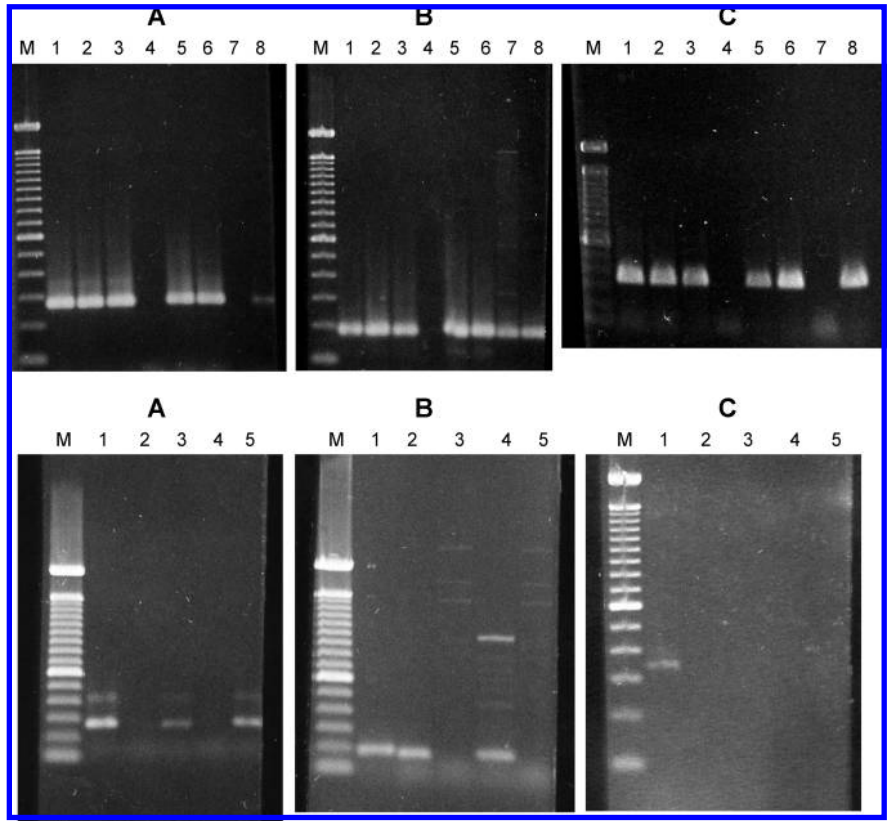

Figure 1. PCR-amplified fragments of *arcA* (**A**), *arcB* (**B**), and *arcC* (**C**) genes (266, 181, and 343 bp, respectively) of different lactic acid bacteria obtained with degenerate primers. (Top) Lanes 1−8 correspond respectively to *Lb. hilgardii* 4786^T, *O. oeni* 217^T, *Lb. brevis* 4246, *P. parvulus* 813^T, *Lb. buchneri* 4111^T, *P. pentosaceus* 4695^T, *Lb. plantarum* 220, and *Lb. plantarum* CB21. (Bottom) Lanes 1−5 correspond respectively to *O. oeni* 217^T, *Lc. mesenteroides* 219^T, *Lc. mesenteroides* V41, *Lc. mesenteroides* BIFI-54, and *Lc. mesenteroides* L17. Lane M is a ladder DNA of 250 bp of Invitrogen (Barcelona, Spain).

With regard to the fragments of *P. pentosaceus* 4214, homologies of >94% were found with the corresponding published sequences for strain 25745 from the same species.

With regard to *Lb. plantarum*, the arginine-degrading strain CB21 presented the bands corresponding to the three genes *arc* (**Figure 1**). The identity of these fragments was confirmed by sequencing, showing high homology with the homologous *arc* genes of *Lb. hilgardii* (**Table 4**). Nevertheless, the non-arginine-degrading strain 220 presented exclusively the band of the OTC gene, lacking *arcA* and *arcC*. The fragment amplified using the *arcB* primers was sequenced to confirm its identity and showed a 94% homology with *Lb. plantarum argF* (**Table 4**), coding for the anabolic counterpart of OTC.

Likewise, the two non-arginine-degrading *Leuc. mesenteroides* strains presented only the *arcB* band (**Figure 1**). In these cases, such as strain 219, the amplification of ornithine transcarbamoyltransferase gene was confirmed by DNA sequencing of the obtained amplicon, presenting a 99% homology with the published sequence (**Table 4**). Surprisingly, the arginine-degrading strains of *Leuc. mesenteroides* presented the *arcA* gene but not the other two (**Figure 1**). The absence of an *arcABC* organization in this strain was confirmed by sequencing the downstream region of *arcA*. In this case, the amplification showed that both V41 and L17 strains had a *ribD* gene downstream of *arcA* (GenBank FJ426345, FJ426343, FJ426344).

DISCUSSION

For the detection of *arcA*, *arcB*, and *arcC* genes in any wine LAB, three pairs of degenerate primers were designed. The available sequences of three LAB species (*O. oeni*, *Lb. sakei*, and *Lactococcus lactis*) were used for the design of the primers. The amplified fragments had the expected sizes, and the designed degenerate primers showed to be useful in detecting *arc* genes in all of these species. The three *arc* genes (*A*, *B*, and *C*) were found in the tested strains of heterofermentative lactobacilli and *Oenococcus*, and also in all tested strains of *P. pentosaceus*. All of these strains were able to degrade arginine.

This work reports the presence of *arc* genes in *Pediococcus* strains isolated from wine. All tested strains of *P. pentosaceus*, including six strains from wine, were able to degrade arginine, and the three *arc* genes were detected in all them. Some *Pediococcus* from other fermented foods were found to degrade arginine (*22*), but those isolated from the wine environment until now did not seem to do so. On the other hand, and according to the previous description of *P. parvulus* (*48*), we did not find the *arc* genes in any of the tested strains of this species, which included three strains from wine.

Until recently, arginine-degrading strains of *Lb. plantarum* had been isolated only from sources other than wine, such as fish (*49*) and orange juice (*12*), but Spano et al. (*24*) demonstrated the presence of *arc* genes in some wine strains of this species, which are able to degrade arginine. Our results agree with that, because strain CB21 of *Lb. plantarum* isolated from wine analyzed in this work degrades arginine and it has the three *arc* genes. On the other hand, we found that strain CECT220 (= ATCC 8014) of *Lb. plantarum* is non-arginine-consuming. This well-studied strain was originally isolated from corn silage, and it is almost more known than the type strain (ATCC 14917) isolated from pickled cabbage. It has also been shown that this type strain is nondegrading and that it lacks the

Table 4. Highest Homologies Found between Gene Fragments Sequenced in This Work and Published Sequences

sequences obtained in this work[a]		highest homology found[b]
Lb. brevis 4246	*arcA* (EF54853)	95% *Lb. brevis* ATCC 367 *arcA* (LVIS_2027)
	arcB (EF54854)	91% *Lb. brevis* ATCC 367 *arcB* (LVIS_2026)
	arcC (EF54855)	96% *Lb. brevis* ATCC 367 *arcC* (LVIS_2023)
Lb. buchneri 4674	*arcA* (EF54856)	79% *Lb. brevis* ATCC 367 *arcA* (LVIS_2027)
	arcB (EF54857)	81% *Lb. hilgardii* X1B *arcB* (AJ421514)
	arcC (EF54858)	69% *Lb. hilgardii* X1B *arcC* (AJ421514)
P. pentosaceus 4214	*arcA* (EF54850)	94% *P. pentosaceus* ATCC 25745 *arcA* (PEPE_1629)
	arcB (EF54851)	94% *P. pentosaceus* ATCC 25745 *arcB* (PEPE_1631)
	arcC (EF54852)	99% *P. pentosaceus* ATCC 25745 *arcC* (PEPE_1630)
Lb. plantarum CB21	*arcA* (FJ426339)	98% *Lb. hilgardii* X1B *arcA* (AJ421514)
	arcB (FJ426340)	95% *Lb. hilgardii* X1B *arcB* (AJ421514)
	arcC (FJ426341)	72% *Lb. hilgardii* X1B *arcB* (AJ421514)
Lb. plantarum 220	*argF* (FJ426338)	94% *Lb. plantarum argF* (X99978.2)
Leuc. mesenteroides 219	*arcB* (FJ426342)	99% *Leuc. mesenteroides* subsp. *mesenteroides* ATCC 8293 *arcB* (LEUM_1457)
Leuc. mesenteroides V41	*arcA* (FJ426345)	100% *Leuc. mesenteroides* subsp. *mesenteroides* ATCC 8293 *arcA* (LEUM_0821)
	ribD (FJ426345)	97% *Leuc. mesenteroides* subsp. *mesenteroides* ATCC 8293 *ribD* (LEUM_0822)
Leuc. mesenteroides L17	*arcA* (FJ426343)	88% *Leuc. mesenteroides* subsp. *mesenteroides* ATCC 8293 *arcA* (LEUM_0821)
	ribD (FJ426344)	94% *Leuc. mesenteroides* subsp. *mesenteroides* ATCC 8293 *ribD* (LEUM_0822)

[a] Nucleotide accession numbers of the sequenced fragments, deposited in the GenBank database, are shown in parentheses. [b] Highest homology found of the sequence of interest against all published sequences. The locus tag or accession number of the NCBI GenBank database of these genes is shown in parentheses.

arc genes (*24*). The absence of arginine consumption in other strains of *Lb. plantarum* from wine has been shown previously (*14*,*50*). All of this suggests that arginine degradation would be strain-characteristic for some wine isolates of this species. On the other hand, we obtained an amplicon when using *arcB* primers in this nondegrading strain 220. According to sequencing results, this DNA fragment belongs to *argF*, coding for the anabolic OTC activity. In the draft sequence genome of *Lb. plantarum* strain WCFS1 available at the GenBank database (AL935263), there is no evidence of an *arc* operon, but an *argF* gene (NP_784310) appears for OTC function near other genes related to arginine metabolism, such as *argD*, coding for an acetylornithine aminotransferase. This is in agreement with the presence of the biosynthetic OTC gene reported in this study in non-arginine-degrading strains.

In relation to *Leuc. mesenteroides*, it was found that some strains of this species are able to degrade arginine. Apparently, all leuconostoc have been always described as non-arginine-degrading (*48*) and the only exception was *O. oeni* (then still considered as *Lc. oenos*) because several strains of this wine LAB had been found to degrade arginine, as reviewed by Liu and Pilone (*22*). The detection of arginine-degrading strains of *Leuc. mesenteroides* reinforces the idea stated by these authors of considering the genus *Leuconostoc* as arginine-variable.

Surprisingly, these arginine-degrading strains (V41 and L17) of *Leuc. mesenteroides* isolated from wine presented the *arcA* gene but not the other two. The sequencing of the region downstream of *arcA* showed the presence of a *ribD* gene, coding for a 5-amino-6-(5-phosphoribosylamino) uracil reductase. The same gene organization has been observed in the complete genome sequence available of *Leuc. mesenteroides* (CP000414). Therefore, these strains have the ability to degrade arginine with ADI but not the complete operon. In consequence, they are not able to catabolyze the citrulline produced and it can be excreted. As confirmation, a molar ratio of 30% citrulline/arginine was found in the supernatant of these strains, significantly higher than that for other arginine-degrading species (<10%).

On the other hand, the two non-arginine-degrading strains of *Leuc. mesenteroides* (219 and BIFI-54) presented only the band of *arcB*, but not *arcA* or *arcC*. In relation to this, in the sequence genome (CP000414) of *Leuc. mesenteroides* ATCC 8293 there is no evidence of an *arc* operon, and there are reported putative genes only for ADI (YP_818302.1) and OTC (YP_818923) distantly located in the genome. Altogether, it seems that the presence of *arc* genes in *Leuc. mesenteroides* is variable. Arginine-degrading strains may have only part of the ADI pathway, whereas nondegrading strains may have lost *arcA*, or part of this gene, or the complete pathway, remaining unable to catabolyze arginine.

With regard to *O. oeni*, the species most used as starter of MLF in wine, the *arc* genes have been found in all tested strains, although there was found variability of degradation, including some strains with very little degradation.

The purified fragments sequenced and compared to published sequences confirm their identity (**Table 4**). These sequences are available in the GenBank database and might be useful for further studies on these LAB species.

This work shows the utility of the detection of the *arc* genes using degenerate primers in a representative group of wine LAB. A good correlation between the presence or absence of the *arc* genes, and mainly with the *arcA* gene, with the degradation or lack of degradation of arginine has been found in the different tested wine-related LAB strains. The sole presence of the ADI gene in some arginine-degrading strains of *Leuc. mesenteroides* requires special consideration because these strains would be able to accumulate higher amounts of citrulline, increasing the potential risk of ethyl carbamate formation. Therefore, to detect strains like these it is necessary to analyze the presence of *arcB*

and *arcC*, meaning that it is worth testing all three genes, not just *arcA*. Finding these strains is clearly an advantage of this genetic methodology over the test of arginine degradation.

These results suggest the usefulness of the detection of the *arc* genes as a tool for screening strains of different wine LAB species for their ability to generate or not ethyl carbamate precursors. Nevertheless, the variability of arginine degradation found in strains of *O. oeni* despite having the *arc* genes, suggests that further studies related with the expression of these genes are needed. In any case, the results of our study contribute to the knowledge of the influence of the different LAB present in wine on the formation of ethyl carbamate precursors, and the methodology developed and used in this work might be also useful for the study of other LAB species relevant for food processing.

LITERATURE CITED

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