International Journal of Food Microbiology 175 (2014) 14–19

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

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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ARTICLE INFO

Article history:
Received 5 October 2013
Received in revised form 7 January 2014
Accepted 16 January 2014
Available online 22 January 2014

Keywords:
Lactic acid bacteria
Food fermentation
Biogenic amine
Putrescine
Acid resistance

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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http://dx.doi.org/10.1016/j.ijfoodmicro.2014.01.009

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