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The putrescine biosynthesis pathway in *Lactococcus lactis* is transcriptionally regulated by carbon catabolic repression, mediated by CcpA

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ABSTRACT

Lactococcus lactis is the lactic acid bacterium most widely used by the dairy industry as a starter for the manufacture of fermented products such as cheese and buttermilk. However, some strains produce putrescine from agmatine via the agmatine deiminase (AGDI) pathway. The proteins involved in this pathway, including those necessary for agmatine uptake and conversion into putrescine, are encoded by the *aguB*, *aguD*, *aguA* and *aguC* genes, which together form an operon. This paper reports the mechanism of regulation of putrescine biosynthesis in *L. lactis*. It is shown that the *aguBDAC* operon, which contains a *cre* site at the promoter of *aguB* (the first gene of the operon), is transcriptionally regulated by carbon catabolic repression (CCR) mediated by the catabolite control protein CcpA.

1. Introduction

Food-fermenting lactic acid bacteria (LAB) are generally considered non-toxic and non-pathogenic. *Lactococcus lactis* is the LAB most widely used in the dairy industry as a primary starter, especially in cheese and buttermilk manufacture. Its main role in dairy fermentations is the production of lactic acid from lactose. This results in a reduction in the pH of the food medium, which inhibits the growth of spoilage and pathogenic microorganisms. Other metabolic activities of *L. lactis* enhance the organoleptic properties of the final product. The long and safe history of use of *L. lactis* in dairy fermentations has resulted in the species being classified as Generally Regarded as Safe (GRAS) in the United States; the European Food Safety Authority (EFSA) has conferred Qualified Presumption of Safety (QPS) status upon it (Bourdichon et al., 2012).

The use of *L. lactis* in dairy factories is, however, not without issues. Some strains have enzymatic activities associated with the production of undesirable flavours (Smit et al., 2005) and even toxic compounds, such as biogenic amines (BAs) (Ladero et al., 2011). BAs are biologically active compounds that are mainly formed in foodstuffs through the microbial decarboxylation of certain amino acids. Producer strains can be either contaminants or belong to the microbial groups participating in the food-making process (Linares et al., 2011). The ingestion of BA-rich foods may lead to respiratory, digestive, neurological and blood pressure disorders (Spano et al., 2010; Russo et al., 2011; Ladero et al., 2010; Linares et al., 2012). The BAs most commonly found in fermented dairy products are histamine and tyramine, but putrescine is also frequently detected and can occasionally accumulate in concentrations of up to 2.5 g per kg of cheese (unpublished data). Although the role and significance of putrescine in food safety and BA poisoning is yet to be established (FDA, 2012), a number of studies have reported neurotoxic and clinical severe effects in rats after treatment with putrescine doses of 200 mg/kg (Til et al., 1997; de Vera et al., 1992; Genedani et al., 1991). This may pose a risk taking into account that levels of 875 mg putrescine per kg have been detected in cheeses (Fernández et al., 2007a). Moreover, putrescine increases the toxic effects of other BAs frequently present in fermented foods and beverages, as histamine, tyramine and phenylethylamine (Russo et al., 2011; Landete et al., 2010). It can also react with nitrite to form carcinogenic nitrosamines (Ten Brink et al., 1990; Ladero et al., 2010). Furthermore, the derivatives of putrescine – the polyamines spermine and spermidine – play an important role in the promotion of malignancy in colorectal tumorigenesis, encouraging cell proliferation and migration (Linsalata and Russo, 2008; Moffatt et al., 2000; Shah and Swiatlo, 2008; Casero and Marton, 2007).

The biosynthesis of putrescine from arginine requires two catabolic reactions: decarboxylation and deimination. Two different production routes have been described depending on the order in which these reactions occur: 1) the agmatine deiminase (AGDI) pathway, in which arginine is first decarboxylated to agmatine and then deiminated to putrescine, and 2) the ornithine decarboxylase (ODC) pathway, in which arginine is first deiminated to ornithine and then decarboxylated

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to putrescine (Landete et al., 2010; Ladero et al., 2011). To date, only LAB strains producing putrescine via the AGDI pathway have been isolated from dairy products; these include *Lactobacillus brevis*, *Lactobacillus curvatus*, *Enterococcus faecalis* (Lucas et al., 2007; Llácer et al., 2007; Ladero et al., 2010) and *L. lactis* (Ladero et al., 2011).

A number of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* strains isolated from traditional cheeses made from raw milk have been genotypically classified by *16S rRNA* sequence comparisons (Fernández et al., 2011). Using PCR- and HPLC-based methods these strains were then screened and selected for their ability to produce putrescine from agmatine (Ladero et al., 2011). The sequencing of the genes related to the putrescine biosynthesis pathway revealed a cluster encoding five proteins: a putative transcriptional regulator (AguR), a putrescine carbamoyl transferase (AguB), an agmatine/putrescine antiporter (AguD), an agmatine deiminase (AguA), and a carbamate kinase (AguC) (Ladero et al., 2011). The catabolic genes *aguB*, *aguD*, *aguA* and *aguC* were found to be cotranscribed in a single mRNA in *L. lactis* subsp. *lactis* CHCC7244 (Ladero et al., 2011). However, little is known about the regulatory mechanism of the AGDI pathway in *L. lactis*.

The present work reports the effect of the carbon source on putrescine production and its influence on the genetic regulation of the putrescine biosynthesis cluster in putrescine-producing *L. lactis* subsp. *cremoris*. A mechanism for carbon catabolite repression by glucose, mediated by the transcriptional regulator CcpA (catabolite control protein A), is presented.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Table 1 shows the *L. lactis* subsp. *cremoris* strains used in the present study. Some of these were originally isolated from traditional cheeses (Fernández et al., 2011) and now belong to the IPLA collection. All strains were grown as standing cultures at 30 °C in M17 medium devoid of sugars (Oxoid, Basingstoke, United Kingdom) subsequently supplemented with desired sugar concentrations. To study the carbon source effect, glucose (or a substitute sugar) was added to the medium at 30 mM, 60 mM or 120 mM. When indicated, cultures were supplemented with 20 mM agmatine (M17 + AG) (Sigma-Aldrich, St. Louis, MO). When needed, chloramphenicol (5 μg/ml) was added to the culture medium.

2.2. Analytical chromatography methods

Cultures were centrifuged at 8000 *g* for 10 min and the resulting supernatants filtered through a 0.2 μm Supor membrane (Pall, NY). Putrescine and agmatine concentrations were then analysed by ultra performance liquid chromatography (UPLC) using a Waters H-Class ACQUITY UPLC apparatus controlled by Empower 2.0 software and employing a UV-detection method based on derivatization with diethyl ethoxymethylene malonate (Sigma-Aldrich) (Redruello et al., 2013). The quantitative analysis of sugars and lactic acid was undertaken by reverse-phase high performance liquid chromatography (RP-HPLC) using a Waters liquid chromatograph controlled by Millennium 32 software (Waters, Milford, MA), as indicated by Fernández et al. (2007b).

2.3. DNA manipulation procedures

The DNA manipulation and recombinant techniques used were essentially those described by Sambrook and Russell (2001). Table 2 shows the sequences of the primers used. Genetic constructions for *L. lactis* were achieved using *L. lactis* NZ9000 as an intermediate host. The plasmid and chromosomal DNA of *L. lactis* was isolated and transformed as described by de Vos et al. (1989). All enzymes for DNA technology were used according to their manufacturers' specifications.

2.4. Plasmid construction

A translational fusion construct was generated by cloning, in pNZ8048 (Kuipers et al., 1997, 1998), the AGDI cluster fragment (including the *aguR* promoter P_{aguR}, the *aguR* gene and the *aguB* promoter P_{aguB}) (Fig. 1B) attached to the reporter gene *gfp* (encoding the green fluorescent protein) in-frame. For this, the *gfp* gene (Geertsma et al., 2008) was first inserted into the *Nco*I–*Sph*I sites of the vector pNZ8048. The P_{aguR}–*aguR*–P_{aguB} fragment was then PCR-amplified using the primers indicated in Table 2, and cloned into the *Bgl*II–*Nco*I sites of the resulting construct, yielding plasmid pAGDI. The fusion was checked by nucleotide sequencing at Macrogen Inc. (Seoul, Republic of Korea).

2.5. RNA extraction

L. lactis was grown in M17 and M17 + AG supplemented with 30 mM, 60 mM or 120 mM glucose. Cells were harvested by centrifugation after 8 h of incubation and disrupted using glass beads (diameter of up to 50 μm) in a Fast-Prep FP120 Instrument (Thermo Savant-BIO101/Q-Biogen, CA) at 4 °C for 6 × 30 s at power setting 6. Total RNA was extracted using the TRI Reagent (Sigma) as recommended by the manufacturer. Purified RNAs were resuspended in RNAse-free water. After extraction, RNA samples were treated with DNase (Fermentas, Vilnius, Lithuania), as described by the manufacturer, to eliminate any DNA contamination. Total RNA concentrations were determined by UV spectrophotometry, measuring absorbance at 260 nm in a BioPhotometer (Eppendorf, Hamburg, Germany).

2.6. Gene expression quantification by reverse transcription quantitative PCR (RT-qPCR)

Gene expression analysis was performed by RT-qPCR in a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using SYBR® Green PCR Master Mix (Applied Biosystems). cDNA samples were synthesized from total RNA using the iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA). After 2-fold dilution of the cDNA, 5 μl was added to 20 μl

Table 1
Strains used in this study.

Strain	Characteristics	Source
L. lactis subsp. *cremoris* NZ9000	*L. lactis* subsp. *cremoris* MG1363 containing *nisR*–*nisK*. AGDI$^-$, put$^-$	Kuipers et al. (1998)
L. lactis subsp. *cremoris* GE2–14	Isolated from traditional cheeses. AGDI$^+$, put$^+$	Fernández et al. (2011)
L. lactis subsp. *cremoris* 2A22	Isolated from traditional cheeses. AGDI$^+$, put$^+$	Fernández et al. (2011)
L. lactis subsp. *cremoris* 2A27	Isolated from traditional cheeses. AGDI$^+$, put$^+$	Fernández et al. (2011)
L. lactis subsp. *cremoris* 4AA28	Isolated from traditional cheeses. AGDI$^+$, put$^+$	Fernández et al. (2011)
L. lactis subsp. *cremoris* 2A5	Isolated from traditional cheeses. AGDI$^+$, put$^+$	Fernández et al. (2011)
L. lactis subsp. *cremoris* 3AA9	Isolated from traditional cheeses. AGDI$^+$, put$^+$	Fernández et al. (2011)
L. lactis subsp. *cremoris* 3AA23	Isolated from traditional cheeses. AGDI$^+$, put$^+$	Fernández et al. (2011)
L. lactis subsp. *cremoris* MG1363Δ*ccpA*	MG1363 strain, chromosomal deletion of *ccpA*. AGDI$^-$, put$^-$	Zomer et al. (2007)

AGDI$^+$: contains the AGDI gene cluster; AGDI$^-$: has no AGDI gene cluster; put$^+$: a putrescine producer; put$^-$: not a putrescine producer.