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Arginine deiminase pathway genes and arginine degradation variability in *Oenococcus oeni* strains

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Abstract Trace amounts of the carcinogenic ethyl carbamate can appear in wine as a result of a reaction between ethanol and citrulline, which is produced from arginine degradation by some bacteria used in winemaking. In this study, arginine deiminase (ADI) pathway genes were evaluated in 44 *Oenococcus oeni* strains from wines originating from several locations in order to establish the relationship between the ability of a strain to degrade arginine and the presence of related genes. To detect the presence of *arc* genes of the ADI pathway in *O. oeni*, pairs of primers were designed to amplify *arcA*, *arcB*, *arcC* and *arcD1* sequences. All strains contained these four genes. The same primers were used to confirm the organization of these genes in an *arcABCD1* operon. Nevertheless, considerable variability in the ability to degrade arginine among these *O. oeni* strains was observed. Therefore, despite the presence of the *arc* genes in all strains, the expression patterns of individual genes must be strain dependent and influenced by the different wine conditions. Additionally, the presence of *arc* genes was also determined in the 57 sequenced strains of *O. oeni* available in GenBank, and the complete operon was found in 83 % of strains derived from wine. The other strains were found to lack the *arcB*, *arcC* and *arcD* genes, but all contained sequences homologous to *arcA*, and some of them had also ADI activity.

Introduction

Oenococcus oeni is a species of lactic acid bacteria (LAB) that is mainly involved in malolactic fermentation (MLF), which usually occurs after alcoholic fermentation (AF) during the winemaking process (Wibowo et al. 1985). MLF involves the decarboxylation of L-malic acid into L-lactic acid and CO_2, which decreases the acidity of the wine and produces a softer taste. *O. oeni* also produces other interesting compounds, such as diacetyl, which can improve the aromatic quality of wine (Henick-Kling 1993; Lonvaud-Funel 1999), and the microbiological stability of wine that has undergone MLF is also improved (Davis et al. 1985; Wibowo et al. 1985).

However, two groups of undesirable substances that are potentially harmful to human health may be released by LAB during and after winemaking: biogenic amines and precursors of ethyl carbamate (or urethane) (Lonvaud-Funel 1999). Wine, like most fermented foods and beverages, can contain trace amounts of ethyl carbamate (EC) (Ough 1976), which is an animal carcinogen (Schlatter and Lutz 1990). The recommended maximum level of EC in table wines is 15 μg/L, and 30 μg/L is the legal limit in some countries, such as Canada. EC is formed by a spontaneous chemical reaction between ethanol and carbamyl group containing compounds such as urea or citrulline (Ough et al. 1988). The urea produced by yeast arginine metabolism is the main precursor in EC production, but there is also evidence that EC levels can increase after MLF (Uthurry et al. 2004; Uthurry et al. 2006). EC-forming reactions are favoured by high temperature and an acidic pH (Stevens and Ough 1993).

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Several studies have examined the role of wine LAB in EC formation (Liu et al. 1994; Liu and Pilone 1998; Arena et al. 1999). Many wine LAB strains are known to degrade L-arginine (Mira de Orduña et al. 2000), which is one of the main amino acids found in grapes and wine (Lehtonen 1996). It has been demonstrated that arginine catabolism by these LAB involves the arginine deiminase (ADI) pathway (Liu et al. 1995; Liu et al. 1996). This pathway is the most widespread anaerobic route for arginine degradation (Cunin et al. 1986), and its presence has been reported in bacteria, archaea, and some anaerobic eukaryotes (Zúñiga et al. 2002).

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)—that catalyse the following reactions:

$$\begin{aligned} &\text{L–arginine} + H_2O \xrightarrow{\text{ADI}} \text{L–citrulline} + NH_3 \\ &\text{L–citrulline} + \text{Pi} \xleftrightarrow{\text{OT}} \text{L–ornithine} + \text{carbamyl–P} \\ &\text{carbamyl–P} + \text{ADP} \xleftrightarrow{\text{CT}} \text{ATP} + CO_2 + NH_3 \end{aligned}$$

Theoretically, this pathway plays a positive role in the growth and viability of LAB because ATP is formed, and the production of ammonia decreases the acidity of the wine (Tonon and Lonvaud-Funel 2000), similar to LAB found in other acidic foods, as the apple surface (Savino et al. 2012). However, this reaction may differ among the different wine LAB strains, and it has been shown that arginine and citrulline do not stimulate the growth of some *O. oeni* strains (Terrade and Mira de Orduña 2009). However, if LAB degrades arginine by the ADI pathway, citrulline can be produced (Mira de Orduña et al. 2000), which can react spontaneously with ethanol to form EC.

It has been shown that *O. oeni* strains can catabolize arginine. Mira de Orduña et al. (2000) tested 23 *O. oeni* strains and found that all strains degraded arginine and excreted citrulline. Pilone et al. (1991) observed that 18 of 24 strains tested produced ammonia from arginine. Granchi et al. (1998) found 30 arginine-consuming strains among 32 that had been isolated from the Chianti region. Liu et al. (1995) found considerable variability among the arginine degradation pathways of 22 *O. oeni* strains (commercially available strains and those isolated in New Zealand), including five non-degradative strains. Finally, Tonon et al. (2001a) also found 13 different arginine-consuming strains among 17 in the Bordeaux Oenology collection.

Arginine degradation is influenced by different wine conditions. Malic acid inhibits this process because MLF, which consumes malic acid, seems to take priority over arginine consumption for LAB (Mira de Orduña et al. 2001; Terrade and Mira de Orduña 2006; Araque et al. 2011). The presence of ethanol has been shown to increase arginine utilization, and the expression of *arc* genes in strains of *Lactobacillus brevis* and *Pediococcus pentosaceus* may occur as a result of a stress response mechanism (Araque et al. 2013).

As with other bacteria (Maghnouj et al. 1998; Griswold et al. 2004), genes encoding the three enzymes in this pathway in LAB are clustered in an operon structure: *arcA* (ADI), *arcB* (OTC) and *arcC* (CK). These genes have been characterized in LAB species such as *Lactobacillus sakei* (Zúñiga et al. 1998), *Lactobacillus hilgardii* (Arena et al. 2002), *Lactobacillus plantarum* (Spano et al. 2004; Spano et al. 2006), and *Lactobacillus fermentum* (Vrancken et al. 2009). They have also been characterized in *O. oeni* (Tonon et al. 2001b).

Other protein-coding genes, such as transcriptional regulators (*arcR*, *ArgR/AhrC*) and arginine/ornithine transporters (*arcD1*, *arcD2*), have been described in some LAB *arc* operons (Kok et al. 2005; Vrancken et al. 2009), including that of *O. oeni* (Divol et al. 2003). Genes for other proteins that are not related to the ADI pathway have also been described in *arc* operons. For example, *arcT* for a putative transaminase and *argS* for arginyl-tRNA synthetase have both been described in *Lactococcus lactis* (Bolotin et al. 2001; Kok et al. 2005). An *argS2* for a putative arginyl-tRNA synthetase has also been described at the 3′ end of the *arc* operon in *O. oeni* (Nehmé et al. 2006).

Although the *arc* genes have been characterized, few studies have analysed whether they are present in *O. oeni* strains (Tonon et al. 2001b; Divol et al. 2003), and no representative screening of *O. oeni* strains from different sources has been reported. The *arc* genes have been detected in several strains of different wine LAB species, including eight *O. oeni*, by means of degenerate primers (Araque et al. 2009). In this work, the *arc* genes were detected in arginine-degrading strains of all assayed heterofermentative lactobacilli, *O. oeni* and *P. pentosaceus* and in some *Leuconostoc mesenteroides* and *Lb. plantarum* strains.

Nevertheless, in the study of Araque et al. (2009), *arc* gene presence was evaluated in a few *O. oeni* strains. For this reason, and given the importance of this species in vinification, in the current study *arc* genes in several *O. oeni* strains have been detected by PCR amplification using species-specific primers. A wide range of strains from different sources has been analysed in order to establish a relationship between the strain's ability to degrade arginine and the presence of *arc* genes.

Additionally, in order to corroborate the results and to study the presence of *arc* genes more globally in *O. oeni*, we have searched for these genes in silico in the sequenced strains available in GenBank, comparing them using BLAST with the published *arc* genes sequences from the type strain of *O. oeni* (Tonon et al. 2001b; Divol et al. 2003).

Materials and methods

Microorganisms and growth conditions

A total of 44 strains of *O. oeni* of different origins (Table 1) were used, including the type culture, 10 strains from CECT (the official Spanish culture collection), 15 strains from around the world (USA, South Africa, and mostly Italy), eight strains from different Catalan cellars, five commercial strains and five strains isolated from the university's experimental cellar in Tarragona, Catalonia, Spain. All strains were grown anaerobically at 27 °C in MRS (De Man et al. 1960) at pH 5.0, supplemented with 4 g/L D,L-malic acid and 5 g/L D(−)-fructose.

Arginine degradation

Cells grown in MRS with malic acid and fructose (see above) were inoculated (1 % *v/v*) in 5 mL of a modified MRS (Tonon et al. 2001b) at pH 4.5, supplemented with 5 g/L of arginine and 5 g/L of glucose, and incubated anaerobically at 27 °C. Samples were taken at the end of the logarithmic phase, and all assays were performed in duplicate. Arginine and citrulline supernatant concentrations were quantified by HPLC, according to the method reported by Gómez-Alonso et al. (2007).

Determination of arginine deiminase activity

Cells grown as above were harvested by centrifugation at 10,000×*g* for 15 min, and the pellet was washed twice with 0.2 mol/L sodium phosphate buffer, pH 6.5 and then resuspended at 2.5 % (*w/v*) in the same buffer. Then, cellular extracts were obtained using a cell disrupter (BASIC Z; Constant Systems Ltd., Daventry, UK) at a pressure of 2.5 kbar. Unbroken cells and cellular debris were removed by centrifugation at 13,000×*g* for 6 min, and the supernatant extract was used. All operations were carried out at 4 °C. Arginine deiminase (ADI) activity of extracts was determined following Spano et al. (2007) by analysing citrulline produced from arginine.

Genomic DNA extraction

DNA was prepared by extraction with chloroform and previous incubation with lysozyme (2.5 mg/mL) with TE buffer, according to the method reported by Persing et al. (1993) with some modifications (see Reguant and Bordons 2003).

Detection of *arc* genes in *O. oeni*

Starting from the published sequences of the *arcABC* cluster in *O. oeni* ATCC 23279^T (Tonon et al. 2001b) [GenBank accession number AF124851, National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov)] and *arcD* genes (Divol et al. 2003) [GenBank accession number AF541253], a pair of primers was designed for each of the following genes: *arcA*, *arcB*, *arcC* and *arcD1*. General recommendations on primer design were followed (Ausubel et al. 1989; Iowa State University 2005). Fragments were verified using the Amplify 1.2 software (Engels 1993) to avoid secondary structure formation. The Basic Local Alignment Search Tool (BLAST) program (Altschul et al. 1990) at NCBI was also used to check that the primers did not hybridize with other fragments from the same microorganism.

Finally, PCR conditions were optimized for four pairs of primers synthesized by Ecogen (Barcelona). This approach yielded amplified fragments of different sizes that were easily discriminated by electrophoresis (Table 2).

Amplification was performed in a GeneAmp PCR System thermocycler (Perkin–Elmer, Applied Biosystems, Foster City, CA, USA). The PCR volume was 20 μL and contained 20 ng of genomic DNA, 2.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 0.5 U Taq DNA polymerase and 0.2 μmol/L of each primer in 1× PCR buffer. The amplification programme was 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, which 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 (Tris–HCl 89 mmol/L, boric acid 89 mmol/L, Na_2-EDTA 2 mmol/L, pH 8.0) gels stained with ethidium bromide and photographed. A 100-bp ladder (Invitrogen SA, Barcelona) was included as a DNA molecular size marker.

Genomic organization of the *O. oeni arc* operon

To confirm the organization of the *arc* operon containing the four genes *arcA*, *arcB*, *arcC* and *arcD1*, corresponding pairs of primers were combined to obtain six PCR-amplified fragments (Fig. 1). DNA was obtained from *O. oeni* 4772. The procedure was the same as above but the amplification products were resolved in 0.8 % (*w/v*) agarose TBE gels, and a 250-bp ladder (Invitrogen S.A.) was included as a DNA molecular size marker.

The presence of *arc* genes in the sequenced strains of *O. oeni*

The abovementioned published sequences of *arc* genes from *O. oeni* ATCC 23279^T (=DSM 20252) (Tonon et al. 2001b; Divol et al. 2003) were compared using the BLASTN 2.2.30+ method to somewhat similar sequences (Altschul et al. 1997) deposited to the NCBI website as of January 2015 from the 57 sequenced *O. oeni* genomes available at the NCBI (Table 3).

Table 1 Arginine degradation and presence of *arc* genes (*arcA*, *arcB*, *arcC* and *arcD1*) in strains of *Oenococcus oeni*

Strain	Source [a]	Place isolated[b]	Arginine consumption (%)[c]	Cell growth[d]	Presence of all *arc* genes
4727	CECT	California, USA	99	0.88	+
4747	CECT	Cigales, SP	99	0.74	+
4744	CECT	Chile	99	0.44	+
4760	CECT	Requena, SP	99	0.24	+
MB42a	CM	Tarragona, SP	99	0.71	+
Vitilactic	MV	Not specified	99	0.98	+
Mf2	URV	Tarragona, SP	99	0.90	+
Mf3	URV	Tarragona, SP	99	0.66	+
Mf23	URV	Tarragona, SP	99	1.18	+
J51	CM	Montsant, SP	98	0.49	+
Mf8	URV	Tarragona, SP	98	0.76	+
MB42b	CM	Tarragona, SP	97	1.67	+
E31	CM	Montsant, SP	94	0.93	+
B-C4a	FD	Bardolino, IT	94	1.64	+
V-A2	FD	Valpolicella, IT	94	1.49	+
X43	CM	Costers Segre, SP	90	1.63	+
MTO1	LAL	Champagne, FR	90	1.74	+
217^T (=ATCC 23279)	CECT	Bordeaux, FR	89	1.50	+
4730	CECT	Australia	89	1.87	+
NCDO 1696	NCDO	Germany	83	0.84	+
4028	CECT	Bordeaux, FR	81	1.79	+
V-O8	FD	Valpolicella, IT	81	1.36	+
NCDO 2121	NCDO	Bordeaux, FR	73	1.86	+
Microenos	JL	Not specified	56	1.41	+
Z42	CM	Montsant, SP	53	1.00	+
V-Q1	FD	Valpolicella, IT	49	1.20	+
Va-S1bb	FD	Valdadige, IT	48	0.96	+
4733	CECT	Barolo, IT	45	1.13	+
V-V7	FD	Valpolicella, IT	45	1.15	+
4029	CECT	Germany	40	1.08	+
I33	CM	Montsant, SP	39	1.06	+
J52	CM	Montsant, SP	32	0.69	+
V-Aa3	FD	Valpolicella, IT	27	1.03	+
V-M2	FD	Valpolicella, IT	30	0.68	+
Aa1	LD	South Africa	26	1.16	+
4729	CECT	Bordeaux, FR	26	0.57	+
Mf15	URV	Tarragona, SP	23	1.05	+
B-C8	FD	Bardolino, IT	19	1.20	+
Viniflora oenos	CH	not specified	19	1.11	+
V-R7	FD	Valpolicella, IT	17	1.07	+
V-A46	FD	Valpolicella, IT	14	1.20	+
V-Q8	FD	Valpolicella, IT	14	1.07	+
4772	CECT	Ribeiro, SP	13	0.64	+
OSU	LAL	Oregon, USA	8	1.44	+

[a] CECT, Colección Española de Cultivos Tipo, Valencia, Spain; CM, MC Masqué, Incavi, Reus, Spain; FD, F. Dellaglio, Department of Biotechnology, University of Verona, Italy; NCDO: National Collection of Dairy Organisms, UK; LAL, Lalvin, Lallemand Inc., Montreal, Canada; JL, J. Laffort y Cia, Renteria, Spain; CH, Chr. Hansen A/S, Hørsholm, Denmark; LD, LMT Dicks, Department of Microbiology, University of Stellenbosch, South Africa; MV, Martin Vialatte Oenologie, Epernay, France; URV, authors' collection, Rovira i Virgili University, Tarragona, Spain

[b] SP, Spain; IT, Italy; FR, France

[c] 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

[d] Absorbance (600 nm, 1-cm path length) at the end of exponential growth

Table 2 Primers used for the detection of *arc* genes in strains of *Oenococcus oeni*

Gene	Primer name	Sequence (5′ to 3′), orientation	T_m (°C)	Fragment amplified (bp)	Localization in the operon
arcA	ADIon3	CATTGAAGGTGGAGACGAAC, forward	51.8	477	2667-3143
	ADIon2	GCATTCGAAACATAGTTCCTGTCAT, reverse	54.4		
arcB	OTCon1	CAAGGTAGAAGTTTGTTGGCTGAA, forward	54.0	892	3332-4223
	OTCon2	CCTCATCGGTCACTTCCATTTCT, reverse	55.3		
arcC	CKon1	CAGGCAGCTTTACGTGAAACAG, forward	54.8	802	4518-5319
	CKon2	CCACAGCCTCTCCACAACCATT, reverse	56.7		
arcD1	arcDon1	CCGCTATCGGAATGATTATGT, forward	50.5	670	6261-6930
	arcDon2	GCCATTGCTGGTTGGCTTAGA, reverse	54.4		

In the search set, "whole-genome shotgun contigs" and "refseq genomic" databases, both for *O. oeni* taxid 1247, were used.

Results

Arginine degradation by *O. oeni* strains

As Table 1 shows, considerable variability was observed in the ability of the different *O. oeni* strains to degrade arginine in a complex medium. More than half of the strains (25 out of 44, i.e., 57 %) degraded more than 50 % of 5 g/L (29 mmol/L) arginine in MRS medium at pH 4.5 at the end of logarithmic phase (approx. 7 days). These strains were of very different origins and included the type culture and some commercial strains. The other 19 strains only partly degraded arginine or exhibited hardly any signs of degradation. Once again, these strains were from different sources and two of them were commercially available. Therefore, there is considerable variability among strains with regard to arginine degradation, and it seems that there is no relationship between the origin of a strain and its ability to degrade arginine.

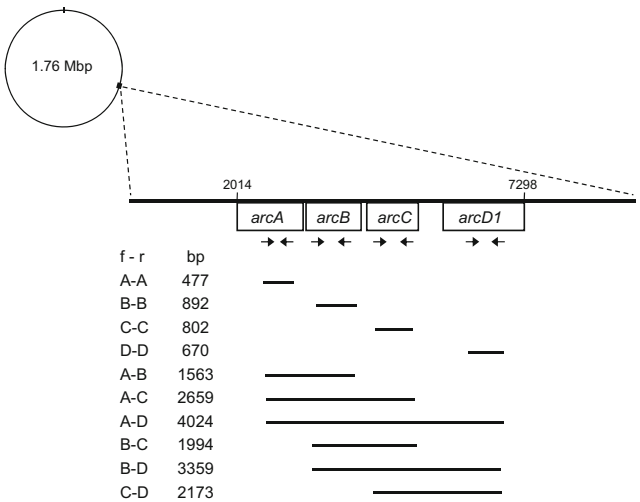

Fig. 1 The *arc* operon of *Oenococcus oeni*. Its location in the chromosome (*left upper circle*) is approximate. Other elements of this gene cluster are not shown. The *arrows* (→forward, ← reverse) indicate the positions of the primers used (see Table 2). Below, the PCR amplified fragments of each gene and those that confirm the operon structure (size in bp) are represented. In the *left-hand column*, the forward (*f*) and reverse (*r*) pairs of primers used for each amplification are indicated: A, B, C and D correspond to *arcA*, *arcB*, *arcC* and *arcD1*, respectively

Table 1 also shows the growth of the strains during the arginine degradation assay, as measured by absorbance at 600 nm. There was no relationship between increased arginine consumption and greater growth because some strains, such as strain 4760, degraded arginine completely and grew slightly, while others consumed very little arginine and grew considerably (strain OSU).

Supernatant citrulline concentrations at the end of assays were slightly higher in some arginine-degrading strains, reaching values over 200 mg/L (see Table S1 in the supplementary data), while they were clearly lower, with values lower than 20 mg/L in most strains with lesser arginine degradation. As can be seen, the ratios of citrulline-produced versus arginine consumed were between 0 and 9 % for the different strains.

Arginine deiminase activity

Enzymatic activity of ADI, corresponding to *arcA* gene, was preliminary studied in some strains of *O. oeni*. Variable values of activity were found in them from 5 U/mg protein (strains *Viniflora oenos*, 4727, MB42b) to 20 U/mg protein (217^T

Table 3 Nucleotide identity (%) of the *arc* genes in the sequenced strains of *Oenococcus oeni* when compared by BLAST with published sequences of these genes (Tonon et al. 2001b; Divol et al. 2003)

% strains	Strains of *Oenococcus oeni*	Sequentiation level	*arcA*		*arcB*		*arcC*		*arcD1*		*arcD2*		Submitter[a]
			%		%		%		%		%		
			Cover	Ident	Cover	Ident	Cover	Ident	Cover	Ident	Cover	Ident	
75	AWRIB202, AWRIB304, AWRIB318, AWRIB418, AWRIB419, AWRIB422, AWRIB429, AWRIB548, AWRIB553, AWRIB568, AWRIB576, DSM 20252^T (=AWRIB129)[b]	Contigs	100	>99	100	>99	100	>99	100	99	100	99	AWRI
	IOEB_0205, IOEB_0502, IOEB_0608, IOEB_1491, IOEB_8417, IOEB_9803, IOEB_9805, IOEB_B10, IOEB_CiNe, IOEB_L18_3, IOEB_L65_2, IOEB_S277, IOEB_S436a, IOEB_S450, IOEB_VF, S11, S12, S14, S15, S19, S23, S28, S161	Contigs	100	>99	100	>99	100	>99	100	>99	100	>99	ISVV
	IOEB_9517, IOEB_B16, S13	Contigs	100	>99	100	>99	100	>99	100	99	88-95	99	ISVV
	OM22, OT25, OT3, OT4, OT5	Contigs	100	>99	100	100	100	100	100	99	100	99	UFog
2	IOEB_0501	Contigs	99	68	100	100	100	100	100	99	100	99	ISVV
23	PSU–1 (representative genome)	Complete	99	68	–		–		–		–		JGI
	ATCC BAA–1163	Scaffold	99	68	–		–		–		–		UBo & ISVV
	IOEB_0607, IOEB_L26_1, IOEB_L40_4, S22, S25	Contigs	99	68	–		–		–		–		ISVV
	IOEB_9304, IOEB_C23, IOEB_C28, IOEB_C52 (strains from cider)	Contigs	99	68	–		–		–		–		ISVV
	OM27	Contig	99	68	–		–		–		–		CRSA
	X2L	Contig	99	68	–		–		–		–		CERELA

The symbol "–" means that the gene was absent since the homology found was lower than 50 %

[a] AWRI: Australian Wine Research Institute, Glen Osmond, South Australia; ISVV: Institut des Sciences de la Vigne et du Vin, Université de Bordeaux, France; UFog: Università di Foggia, Italy; JGI: Joint Genome Institute, California, USA; UBo: Université de Bourgogne, Dijon, France; CRSA: Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Piacenza, Italy; CERELA: Centro de Referencia para Lactobacilos, Conicet, Tucumán, Argentina

[b] Sequenced genome of type strain DSM 20252=ATCC 23279=AWRIB129 was also submitted by JGI

strain). Additionally, PSU-1 strain, which has only *arcA* according its sequenced genome (see below), was analysed and it had an ADI activity of 9.5 U/mg protein.

Detection of *arc* genes in *O. oeni*

Four pairs of primers were designed for each *arc* gene cluster from the known sequences in *O. oeni*, and PCR conditions were optimized so that different fragments were obtained. As Table 2 shows, amplified fragments of different sizes were easily discriminated by electrophoresis.

The four pairs of primers were assayed with DNA isolated from 44 strains of different origins (Table 1). All of these strains contained the four genes of the ADI pathway. As an example, the electrophoretic bands obtained for two strains of *O. oeni* are shown in Fig. 2. These bands presented the expected sizes of the four amplified fragments and were in agreement with those described in Table 2.

Genomic organization of the *arc* operon in *O. oeni*

When the four pairs of primers used to detect the *arc* genes of *O. oeni* (Table 2) were combined to obtain six PCR-amplified fragments including two or more genes of the same operon (Fig. 1), the sizes obtained by electrophoresis were as expected (Fig. 3). The sizes of these fragments were the same as the

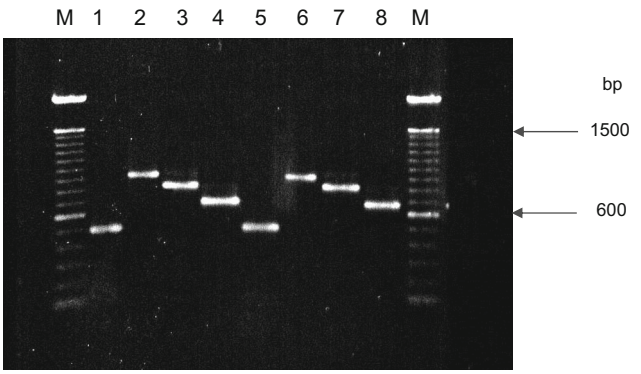

Fig. 2 The *arcABCD* genes in two strains of *Oenococcus oeni* (217^T and Mf15). Genes are arranged (*lanes 1* to *4*, strain 217^T; *lanes 5* to *8*, strain Mf2): *arcA*, *arcB*, *arcC* and *arcD1* and sizes are as expected: 477, 892, 802 and 670 bp, respectively. An Invitrogen DNA 100-bp ladder was used

theoretical values obtained with the Amplify 1.2 software (Engels 1993). As shown, there were no secondary products except for some amplification between the *arcB* forward and *arcD* reverse primers, where a secondary band of approx. 750 bp appeared and was most likely due to unspecific hybridization.

Presence of *arc* genes in the sequenced *O. oeni* strains

As seen in Table 3, there were published genome sequences available for 57 strains of *O. oeni* that had been deposited in GenBank from 2006 until 2014. Only one strain had a complete sequence reported (PSU-1), and as this was the first sequenced it was used as the representative genome. The sequence of another strain (ATCC BAA-1163) was at the scaffold level, and all other strains were at the contig draft level.

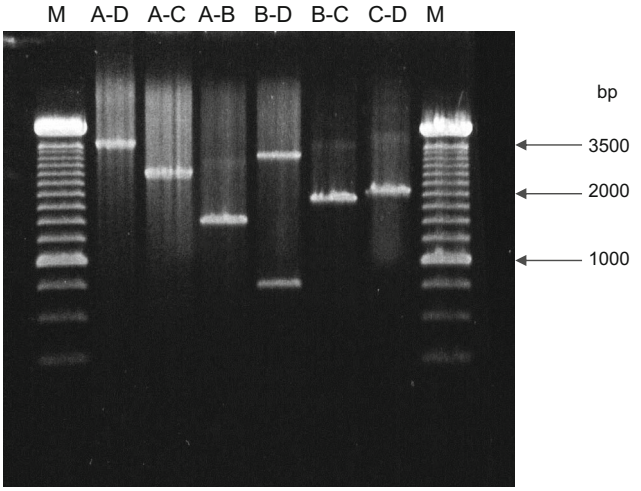

Fig. 3 PCR-amplified fragments of the arc operon of *Oenococcus oeni*, which confirm the operon structure by combining pairs of primers of the four genes. For each pair, the *first letter* corresponds to the forward primer and the *second letter* corresponds to the reverse one, where A, B, C and D are the genes *arcA*, *arcB*, *arcC* and *arcD1*, respectively. *Lane M* is an Invitrogen DNA 250-bp ladder

Sequenced strains in Table 3 are grouped by the presence of the different *arc* genes and by the submitter organization, which promoted the corresponding Genome Annotation Project. As expected, the sequence identity found for type strain DSM 20252 (=AWRIB129=ATCC 23279) in all genes was 100 % because the *arc* gene sequences reported were derived from this strain.

The *arcA* gene (Table 3) was present in all of the sequenced strains, with a query coverage and identity of 99–100 %, but in 14 strains (25 % of all evaluated), including the representative genome of PSU-1 strain, the identity observed was just 68 %. The other *arc* genes (*arcB*, *arcC*, *arcD1* and *arcD2*) were found to be present in 44 strains (77 % of the total strains evaluated). The query coverage and identity was 99–100 % in all of these strains for the four genes examined, but a slightly lower coverage of 88–95 % for the *arcD2* gene was observed in three strains.

The query coverage for these four genes in the other 13 strains (23 % of total) was lower than 50 %, and thus, these genes are likely absent in these strains. These are the same strains for which the *arcA* gene identity was 68 %, with the exception of strain IOEB_0501, which contains all the other genes. As shown, the four sequenced strains isolated from cider and included in this group lack the genes *arcB*, *arcC*, *arcD1* and *arcD2*. All other sequenced *O. oeni* strains were isolated from wine.

Discussion

This work shows that there is considerable variability in the ability of different *O. oeni* strains to degrade arginine. The arginine consumption of different strains was studied in a rich media (MRS medium), as other authors have done (Liu et al. 1995; Tonon et al. 2001b). Relatively high levels of arginine (5 g/L) were used because the cell's ability to consume is more clearly revealed at this concentration, as other authors have reported (Pilone et al. 1991). Similarly, a pH of 4.5 was used in spite of being too high for wine conditions because arginine metabolism occurs more readily at this pH and is inhibited at pH values lower than 3.5, as shown by Terrade and Mira de Orduña (2006). Previous studies have also found variability in the arginine consumption of different *O. oeni* strains, but in this respect, the main contribution of this work is that several strains from very different sources have been used.

Citrulline levels have been considered as good indicators of the final EC concentrations found in wine. The possible production of citrulline from arginine during MLF therefore strongly depends on the strain (Romero et al. 2009). This reinforces the idea that the ability of each strain used in MLF to degrade arginine needs to be assessed, and this information should be used to select the starter cultures. Here, we have seen some relationship between the ability of arginine

degradation and the production of citrulline, which varies depending on the strain. The ratios of citrulline produced versus arginine consumed were lower than 10 % for all strains, and there was no clear relationship of these ratios to the level of arginine consumption or the origin or characteristics of strains. Similar ratios have been found in previous works for strains of *O. oeni*, such as 4 % (Terrade and Mira de Orduña 2009) or 6 % (Mira de Orduña et al. 2001), but higher ratios have been found for other LAB species: 10 % for *Lactobacillus buchneri* (Liu and Pilone 1998) or near 20 % for *Lb. hilgardii* (Araque et al. 2011).

This is the first time that a large number of *O. oeni* strains have been screened for the presence of the *arc* genes. It has been shown that all 44 strains tested contained the four genes of the *arc* operon. Because these strains were from very different sources, and included some commercial MLF starters, we can suggest that all *O. oeni* strains would have these genes. As a positive control, there were included strains such as the type strain in which the presence of these genes has already been demonstrated (Tonon et al. 2001b).

The presence of the *arc* operon in all of the tested strains is consistent with the fact that all strains consume arginine, although some to a very small extent. Other authors have reported that some *O. oeni* strains with no *arc* genes are unable to degrade arginine (Tonon et al. 2001b; Divol et al. 2003). Nevertheless, it has been shown that some of these non-arginine-degrading strains can sometimes have a faint amplification of the *arcA* gene (Tonon et al. 2001b). Therefore, the use of arginine can vary, including some cases of absent or very minor arginine degradation regardless the presence of the *arc* genes. This variability supports the idea that environmental factors are essential for the expression of these genes, depending on the strain.

Using all of the combinations of forward and reverse primers for the four genes *arcA*, *arcB*, *arcC* and *arcD1* in *O. oeni* strains, amplified fragments of the expected sizes (Fig. 3) from the theoretical values were obtained (Fig. 1). As revealed by Divol et al. (2003), there is another *arcD2* gene, but it was not included in the current study because *arcD1* and *arcD2* have been shown to be very similar, both codifying for the arg/orn antiport, and because *arcD1* is the one closely located to arcABC. These data confirm that the four genes are organized in an *arcABCD1* operon in the tested strains and correlate well with previously reported information (Divol et al. 2003). Other LAB that have been studied, such as *Lb. sakei* (Zúñiga et al. 1998), *Lb. plantarum* (Spano et al. 2004) and *Lc. lactis* (Bolotin et al. 2001; Kok et al. 2005), have slightly different operon organizations.

In other wine LAB, such as *Lb. plantarum*, a great variability in ADI activity was observed among different strains (Spano et al. 2007). Those strains with higher ADI activity and lower OTC activity accumulate greater amounts of citrulline and thus produce more EC. In the current work, we observed a variable ADI activity among tested strains of *O. oeni*. Interestingly, the representative sequenced strain PSU-1, which has only the *arcA* gene (see Table 3), presented similar ADI activity to other strains having all the *arc* operon, demonstrating that this separate *arcA* gene codifies also for an active enzyme.

To determine whether *arc* genes are present in other strains of *O. oeni* besides those tested in this study, we searched for their presence in the sequenced strains available in GenBank. There was found that all 57 strains (Table 3) have sequences that are homologous to *arcA*; in spite of this information, 14 strains (25 % of them) have an identity of only 68 % for this gene. With the exception of strain IOEB_0501, all of these strains (23 % of total) that have a partial identity to *arcA* lack the other four genes of the *arc* operon. The only four sequenced strains of *O. oeni* isolated from cider are included in this group and were shown to be phylogenetically clustered and separated from other strains upon SNP analysis (El Khoury 2014).

As seen in the GenBank data, the sequenced strains containing all *arc* genes have localized them to the same contig with one gene after another structured as an operon, which is the same as that seen in the strains of this work. Nevertheless, the strain IOEB_0501 presents the gene *arcA* (with an identity of 68 %) in a different genome location of genome separated from *arcB*, *arcC*, *arcD1* and *arcD2*, as reported previously (Mills et al. 2005; Bon et al. 2009). On the other hand, these four genes are placed together, and upstream of that cluster appears a sequence with certain homology to *arcA* (38 % query coverage) which could be a nonfunctional evolutionary remnant of this gene.

Therefore, it can be concluded that all strains of *O. oeni*, those sequenced and deposited in GenBank and those studied in this work, contain the *arcA* gene. Regarding the other genes of *arc* operon, despite the fact that all strains tested here contained all of the operon genes, it has been observed that some of the sequenced strains are lacking these genes. In this way, we can suggest that the presence of the *arcB*, *arcC* and *arcD* genes is not universal in *O. oeni* because they can be found in approximately 77 % of sequenced strains. Excluding the cider-derived strains, the absence of those genes is limited to nine sequenced strains and the presence of all *arc* genes in sequenced strains of *O. oeni* isolated from wine increases to 83 %.

The results of the current study leads to a better understanding of how the different *O. oeni* strains can degrade arginine and in this way influence the formation of ethyl carbamate precursors. Having observed that the *arcA* gene is present in all studied strains of this species, a more in-depth study of the expression of this gene in different conditions is now required. Screening for safer strains should not only analyse arginine degradation but also seek strains having the complete operon or those with maximum specific activity for the final enzyme in the pathway, CK, so that citrulline does not accumulate.

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