Putrescine biosynthesis in *Lactococcus lactis* is transcriptionally activated at acidic pH and counteracts acidification of the cytosol

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Abstract

*Lactococcus lactis* subsp. *cremoris* CECT 8666 is a lactic acid bacterium that synthesizes the biogenic amine putrescine from agmatine via the agmatine deiminase (AGDI) pathway. The AGDI genes cluster includes *aguR*. This encodes a transmembrane protein that functions as a one-component signal transduction system, the job of which is to sense the agmatine concentration of the medium and accordingly regulate the transcription of the catabolic operon *aguBDAC*. The latter encodes the proteins necessary for agmatine uptake and its conversion into putrescine. This work reports the effect of extracellular pH on putrescine biosynthesis and on the genetic regulation of the AGDI pathway. Increased putrescine biosynthesis was detected at acidic pH (pH 5) compared to neutral pH. Acidic pH induced the transcription of the catabolic operon via the activation of the *aguBDAC* promoter P*aguB*. However, the external pH had no significant effect on the activity of the *aguR* promoter P*aguR*, or on the transcription of the *aguR* gene. The transcriptional activation of the AGDI pathway was also found to require a lower agmatine concentration at pH 5 than at neutral pH. Finally, the following of the AGDI pathway counteracted the acidification of the cytoplasm under acidic external conditions, suggesting it to provide protection against acid stress.

**Keywords:** *Lactococcus lactis*, Biogenic amines, Putrescine, AGDI cluster, pH induction, Acid stress.
1 Introduction

Dairy products, especially cheese, can accumulate large amounts of biogenic amines (BA) (Linares et al., 2011; Spano et al., 2010) particularly putrescine (Ladero et al., 2012), tyramine (Ladero et al., 2010b) and histamine (Fernandez et al., 2006; Ladero et al., 2008). Putrescine is one of the BAs most commonly found in cheese (Fernandez et al., 2007b; Ladero et al., 2012). It is mainly produced by lactic acid bacteria (LAB) via the enzymatic deamination of agmatine (Linares et al., 2012), a metabolite derived from the decarboxylation of arginine (Simon and Stalon, 1982). High concentrations of putrescine not only have a negative affect on the organoleptic properties of fermented foods, but can induce toxicological reactions, including increased cardiac output, tachycardia and hypotension. It might even be involved in the malignant transformation of cells (Ladero et al., 2010a; Linares et al., 2011).

The biosynthesis of putrescine by LAB can occur via the ornithine decarboxylase (ODC) and agmatine deiminase (AGDI) pathways. The AGDI pathway, via which agmatine is deaminated to putrescine with the concomitant production of CO₂, ATP and ammonium ions, is used by LAB such as Enterococcus faecalis (Ladero et al., 2012), Lactobacillus brevis (Lucas et al., 2007), Lactobacillus hilgardii (Alberto et al., 2007), Streptococcus mutans (Griswold et al., 2004), and even some Lactococcus lactis strains (del Rio et al., 2015a; del Rio et al., 2015b; Ladero et al., 2011; Linares et al., 2013). Putrescine biosynthesis via the AGDI pathway has been suggested a natural defence mechanism used by some LAB to withstand acidic environments; it is thought both to provide bio-energetic advantages to the cells (del Rio et al., 2015b) and to increase their acid
Dairy *L. lactis* subsp. *cremoris* CECT 8666 produces putrescine via the AGDI route (Linares et al., 2013); this increases its growth and induces the alkalinization of the culture medium (del Rio et al., 2015b). The AGDI gene cluster of this strain is composed of *aguR* (a regulatory gene) followed by the *aguBDAC* operon, which codes for the catabolic enzymes of the reaction and the agmatine/putrescine antiporter (Linares et al., 2013). The *aguR* gene is transcribed as a monocistronic mRNA from its own promoter $P_{aguR}$, independently of the *aguBDAC* genes. These latter genes are co-transcribed in a single polycistronic mRNA from the promoter of the first gene of the operon ($P_{aguB}$) (Linares et al., 2015). AguR is a transmembrane protein that functions as a one-component signal transduction system: it senses the agmatine concentration of the medium and regulates the transcription of the *aguBDAC* operon accordingly via a C-terminal cytoplasmic DNA-binding domain (Linares et al., 2015). The *aguBDAC* operon, which contains a cre site in the promoter $P_{aguB}$, is transcriptionally regulated by carbon catabolic repression (CCR) mediated by the catabolite control protein CcpA (Linares et al., 2013). The putrescine biosynthesis pathway in the strain used in this work (*L. lactis* subsp. *cremoris* CECT 8666) is regulated by CCR via glucose, but not by other sugars such as lactose or galactose (del Rio et al., 2015a; Linares et al., 2013).

The aims of the present work were to analyze the effect of extracellular pH on putrescine biosynthesis and its genetic regulation in *L. lactis*, and to examine the involvement of the AGDI pathway in the homeostasis of the cytosolic pH under acidic external conditions.
2 Material and Methods

2.1 Bacterial strains, plasmids and culture conditions

Table 1 shows the lactococcal strains and plasmids used in this study. *L. lactis* subsp. *cremoris* CECT 8666 (formerly *L. lactis* subsp. *cremoris* GE2-14) - the putrescine-producing strain used in this study - was previously isolated from Genestoso cheese, a Spanish artisanal cheese made from raw milk without the addition of any commercial starter culture (Fernandez et al., 2011; Ladero et al., 2011). *L. lactis* CECT 8666 (referred to as wild-type "wt") and the agmatine deiminase mutant *L. lactis* ∆agdi (referred to as "∆agdi") (del Rio et al., 2015b) were grown in M17 (Oxoid, Basingstoke, UK) supplemented with 0.5% (w/v) glucose (GM17) or 1% galactose (GalM17) (with no CCR regulation in either case) at 32°C without aeration. Where indicated, the medium was supplemented with agmatine (Sigma-Aldrich, Madrid, Spain). *L. lactis* subsp. *cremoris* NZ9000 strains harbouring plasmids pAG1, pAG2 or pAGDI were grown in GM17 medium supplemented with 5 µg ml⁻¹ chloramphenicol (Sigma-Aldrich). Overnight cultures of *L. lactis* strains were used as inocula (1% v/v) in 30 ml of culture medium. Microbial growth was monitored by measuring the optical density of cultures at 600 nm (OD₆₀₀) using a spectrophotometer (Eppendorf, NY, USA). pH-controlled cultures were produced in a Six-Fors® bioreactor (Infors AG, Bottmingen, Switzerland) containing 300 ml of culture medium supplemented with agmatine at the concentration indicated in each case, at a fixed pH (pH 5 or 7, maintained by the automatic addition of 1 N NaOH or 1 N HCl as needed). The reactor was maintained at 32°C, stirring at 50 rpm and with zero air input.

The pAG1, pAG2 and pAGDI plasmids were constructed as previously reported (Linares et al., 2013; Linares et al., 2015). pAG1 bears a fusion of the *aguR* promoter P₉₉ and the
reporter gene *gfp* (coding for green fluorescent protein GFP), pAG2 bears a fusion of the *aguB* promoter (P\_aguB) and *gfp*, and pAGDI bears a fusion of the AGDI cluster cassette P\_aguR-aguR-P\_aguB and *gfp*.

2.2 Putrescine analysis by ultra-high performance liquid chromatography

Culture supernatants were obtained by centrifugation (2000 g for 5 min). Putrescine was analyzed by ultra-high performance liquid chromatography (UHPLC) 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), following the protocol of Redrueño et al. (2013).

2.3 Quantification of gene expression by reverse transcription quantitative PCR

For all reverse transcription quantitative PCR (RT-qPCR) experiments, *L. lactis* cultures were grown in pH-controlled conditions in a Six-Fors® bioreactor as described in section 2.1. Total RNA was extracted from 2 ml of cultures collected at the end of the exponential phase of growth as previously described (del Rio et al., 2015a). RNA samples (2 µg of total RNA) were treated with 2 U of DNase (Fermentas, Vilnius, Lithuania) for 30 min at 37°C to eliminate any contaminating DNA. The reaction was stopped by adding 3 µl of 25 mM EDTA at 65°C for 1 h. The absence of contaminating DNA was checked for by quantitative real-time PCR (qPCR) using the corresponding RNA as a template, Power SYBR® Green PCR Master Mix (Applied Biosystems, UK), and a primer pair to amplify *tufA* (the reference gene) (Table 2) (Linares et al., 2013). cDNA was then synthesized from DNase-treated RNA samples using the iScript™ cDNA Synthesis Kit (Bio-Rad, Barcelona, Spain) following the manufacturer’s recommendations. cDNA samples were analyzed by qPCR using the primers listed in Table 2. The primer pairs used to amplify the *aguR* and
*aguB* genes of the AGDI cluster of *L. lactis* CECT 8666 and the *tufA* reference gene were those previously described (Linares et al., 2013). The primer pair used to amplify the *rpoA* reference gene was that previously described by Taibi et al. (2011). All qPCR reactions were performed following the protocol described by del Rio et al. (2015). Threshold cycle (Ct) values were calculated automatically using 7500 Software v.2.0.4 (Applied Biosystems). No-template samples were included in each run as negative controls. Relative gene expression was calculated using the $\Delta\Delta$Ct comparative method as previously described (Livak and Schmittgen, 2001).

### 2.4 Whole-cell fluorescence measurements

*L. lactis* NZ9000 cells harbouring the pAG1, pAG2 or pAGDI plasmids were grown in the bioreactor at pH 5 or pH 7 (see section 2.1) in GM17 supplemented with 1 mM agmatine and 5 µg ml$^{-1}$ chloramphenicol, for 7 h at 32°C. Whole-cell fluorescence was examined following the protocol described by Linares et al. (2013). Background fluorescence levels of *L. lactis* NZ9000 harbouring pAG1, pAG2 or pAGDI were assessed in cultures grown in GM17 supplemented with chloramphenicol in the absence of agmatine; these basal values were subtracted from the fluorescence results of the corresponding culture.

### 2.5 Measurement of cytosolic pH

Cytosolic pH measurements were performed using carboxyfluorescein succinimidyl ester (cFSE) (Sigma-Aldrich) (an internally conjugated fluorescence pH probe) following the protocol described by Perez et al. (2014) with minor modifications. The wt and Δ*agdi* strains were grown in GM17 in the presence or absence of 20 mM agmatine for 7 h. Cells collected by centrifugation from 1 ml of culture were washed twice in CPK buffer (citric acid
50 mM, disodium phosphate 50 mM and potassium chloride 50 mM) at pH 7.0. They were resuspended in 1 ml of CPK buffer at pH 4.5, pH 5, pH 5.5, pH 6.0, pH 6.5 or pH 7.0, and incubated at 32°C for 30 min in the presence of the cFSE probe (1 µM). The cells were then washed with CPK buffer at the required pH and resuspended in 1 ml of the same buffer supplemented with 10 mM lactose, and maintained for 15 min at 32°C. They were then washed once again in CPK buffer at the required pH. The cells grown in GM17 without agmatine were resuspended in 100 µl of CPK buffer at the required pH, while those that were grown in GM17 with agmatine were resuspended in 100 µl of CPK buffer at the required pH and supplemented with 20 mM agmatine. Fluorescence intensities were monitored for 10 min in a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA, USA) (excitation wavelengths 490 and 440 nm, emission wavelength 525 nm, excitation and emission slit widths 5 and 10 nm respectively). The value shown for each condition is the mean of three independent replicates (each the mean of values obtained over 10 min of monitoring). Background fluorescence levels were assessed in control cells not incubated with the cFSE probe; these values were subtracted from the fluorescence results. Cytosolic pH values were determined from the ratio of the fluorescence signal at 440/490 nm taken from a calibration curve constructed using buffers in the pH range 4.5-8.0 after equilibrating the cytosolic pH (pH_{in}) and external pH (pH_{out}) with 1µM valinomycin and 1µM nigericin respectively (Breeuwer et al., 1996).

2.6 Statistical analysis

Means ± standard deviations were calculated from at least three independent replicates. Means were compared using the Student t test. Significance was set at p<0.05.
3 Results

3.1 Acidic pH increases putrescine production in L. lactis CECT 8666

The influence of the pH of the medium on putrescine biosynthesis was studied in L. lactis CECT 8666 wt and Δagdi cultures grown in a bioreactor in GM17 supplemented with 20 mM agmatine for 12 h at 32°C and at a fixed pH of 5 or 7. Putrescine production and bacterial growth (OD$_{600}$) were monitored every hour (Fig. 1). The wt culture grown at pH 5 accumulated 16.7 mM putrescine 12 h after inoculation (Fig. 1A), while in the culture grown at pH 7 only 0.5 mM was accumulated (some 33 times less) (Fig. 1B). As expected, no putrescine production was observed in the Δagdi cultures, either at pH 5 or pH 7 (Fig. 1A and 1B, respectively). The wt culture showed greater growth compared to the Δagdi mutant at pH 5 (Fig. 1A), while no differences in growth were observed between the wt and the Δagdi cultures when the fermentation was performed at pH 7 (Fig. 1B).

3.2 The transcription of aguBDAC, but not aguR, is activated at acidic pH

Figure 2 shows the influence of the pH of the medium on the transcriptional activity of the AGDI cluster. The expression profiles of aguR and aguB (the first gene of the aguBDAC operon and used to represent the entire aguBDAC mRNA) were analyzed by RT-qPCR using RNA obtained from the wt cultures described in section 3.1. The reference condition was that for cultures grown at pH 7; tufA was chosen as the reference gene. No difference was seen in aguR expression when growth proceeded at pH 5 or pH 7 (Fig. 2A). Transcriptional analysis of the aguB mRNA showed a 170-fold up-regulation at pH 5 compared to that recorded in cultures grown at pH 7 ($p<0.001$) (Fig. 2B). Similar results were obtained using rpoA as the reference gene (data not shown). These results indicate
the transcriptional induction of the catabolic genes of the AGDI cluster at pH 5, with no upregulation of \textit{aguR}.

3.3 \textit{The aguB promoter} $P_{aguB}$ \textit{is induced at acidic pH}

The effect of pH on the activity of the \textit{aguR} and \textit{aguB} promoters ($P_{aguR}$ and $P_{aguB}$) was examined using the $P_{aguR}$-$\textit{gfp}$ and $P_{aguB}$-$\textit{gfp}$ fusions, in which the \textit{aguR} or \textit{aguBDAC} genes respectively were substituted by the \textit{gfp} reporter gene (Linares et al., 2015). Constructs were assayed in strain \textit{L. lactis} NZ9000 grown in GM17 with agmatine (in a bioreactor) grown at pH 5 or 7 for 7 h at 32°C, measuring whole-cell fluorescence (Fig. 3). Neither $P_{aguR}$-$\textit{gfp}$ nor $P_{aguB}$-$\textit{gfp}$ showed any detectable activity at either pH 5 or pH 7 (Fig. 3A and 3B). However, detectable activity was recorded for the $P_{aguR}$-$\textit{aguR}$-$P_{aguB}$-$\textit{gfp}$ construct (which includes $P_{aguR}$, the \textit{aguR} gene and $P_{aguB}$ attached to the \textit{gfp} gene) at both pH 5 and pH 7, an activity that was significantly greater ($p<0.05$) at the acidic pH (Fig. 3C).

3.4 \textit{Effect of the pH of the medium on the concentration of agmatine required to activate the transcription of the aguBDAC genes}

\textit{L. lactis} CECT 8666 cultures were grown in GalM17 in a bioreactor at pH 5 or pH 7 in the presence of small amounts of agmatine (0, 0.05, 0.1 or 0.2 mM) for 10 h at 32°C. The expression profiles of \textit{aguR} and \textit{aguB} were then analyzed by RT-qPCR in samples taken at the end of the exponential phase of growth (the reference condition was that of cultures grown with 0 mM agmatine at pH 7; \textit{tufA} was set as reference gene); putrescine production was also determined at the end fermentation. Figure 4A shows that a lower agmatine concentration was needed to activate the transcription of the catabolic genes at pH 5 than at pH 7. Cultures grown in the presence of 0.05 mM agmatine showed little
transcription of the *aguBDAC* operon at pH 7, but 10 times as much at pH 5. In the presence of 0.1 mM agmatine, transcription at pH 5 was about 30 times that detected at pH 7, and about 6 times that seen in cultures grown with 0.2 mM agmatine (*p*<0.05). The transcriptional activation of the *aguBDAC* operon at acidic pH correlated with a greater accumulation of putrescine in cultures grown at pH 5 than at pH 7 (Fig. 4B). The comparative transcriptional analysis of *aguR* confirmed that, independent of the pH of the medium, the agmatine concentration had no influence on the transcription of *aguR* (data not shown).

3.5 *The AGDI pathway counteracts the acidification of the cytosol under acidic external conditions*

Changes in cytosolic pH (pH\textsubscript{in}) in the wt and the Δ*agdi* mutant strains were monitored when cells were incubated at different extracellular pHs (pH\textsubscript{out}) (from 7.0 to 4.5), in the presence and absence of agmatine. Figure 5 shows the variations detected in pH\textsubscript{in} at different pH\textsubscript{out}. The wt cultures grown in the presence of agmatine maintained a significantly higher pH\textsubscript{in} (0.3 to 0.4 pH unit) when exposed to an acidic pH\textsubscript{out} (6.0, 5.5, 5.0 or 4.5) than cultures grown in the absence of agmatine (*p*<0.05) (Fig. 5A). In contrast, the Δ*agdi* cultures grown with or without agmatine showed no significant differences in pH\textsubscript{in} at any pH\textsubscript{out} tested (Fig. 5B). These results reveal the involvement of the *L. lactis* AGDI pathway in the maintenance of cytosolic pH when cells are exposed to acidic external environments.
4 Discussion

In dairy fermentations, the metabolism of lactose into lactic acid leads to the production of acidic external environments, which in turn are associated with greater BA production (Fernandez et al., 2007b; Marcobal et al., 2006). There has always been much interest, therefore, in how the external pH affects the regulation of pathways leading to the production of different BAs. Certainly, it is known that an acidic pH activates the expression of genes involved in different BA biosynthesis pathways (Arena et al., 2011; Griswold et al., 2006; Linares et al., 2009; Perez et al., 2014), and that some enzymes (decarboxylases and deiminases) are active at acidic but not at neutral or alkaline pHs (Griswold et al., 2006; Moreno-Arribas and Lonvaud-Funel, 2001; Schelp et al., 2001).

The present work examines the effect of the external pH on putrescine biosynthesis in *L. lactis* - undoubtedly the most important dairy starter. The results show that putrescine production is higher in acidic media (pH 5) than neutral media (Fig. 1). The increase in putrescine production at pH 5 suggests that the AGDI cluster is regulated by the pH of the culture medium, and indeed, an acidic pH was found to significantly increase the expression of the *aguBDAC* catabolic genes (Fig. 2B). In addition, the transcriptional activity of the *aguBDAC* operon promoter was found to be increased when the transcriptional fusion P$_{aguR-aguR-P_{aguB}-gfp}$ was assayed in *L. lactis* NZ9000 cells growing at pH 5 compared to those growing at pH 7 (Fig. 3C). These results show that acidic pH activates the transcription of the *aguBDAC* catabolic genes, increasing the production of putrescine. A stimulatory effect of acidic pH on the production of putrescine has previously been described in *S. mutans* (Griswold et al., 2006). In contrast, Suarez et al. (2013) found the AGDI route of *E. faecalis* JH2-2 not to be induced by acidic pH. To our knowledge, the effect has not been studied in any other LAB, although it has been verified that acidic pH
induces transcriptional activity involved with other BA production pathways (e.g., tyramine biosynthesis) in *E. durans* 655 (Linares et al., 2009) and *E. faecalis* V583 (Perez et al., 2014).

The present work also examined the possible role of *aguR*, the regulatory gene of the AGDI cluster, in the regulation of cytosolic pH. The extracellular pH had no effect on the expression of *aguR* (Fig. 2A) or on the activity of its promoter $P_{aguR}$ (Fig. 3A). Neither did it have any effect on the activity of $P_{aguB}$ in the absence of *aguR* (Fig. 3B). These results agree with the previously proposed constitutive expression of *aguR* in *L. lactis* (Linares et al., 2015). Thus, regardless of the environmental conditions, AguR is constantly present at the cell surface of *L. lactis* AGDI-positive strains, sensing the agmatine concentration of the medium and regulating the transcription of the catabolic operon *aguBDAC* via the activation of $P_{aguB}$. The effect of pH on catabolic gene expression might occur via conformational changes in the structure of AguR, as proposed by Liu and Burne (2009) for its orthologue in *S. mutans* UA159. It may be that an acidic pH favours an AguR conformation that renders signal transduction to the DNA binding domain more efficient. Similarly, in *L. lactis*, an acidic pH might induce conformational changes in AguR that enhance the activator signal of $P_{aguB}$ and consequently the transcription of the *aguBDAC* operon. In fact, the present results show that, at acidic pH, the concentration of agmatine needed for maximum transcriptional activation of the *aguBDAC* operon is lower than at neutral pH (Fig. 4A).

Putrescine biosynthesis in *L. lactis* at pH 5 correlated with an increase in bacterial growth (Fig. 1), as previously described in cultures grown at uncontrolled pH (del Rio et al., 2015b). Agmatine catabolism via the AGDI pathway produces ATP, but also ammonium ions, the accumulation of which in the extracellular medium leads to its alkalinization (del
Rio et al., 2015b). The ATP generated, rather than the alkalinization of the culture medium, is the main factor behind the increase in bacterial growth (del Rio et al., 2015b). However, the induction of the AGDI pathway at acidic pH supports the hypothesis that the system may be a component of an adaptive acid tolerance response in *L. lactis*, as has been described for other LAB such as *L. brevis* IOEB 9809 (Lucas et al., 2007), *E. faecalis* JH2-2 (Suarez et al., 2013) and *S. mutans* UA159 (Griswold et al., 2006; Liu and Burne, 2009). In the last of these, the AGDI route is involved in the increase of acid resistance, and thus in improving the competitive fitness of the organism at low pH, via the generation of ammonium ions which alkalinize the cytoplasm, and via the production of ATP, which could be used for growth and for powering proton extrusion (Griswold et al., 2006). Alkalinization induced by ammonium ions has been suggested to contribute towards acid resistance in *E. faecalis* JH2-2 (Suarez et al., 2013) and *L. hilgardii* (Alberto et al., 2007).

The present results indicate that, when agmatine is available, the AGDI pathway participates in the maintenance of the cytosolic pH in *L. lactis* cells faced with an acidic (pH 4.5-6) culture medium (Fig. 5A). This effect was abolished in the ∆agdi cultures (Fig. 5B), further implicating the involvement of the pathway. A role in cellular pH homeostasis has also been suggested for the arginine deiminase (ADI) pathway of *L. lactis*, via which arginine is catabolized to ornithine by reactions that closely resemble those of the AGDI pathway. The ADI route has been widely studied and is considered to be a pathway via which *L. lactis* obtains additional ATP while at the same time combating acid stress through ammonium ion production (Larsen et al., 2004). Similarly, in *Staphylococcus epidermidis*, the ammonium ions generated via the ADI pathway have been linked to the alkalinization of the cytosolic pH as a response to acidic external environments (Lindgren et al., 2014). Thus, the ADI and AGDI pathways appear to be similar and to have similar functions.
While the AGDI pathway provides important advantages to *L. lactis* growing in acidic environments such as dairy fermentations, the product is putrescine, an unwanted product from a food safety point of view. The *L. lactis* strains used in starter cultures therefore need to be thoroughly examined to make sure that no AGDI-positive strains are present.

5 Acknowledgements

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6 References


Lindgren, J.K., Thomas, V.C., Olson, M.E., Chaudhari, S.S., Nuxoll, A.S., Schaeffer, C.R., Lindgren, K.E., Jones, J., Zimmerman, M.C., Dunman, P.M., Bayles, K.W., Fey, P.D.,


7 Figure legends

**Figure 1.** Effect of external pH on putrescine production. wt and Δagdi mutant strains were grown in a bioreactor at pH 5 (A) or pH 7 (B) in GM17 in the presence of 20 mM agmatine for 12 h. Supernatants of samples taken each hour were analyzed by UHPLC to determine the concentrations of putrescine in the extracellular medium. Bacterial growth was determined by measuring the absorbance of the culture at 600 nm (OD\textsubscript{600}). A representative experiment of the duplicates performed is shown.

**Figure 2.** Effect of external pH on the transcriptional activity of the AGDI cluster, as determined by RT-qPCR. *L. lactis* CECT 8666 was grown in a bioreactor at pH 5 or pH 7 in GM17, in the presence of 20 mM agmatine. The relative gene expression of *aguR* (A) and *aguB* (B) (representing the whole *aguBDAC* operon) was determined by RT-qPCR. The data represent the mean of three RNA extractions; error bars represent standard deviations. *Significantly different from cells grown at pH 7 (*p*<0.001).

**Figure 3.** Effect of external pH on the activity of the P\textsubscript{aguR} and P\textsubscript{aguB} promoters as determined by whole-cell fluorescence. *L. lactis* NZ9000 cells harbouring either the P\textsubscript{aguR-\textasciitilde\textit{gfp}} (A), the P\textsubscript{aguB-\textit{gfp}} (B), or the P\textsubscript{aguR-\textit{aguR}-P\textsubscript{aguB-\textit{gfp}} (C) genetic fusion, were grown in a bioreactor at pH 5 or pH 7 in GM17 supplemented with 5 µg ml\textsuperscript{-1} chloramphenicol, in the presence of 1 mM agmatine for 7 h (after which time GFP fluorescence was monitored). The values shown are the means of triplicates. Vertical bars show the standard deviation. *Significantly different from *L. lactis* NZ9000 harbouring the construction P\textsubscript{aguR-\textit{aguR}-P\textsubscript{aguB-\textit{gfp}}
gfp and grown at pH 7 (p<0.05). P_{aguR}: aguR promoter, P_{aguB}: aguB promoter, gfp: gene encoding green fluorescent protein (GFP). a.u.: arbitrary units.

**Figure 4.** Effect of the pH of the medium on the concentration of agmatine required to activate the transcription of the aguBDAC catabolic genes, as determined by RT-qPCR. *L. lactis* CECT 8666 cultures were grown in a bioreactor at pH 5 or pH 7 in GalM17 medium supplemented with 0 mM, 0.05 mM, 0.1 mM or 0.2 mM agmatine for 10 h at 32°C. (A) The expression of aguB was assessed by RT-qPCR and calculated relative to the transcript level detected in samples grown in GM17 with no agmatine and at pH 7. The data represent the mean of three different RNA extractions; error bars represent standard deviations. (B) Putrescine production was determined by UHPLC in supernatants of cultures collected after 10 h of fermentation. The data represent the mean of three different cultures; error bars represent standard deviations. *Significantly different from the transcriptional activation of aguB at pH 7 (p<0.05).

**Figure 5.** Variations in the cytosolic pH (pHₘ) at different external pHs (pHₜₐₓ) (7, 6.5, 6, 5.5, 5 and 4.5), measured using a cFSE probe in resting wt (A) or Δagdi (B) cells grown in the absence (white circles; control condition) or presence of 20 mM agmatine (black circles). The data represent the mean of triplicates; error bars represent standard deviations. *Significantly different from wt cultures grown in the absence of agmatine (p<0.05). GM17+A: GM17 medium supplemented with 20 mM agmatine.
### Table 1. Bacterial strains and plasmids used in this study

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<th>Material</th>
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<td>(Fernandez et al., 2011), CECT*</td>
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<td>CECT 8666 knock-out for the AGDI cluster</td>
<td>(del Rio et al., 2015b)</td>
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*CECT: Colección Española de Cultivos Tipo (Spanish Collection of Type Cultures)

P$_{aguR}$: aguR promoter; P$_{aguB}$: aguB promoter; gfp: gene encoding green fluorescent protein; Cm': chloramphenicol resistance
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<th>Primer</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>aguR^a</td>
<td>qAguR-F</td>
<td>CTATCGACAGGTTAAGCAAGAGCAGTT</td>
<td>(Linares et al., 2013)</td>
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<td>qAguR-R</td>
<td>TCCAAAGATGAGGCCATTATGC</td>
<td>(Linares et al., 2013)</td>
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<tr>
<td>aguB^a</td>
<td>AguB-F</td>
<td>ACTTGGTGACATGAAACAATAGAGAT</td>
<td>(Linares et al., 2013)</td>
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<tr>
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<td>AguB-R</td>
<td>GTCAACACGTGCCATTATGATATCG</td>
<td>(Linares et al., 2013)</td>
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<tr>
<td>tufA^5</td>
<td>qtufF</td>
<td>TCTTCATCTGACATGAAACAATAGAGAT</td>
<td>(Linares et al., 2013)</td>
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<td>qtufR</td>
<td>GAACACATCTGACATGAAACAATAGAGAT</td>
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<tr>
<td>rpoA^5</td>
<td>rpoA-F</td>
<td>CACGGGCAGGTTCAACTTG</td>
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<td></td>
<td>rpoA-R</td>
<td>TTCGGCTGACGAAATAAAG</td>
<td>(Taibi et al., 2011)</td>
</tr>
</tbody>
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^ Target genes
^ Reference genes
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5