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Putrescine production via the ornithine decarboxylation pathway improves the acid stress survival of *Lactobacillus brevis* and is part of a horizontally transferred acid resistance locus

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ABSTRACT

Decarboxylation pathways are widespread among lactic acid bacteria; their physiological role is related to acid resistance through the regulation of the intracellular pH and to the production of metabolic energy via the generation of a proton motive force and its conversion into ATP. These pathways include, among others, biogenic amine (BA) production pathways. BA accumulation in foodstuffs is a health risk; thus, the study of the factors involved in their production is of major concern. The analysis of several lactic acid bacterial strains isolated from different environments, including fermented foods and beverages, revealed that the genes encoding these pathways are clustered on the chromosome, which suggests that these genes are part of a genetic hotspot related to acid stress resistance. Further attention was devoted to the ornithine decarboxylase pathway, which affords putrescine from ornithine. Studies were performed on three lactic acid bacteria belonging to different species. The ODC pathway was always shown to be involved in cytosolic pH alkalinisation and acid shock survival, which were observed to occur with a concomitant increase in putrescine production.

1. Introduction

Lactic acid bacteria (LAB) inhabit a wide variety of environments, including the mucosal surfaces of humans and animals, milk, meat, fruits, grains, vegetables, and fermented foods. The adaptation to some ecological niches has required the capability to resist acid stress. One of the bacterial mechanisms that have been described to counteract low pH is the decarboxylation of amino acids and organic acids (Molenaar et al., 1993). Typical decarboxylation systems consist of a specific decarboxylase and a precursor/product transmembrane exchanger (Poolman, 1990). This pathway results in alkalinisation of the cytosol and the generation of a proton motive force, which can be exploited for acid stress resistance and/or the production of metabolic energy in the form of ATP (Molenaar et al., 1993). Amino acid decarboxylases in LAB have received special attention because their activity results into biogenic amine (BA) production and accumulation in fermented foods and beverages (Linares et al., 2011; Spano et al., 2010). The ingestion of foodstuffs containing high levels of BAs, such as histamine or tyramine, is associated with several toxicological problems derived from their vasoactive and psychoactive properties (Ladero et al., 2010a). Polyamines such as putrescine or spermidine are associated to key biological functions (Igarashi et al., 2001), but they can also combine with nitrates to form carcinogenic nitrosamines (Ten Brink et al., 1990).

Putrescine is one of the most abundant BAs in fermented foods, such as cheese (Linares et al., 2011), sausages (Suzzi and Gardini, 2003), meat (Ruiz-Capillas et al., 2004), and alcoholic beverages, such as cider (Garai et al., 2006; Ladero et al., 2011a) and wine (Ancin-Azpilicueta et al., 2008). Two different metabolic routes have been described in LAB for the biosynthesis of putrescine. The ornithine decarboxylase (ODC) pathway is a typical decarboxylation system consisting of an ODC and an ornithine/putrescine exchanger (Coton et al., 2010a; Marcobal et al., 2006; Romano et al., 2012a). In contrast, the agmatine deiminase (AgDI) pathway is a more complex system, comprising AgDI, a putrescine transcarbamylase, a carbamate kinase, and an agmatine/putrescine exchanger (Griswold et al., 2004; Ladero et al., 2011b; Lucas et al., 2007). It has been shown that the prevalence of either pathway in the accumulation of putrescine depends on the type of foodstuff. In cider and cheese, the AgDI pathway has a predominant role (Ladero et al., 2011a, 2012a), whereas in wine, putrescine is mainly produced through the ODC pathway (Nannelli et al., 2008).

The AgDI pathway is relatively frequent in LAB. It was detected in 14% of the strains of multiple species during the screening of an LAB collection (Coton et al., 2010b), and it is even considered a species trait in some enterococci (Ladero et al., 2012b). The pathway genes were occasionally detected in a putative acid resistance locus in diverse LAB species (Lucas et al., 2007). In this locus, the AgDI genes are found on the chromosome in a position adjacent to the genes associated with the tyrosine decarboxylase (TDC) pathway, which converts tyrosine into tyramine (Lucas et al., 2003). Furthermore, in the *Lactobacillus brevis* ATCC 367 strain, whose

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genomic sequence is publicly available (accession number CP000416), the genes of the malolactic enzyme (MLE) pathway are placed immediately downstream of the TDC and AgDI genes. The MLE pathway promotes the decarboxylation of malate into lactate and CO_2 and is also known to contribute to the survival and growth of certain LAB in acidic environments rich in malate, such as wine (Salema et al., 1996).

Unlike the genes of the AgDI pathway, the ODC genes have to date been detected in a limited number of strains of different LAB species. In a recent screening of wine LAB only strains belonging to species *Oenococcus oeni* were found ODC positive and with frequencies as low as 2% (Coton et al., 2010b). This phylogenetic distribution likely results from horizontal gene transfer (HGT) (Marcobal et al., 2006). The first ODC gene was described in *Lactobacillus saerimneri* 30a (formerly known as Lactobacillus sp. 30a) isolated from horse stomach (Hackert et al., 1994) and was later detected in strains of *O. oeni* from wine (Marcobal et al., 2006; Romano et al., 2012a) and in *L. brevis* IOEB 9906 from sugarcane molasses (Coton et al., 2010b). A second type of ODC shares some sequence similarities with the aforementioned ODC of *L. saerimneri* 30a, *O. oeni*, and *L. brevis* IOEB 9906. These ODCs form a distinct phylogenetic family. In addition, they do not release significant amounts of putrescine into the extracellular matrix but are supposedly involved in the production of cytosolic putrescine used in diverse cellular processes (Romano et al., 2012a). Lastly, the genome sequence of *L. saerimneri* 30a (Romano et al., 2013a) disclosed the unique structure of its ODC locus. This strain hosts a peculiar three-component decarboxylation system composed of an ODC, a lysine decarboxylase and a dual specificity antiporter promoting both ornithine/putrescine and lysine/cadaverine exchanges (Romano et al., 2013b).

This work represents a follow-up on previous studies on ODC systems and acid resistance in LAB. Our investigation was prompted by the surprising observation that the *L. brevis* IOEB 9906 strain presents both known pathways for putrescine biosynthesis (i.e., ODC and AgDI). The ODC genes were encountered within the previously described acid resistance locus and in adjacent position to the AgDI and MLE genes. An in-depth analysis of this locus was performed in numerous strains of *L. brevis* and other LAB species to evaluate its diversity and boundaries. This led us to disclose a genetic hotspot wherein genes encoding the BA pathways and MLE are most likely acquired, lost, and transferred among strains. A more detailed investigation was conducted on the ODC system in three different LAB strains that originate from different environments: *L. saerimneri* 30a, *L. brevis* IOEB 9906, and *O. oeni* S22. The involvement of the ODC system in acid shock resistance and cytosolic pH homeostasis was shown.

2. Materials and methods

2.1. Bacterial strains and cultures

The LAB used in this work were from the collections of the Institut d'Œnologie de Bordeaux (IOEB), the Instituto de Productos Lácteos de Asturias (IPLA), and the Laboratoire SARCO (Floirac, France); the others were obtained from public or laboratory collections as mentioned in the footnote of Table 2. Strains originated mostly from food matrices and namely wine (10), cheese (6), sugarcane (5), apple cider, olives, silage, and sourdough (one strain each); two more strains were isolated in human milk and faeces. The lactobacilli were grown at 30 °C in MRS broth (De Man et al., 1960), with the exception of *L. saerimneri* 30a, which was grown at 37 °C. *O. oeni* SARCO S22 was cultured at 25 °C in grape juice medium containing 25% (v/v) commercial red grape juice, 0.5% (w/v) yeast extract, and 0.1% (v/v) Tween 80 and adjusted to pH 5.0.

2.2. Acid shock experiments

The cells were cultured in half-strength MRS (grape juice medium at pH 5.0 for *O. oeni*). The cultures were harvested at the mid-exponential phase (OD 0.5–2.0 units ml^{-1}, depending on the strain) and centrifuged (4500 $g \times 10$ min). The pellets were resuspended in fresh half-strength MRS at pH 5.0 (grape juice medium at pH 5.0 for *O. oeni*) at the concentration of 1.0 OD units ml^{-1}, and the pH was immediately adjusted to 2.0 by the addition of 1 M HCl. The cell suspensions were incubated at 37 °C, and aliquots were collected at 1 and 4 h. The aliquots were serially diluted, and a 5-μl droplet of for each dilution was plated in triplicate on MRS medium (grape juice medium for *O. oeni*). The plates were incubated at 25/30/37 °C, and a positive/negative response was obtained for each droplet. The original viable cell concentration (in CFU ml^{-1}) was estimated through the most probable number (MPN) method (Harrigan, 1998). For each strain, a trial was performed in the presence of 50 mM ornithine both during growth and acid shock, and a control experiment was conducted in the absence of ornithine. At the end of each acid shock trial, the amount of putrescine was quantified through thin-layer chromatography/densitometry (Romano et al., 2012b). Three biological replicates of each experiment were carried out.

2.3. Measurement of cytosolic pH

This measurement was performed by adapting the protocol developed by Molenaar et al. (1991) based on the pH-sensitive fluorescent probe 2′,7′-bis-(2-carboxyethyl)-5-carboxyfluorescein (BCECF). Briefly, the cells were cultured in half-strength MRS (grape juice medium at pH 5.0 for *O. oeni*). The cultures were harvested at the mid-exponential phase (OD 0.5–2.0 units ml^{-1} depending on the strain) and centrifuged (4500 $g \times 10$ min). The pellets were resuspended in fresh half-strength MRS at pH 5.0 (grape juice medium at pH 5.0 for *O. oeni*) at the concentration of 1.0 OD units ml^{-1}. Then, 3.5% (v/v) saturated BCECF (Sigma-Aldrich, St. Quentin Fallavier, France) solution was added, and the pH was immediately adjusted to 3.0 by the addition of 1 M HCl. The cell suspensions were incubated at 37 °C for 1 h to allow the incorporation of the probe into the cytoplasm. Verifications were performed to ensure that the aforementioned treatment did not significantly reduce the viable cell counts. The cell suspensions were washed twice with potassium phosphate buffer (0.05 M, pH 5.2) and resuspended in the same buffer at the final concentration of 5.0 OD units ml^{-1}. The suspensions were divided into 200-μl aliquots that were transferred to a multiwell plate (Fisher Scientific, Illkirch, France). The plate was maintained under shaking at 37 °C, and the fluorescence was measured using a microplate reader (Synergy HT BioTek, France). The evolution of the intracellular pH was monitored after the addition of 50 mM ornithine (phosphate buffer in the control).

2.4. Determination of the sequence of the ODC locus of L. brevis IOEB 9906

Starting from the previously published ODC locus (Romano et al., 2012a), the sequences of the neighbouring genomic regions were obtained through conventional molecular biology techniques. Briefly, the genomic DNA was digested and subjected to enzymatic ligation. The ligation mixtures were then purified and employed as matrices for inverted PCR reactions. The PCR products were sequenced, and the sequence data were used for further inverted PCR reactions. The alignment of all contigs resulted in a 9223-bp concatenated sequence, which was deposited in GenBank under accession number JN120479.

2.5. PCR reactions

The detection of the ODC, AgDI, and TDC pathway genes was performed as previously described (Coton et al., 2010b). The detection of the MLE pathway genes was performed using the primers MaloF and MaloR (Table 1). Details of the primers are provided in Table 1. The PCR reactions were performed in 25-μl reaction volumes that included 1 μl of DNA template (typically 100 ng of purified genomic DNA), 400 nM of each primer, 200 μM of each dNTP, the reaction buffer, and 1 U of Taq polymerase (Dream Taq, Germany). Typically, the amplification was performed under the following conditions (annealing temperature and

Table 1
Sequence and designation of the primers used in the present work and their respective target genes.

Primer name/target gene	Sequence (5′-3′)	Source
Detection of acid resistance pathways		
Td2/LVIS_2213	ACATAGTCAACCATRTTGAA	[a]
Td5/LVIS_2213	TAGTCAACCATATTGAAATCTGG	[a]
PTC01/LVIS_2210	GGWCAAATTCAIYTIGG	[c]
PTC02/LVIS_2210	CCRTACCAWACATGIGTRTA	[c]
AgD1/LVIS_2208	CAYGTNGAYGGHSAAGG	[b]
AgD2/LVIS_2208	ATTCACTGYATYACNCAACA	[b]
MaloF/LVIS_2204	GCAGTACGCGTTCTTGAACA	[c]
MaloR/LVIS_2204	GGATCATAAACGATTGGCATAA	[c]
LbreODC04/ODC	ACAGATGACGCCTTTGGATG	[c]
LbreODC05rev/ODC	AGCAGCCACAGCCACATTAT	[c]
Genetic organisation of acid resistance loci[d]		
A/LVIS_2192	CACGACCAACACATGAGGTC	[c]
B/LVIS_2196	ACCTCTTCCGTGTTGATTGG	[c]
C/LVIS_2196	AACGACCATCGACCAGAAAC	[c]
D/LVIS_2200	CTGGGGTCATTGTAGCGAAT	[c]
E/LVIS_2200	TCAAAGATATCGCCCAAAGG	[c]
F/LVIS_2203	TGATGAACAAGCCACTCAGG	[c]
G/LVIS_2203	GGATCATAAACGATTGGCATAA	[c]
H/LVIS_2209	GAACGACTAGCAGCTAGTTAT	[c]
I/LVIS_2214	CCATTAATCGTAATCGCACC	[c]
J/LVIS_2218	AACGAAGTGCGAAAGCATCT	[c]
K/*potE*	GATTGCTTTTCTGGCAGCTC	[c]
L/LVIS_2209	GCCATAGTCGCAACCACTTC	[c]

[a] Coton and Coton, 2009.
[b] Coton et al., 2010b.
[c] This study.
[d] Capital letters also refer to primer designations as in Fig. 1.

elongation time were occasionally modified): 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 4 min, and a final extension at 72 °C for 10 min.

3. Results

3.1. In L. brevis, AgDI and MLE loci are found adjacent to the ODC locus

Although the *L. brevis* ODC pathway genes were identified in a previous study (Romano et al., 2012a, 2012b), the genetic environment of these genes remains unknown. The regions surrounding the ODC system in *L. brevis* IOEB 9906 were determined using a strategy based on inverted PCR (Materials and methods). The sequence obtained was compared to the published genome of *L. brevis* ATCC 367, an ODC-negative strain that displays an acid resistance locus containing the MLE, AgDI, and TDC pathways genes. The sequence data from *L. brevis* IOEB 9906 revealed regions lying downstream and upstream of the ODC genes with 100% identity to the *L. brevis* ATCC 367 LVIS_2210 and LVIS_2218 genes, respectively. The region specific to the ODC-positive strain only comprised three genes, which encode the ornithine decarboxylase (ODC), the ornithine-putrescine exchanger (*potE*), and a putative transcriptional regulator of the LacI family (gene 33). Interestingly, the aforementioned genes had replaced those of the TDC pathway (Fig. 1).

3.2. BA pathways and MLE genes are part of a genetic hotspot

The structure of the acid resistance locus was further investigated in LAB strains belonging to five different species isolated from several food- and human-related environments (Table 2). The strains were identified as biogenic amine producers during previous genotypic and/or phenotypic screenings (Coton et al., 2010b; Ladero et al., 2010b; Lucas et al., 2007; and unpublished results). *L. brevis* IOEB 7702 and *L. brevis* IOEB 8404 resulted ODC negative to PCR and did not produce BAs according to TLC. The two strains were also included in the analysis. Using a PCR-based strategy (Materials and methods), it was possible to verify whether the MLE gene cluster was present and to determine its relative position with respect to the genes encoding biogenic amine-producing pathways (AgDI, TDC, and ODC). Using the primers listed in Table 1, the gene disposition was investigated over a 30-kb region (Fig. 1). Whenever a difference in the PCR product size was encountered, it was further investigated through sequencing.

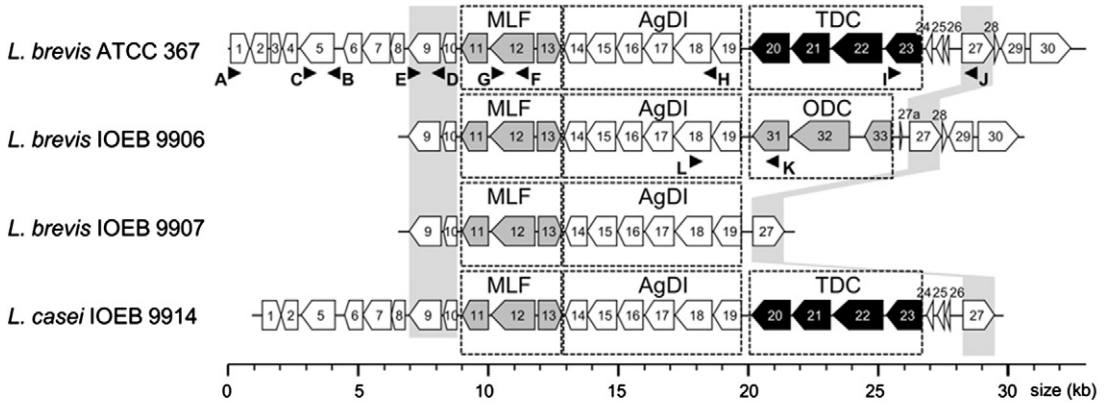
The results showed how the acid resistance locus is characterised by great diversity in terms of presence/absence and disposition of the gene clusters (Table 2). In the *L. brevis* IOEB 8404 strain, none of the clusters (including the malolactic pathway genes) was present. Two strains exhibited only one gene cluster (either AgDI or MLE). Strains from three different species presented two gene clusters (either MLE/AgDI or TDC/AgDI). Of the strains presenting two gene clusters, *L. brevis* IOEB 9907 was studied in more detail. The size of the amplified fragments indicated that the region lying downstream of the MLE cluster was identical to the one found in the reference strain *L. brevis* ATCC 367. Upstream of AgDI, the whole TDC cluster and a few pseudogenes upstream of LVIS_2214 appeared to be missing (Fig. 1). This result was confirmed by sequencing the junction region between LVIS_2210 and LVIS 2218 (data not shown). The gene disposition of *L. brevis* ATCC 367 (MLE/AgDI/TDC) was the most common among the tested strains (Table 2). This configuration was encountered in a number of *L. brevis* strains and in several strains of different species from a great variety of origins (Table 2). Lastly, the cluster configuration MLE/AgDI/ODC was encountered in two *L. brevis* strains isolated from two markedly different environments, namely sugarcane and human milk. The comparison of *L. brevis* ATCC 367, *L. brevis* IOEB 9906, and *Lactobacillus casei* IOEB 9914 revealed a high level of synteny over a region of approximately 30 kb. A minor difference in gene disposition was encountered in *L. casei* IOEB 9914, where the LVIS_2194 and LVIS_2195 genes are absent (Fig. 1).

3.3. The ODC pathway improves acid resistance in LAB strains from three different species

The diversity of the ODC pathways detected in *L. brevis*, *O. oeni*, and *L. saerimneri* prompted us to examine whether they play the same role in these LAB. It is assumed that these pathways are involved in acid resistance, although this has not been demonstrated. The role of the ODC pathway in acid resistance was investigated in three strains from three different species and environments. *L. saerimneri* 30a, isolated from horse stomach, is a well-known putrescine producer (Guirard and Snell, 1980; Rodwell, 1953). *L. brevis* IOEB 9906, isolated from sugarcane, originated from a screening for foodborne biogenic amine producers (Coton et al., 2010b), and its ornithine decarboxylase was previously purified and characterised (Romano et al., 2012a). *O. oeni* SARCO S22 was isolated during a wine screening for biogenic amine producers; upon genome sequencing (unpublished results), it was possible to elucidate the structure of its ODC locus, which was identical to that reported for other *O. oeni* strains (Marcobal et al., 2006; Romano et al., 2012a). After a growth step in MRS at pH 5.0, the three strains were resuspended in fresh MRS, and the pH was adjusted to 2.0. Their acid survival was monitored on MRS plates through a droplet test (Materials and methods). In the absence of ornithine, no colonies were obtained, which, in comparison to an untreated control, corresponded to less than 0.001% survival (Fig. 2A). A marked increase in acid resistance was observed in the presence of ornithine. The survival of the three strains was estimated to be 7–10% after 1 h. In *L. saerimneri* 30a, this survival remained unchanged for up to 4 h, while the survival of *L. brevis* and *O. oeni* decreased to 5 and 1%, respectively. A concomitant production of 6–43 mM putrescine (depending on the strain) was observed after 4 h of acid shock (Fig. 2B).

3.4. The ODC pathway allows for alkalinisation of the cytosol

The mechanism underlying acid resistance in the three strains was investigated by measuring the evolution of the cytosolic pH after ornithine was added to the medium. All of the strains exhibited an increase in intracellular pH upon ornithine addition (Fig. 3), indicating that the



Gene annotation. 1. LVIS_2192: Predicted hydrolase (HAD superfamily), 2. LVIS_2193: Hypothetical protein, 3. LVIS_2194: Phage envelope protein, 4. LVIS_2195: Phospholipid binding protein, 5. LVIS_2196: D-Xylose/proton symporter, 6. LVIS_2197, Antimicrobial peptide ABC transport system, ATPase, 7. LVIS_2198: Antimicrobial peptide ABC transport system, permease, 8. LVIS_2199: Transcriptional regulator, 9. LVIS_2200: Permease (major facilitator superfamily), 10: LVIS_2201. Predicted membrane protein, 11. LVIS_2202: Malate permease, 12. LVIS_2203: Malolactic enzyme, 13. LVIS_2204: Malolactic transcription activator, 14. LVIS_2205: Agmatine deiminase transcription regulator, 15. LVIS_2206: Agmatine deiminase (aguA2), 16. LVIS_2207: Carbamate kinase, 17. LVIS_2208: Agmatine deiminase, 18. LVIS_2209: Putrescine/agmatine antiporter, 19. LVIS_2210: Putrescine transcarbamylase, 20. LVIS_2211: Na^+/H^+ antiporter, 21. LVIS_2212: Tyrosine/tyramine antiporter, 22. LVIS_2213: Tyrosine decarboxylase, 23. LVIS_2214: Tyrosyl-tRNA synthetase, 24. LVIS_2215: Hypothetical protein, 25. LVIS_2216: Hypothetical protein, 26. LVIS_2217: Hypothetical protein, 27. LVIS_2218: Ornithine acetyl-transferase, 27a. Ornithine acetyl-transferase (pseudogene), 28. LVIS_2219: Hypothetical protein, 29. LVIS_2220: Permease of the drug-metabolite transport, 30. LVIS_2221: Alpha-arabinofuranosidase, 31. Ornithine/putrescine antiporter (*potE*), 32. Ornithine decarboxylase (*odc*), 33. Putative transcriptional regulator.

Fig. 1. Acid-resistance loci of four LAB strains analysed in this work. *L. brevis* ATCC 367 was used as a reference strain. The genes are numbered according to the annotation provided below. The gene clusters are highlighted (MLE: malolactic fermentation, AgDI: agmatine deiminase, TDC: tyrosine decarboxylase, ODC: ornithine decarboxylase). The DNA regions bordering the loci and conserved among the four bacteria are highlighted with a light grey background. Dark triangles and capital letters indicate the orientation and approximate placement of the primers used to check the organisation of each locus.

three different ODC systems function through the same general mechanism. Interestingly, the initial pH values were different, and the responses to ornithine addition varied in a strain-specific manner. *L. saerimneri* 30a exhibited the highest gap between the extracellular and intracellular pH values (5.2 versus 6.7), whereas *O. oeni* and *L. brevis* presented lower intracellular pH values (6.3 and 5.6, respectively). In the lactobacilli, the variation in intracellular pH over time was relatively slow with an increase of 0.5–0.9 pH units (for *L. brevis* and *L. saerimneri* 30a, respectively) over a period of 20 min. In *O. oeni*, the increase in pH was fairly abrupt with a rise of approximately 0.3 units in 1.5 min.

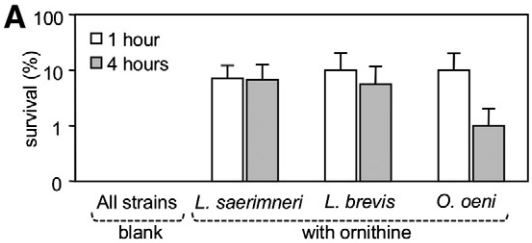
Table 2
Distribution and structure of acid-resistance related gene clusters in LAB strains.

Species	Strain[a]	Origin	Acid resistance locus		
L. brevis	IOEB 8404	Wine	Non detected		
L. brevis	IOEB 7702	Wine	MLE		
L. brevis	IOEB 9301	Apple cider		AgDI	
L. brevis	IOEB 9907	Sugarcane	MLE	AgDI	
L. brevis	CECT 3824	Cheese	MLE	AgDI	
L. brevis	IPLA C311	Cheese		AgDI	TDC
L. casei	IOEB 9920	Sugarcane		AgDI	TDC
L. curvatus	IPLA Vi14	Cheese		AgDI	TDC
L. brevis	ATCC 367	Silage	MLE	AgDI	TDC
L. brevis	IOEB 8511	Wine	MLE	AgDI	TDC
L. brevis	IOEB 8907	Wine	MLE	AgDI	TDC
L. brevis	IOEB 9112	Wine	MLE	AgDI	TDC
L. brevis	IOEB 9809	Wine	MLE	AgDI	TDC
L. brevis	IOEB 9901	Wine	MLE	AgDI	TDC
L. brevis	IOEB 0019	Wine	MLE	AgDI	TDC
L. brevis	IOEB 0402	Wine	MLE	AgDI	TDC
L. brevis	IOEB 9925	Sugarcane	MLE	AgDI	TDC
L. brevis	IPLA 3810	Cheese	MLE	AgDI	TDC
L. brevis	IPLA 3811	Cheese	MLE	AgDI	TDC
L. brevis	IPLA OL5	Olives	MLE	AgDI	TDC
L. brevis	LMG 7944	Faeces	MLE	AgDI	TDC
L. casei	IOEB 9914	Sugarcane	MLE	AgDI	TDC
L. curvatus	IPLA Vi6	Cheese	MLE	AgDI	TDC
L. fructivorans	IOEB 9107	Wine	MLE	AgDI	TDC
L. sanfranciscensis	ATCC 27651	Sourdough	MLE	AgDI	TDC
L. brevis	IOEB 9906	Sugarcane	MLE	AgDI	ODC
L. brevis	ZL95-24	Human milk	MLE	AgDI	ODC

[a] IOEB: Institut d'Œnologie de Bordeaux, IPLA: Instituto de Productos Lácteos de Asturias, CECT: Colección Española de Cultivos Tipo, ATCC: America Type Culture Collection, LMG: Laboratorium voor Microbiologie, Universiteit Gent. *L. brevis* ZL95-24: Laboratory Prof J. M. Rodríguez, Universidad Complutense, Madrid.

4. Discussion

The clustering of BA pathways was previously reported in LAB for the genes encoding TDC and AgDI (Lucas et al., 2007); moreover, in the genome sequence of the *L. brevis* ATCC 367 strain, a third decarboxylation pathway (i.e., MLE) was encountered. In the present work, this phenomenon was further inspected: the clustering of decarboxylation pathways was shown to be a relatively common feature within LAB because the



B

Strain	*L. saerimneri*	*L. brevis*	*O. oeni*
Putrescine (mM)	43 ± 12	31 ± 6	6 ± 1

Fig. 2. Cell survival and putrescine production during exposure to acid shock. (A) Survival of three LAB strains incubated at pH 2.0 for 1 to 4 h in the presence or absence of 50 mM ornithine. The cell survival is expressed as a percentage with respect to that of non-stressed cultures. The standard deviations are represented by error bars. (B) The production of putrescine (±SD) after acid shock for 4 h. All of the results are the means of triplicate experiments.

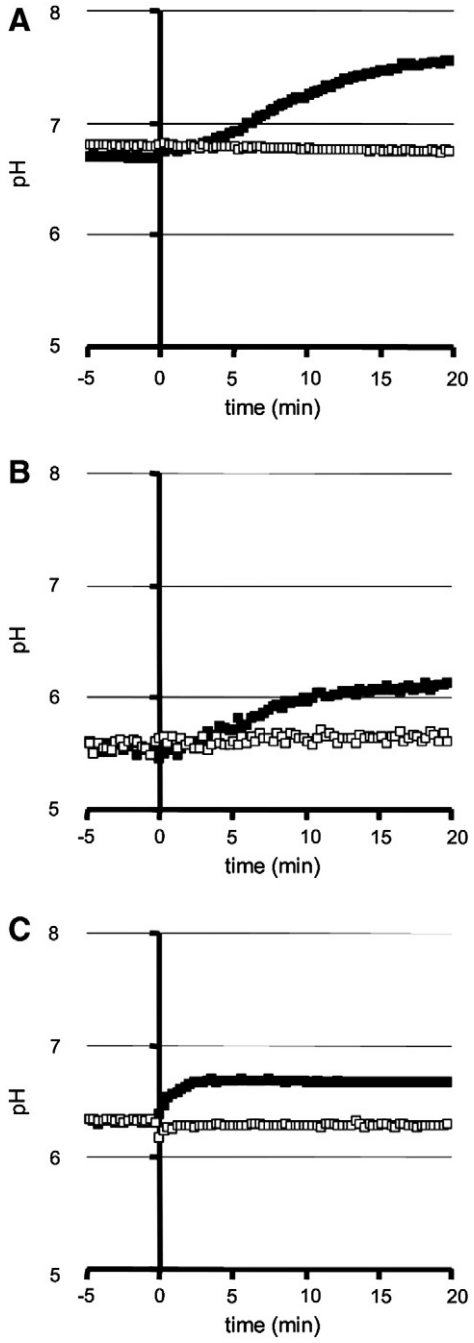


Fig. 3. Modulation of cytosolic pH by ornithine in strains carrying an ODC pathway. The *L. saerimneri* 30a (A), *L. brevis* IOEB 9906 (B), and *O. oeni* SARCO S22 (C) strains were suspended in medium supplemented with 50 mM ornithine at time zero (■). The pH results are compared to that of untreated controls (□). The experimental points are the means of four separate trials.

presence of a MLE/AgDI/TDC gene cluster was witnessed in LAB strains originating from diverse environments and as many as six different species. The disposition of the genes within the clusters and in the immediately surrounding regions was verified by PCR and sequencing. A high degree of gene synteny between strains from different species (i.e., *L. brevis* and *L. casei*) was observed over a region as large as 30 kb. In addition, this locus was characterised by a remarkable level of internal plasticity. By analysing 27 LAB strains from five different species, seven possible pathway arrangements were detected: no pathway, only MLE, only AgDI, MLE/AgDI, AgDI/TDC, MLE/AgDI/TDC, and MLE/AgDI/ODC. Of particular interest are *L. brevis* IOEB 9906 and *L. brevis* ZL95-24. These strains, albeit originating from intrinsically different environments (sugarcane and human milk), both contain an ODC pathway located in a genomic region comprising several other decarboxylation pathways. The simultaneous presence of both pathways leading to putrescine biosynthesis (i.e., AgDI and ODC) in a bacterial strain is – to the best of the authors' knowledge – unprecedented.

The peculiar gene distribution described in this manuscript was likely generated by multiple HGT events involving either the transfer of single pathways or larger chromosomal regions. The pathway genes have been sometimes detected on plasmids (Coton et al., 2010a) and occasionally found to have transposase genes in their vicinity (Coton and Coton, 2009). These elements most likely contributed to the grouping of the pathways, but the mechanism underlying their assemblage remains unknown.

Bacterial amino acid decarboxylation pathways contribute to the maintenance of the intracellular pH of cells exposed to acid stress (Meng and Bennett, 1992; Molenaar et al., 1993; Park et al., 1996). Pereira et al. (2009) previously showed that the LAB-related microorganism *Weissella halotolerans* W22 regulates its intracellular pH by converting ornithine into putrescine. The present research work provides evidence of a direct correlation between the presence of the ODC pathway, survival to acid stress, putrescine biosynthesis, and modulation of cytosolic pH for three different LAB strains. Interestingly, the intracellular pH alkalinisation resulting from the activity of the ODC system was different in the three assayed LAB. Further research is required to ascertain whether these differences play an adaptive role. The microorganisms originated from environments that are intrinsically different, even though they share the presence of an acid stress component (i.e., sugarcane must, wine, and the gut for *L. brevis*, *O. oeni* and *L. saerimneri*, respectively). In the case of strains that have a food origin, the use of the ODC pathway to increase their survival in acidic environments, such as is found in fermented foods and beverages, implies a concomitant production of putrescine and therefore a potential health risk for the consumers (Ladero et al., 2010a).

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