AguR is a transmembrane transcription activator of the putrescine biosynthesis operon in *Lactococcus lactis*, and acts in response to agmatine concentration

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Abstract

Dairy industry fermentative processes mostly use *Lactococcus lactis* as a starter. However, some dairy *L. lactis* strains produce putrescine - a biogenic amine that raises food safety and spoilage concerns - via the agmatine deiminase pathway (AGDI). The enzymatic activities responsible for putrescine biosynthesis in this bacterium are encoded by the AGDI gene-cluster. The role of the catabolic genes *aguB*, *aguD*, *aguA* and *aguC* has been studied, but knowledge regarding the role of *aguR* (the first gene in the cluster) remains limited. In the present work, *aguR* was found to be a very low-level constitutively expressed gene that is essential for putrescine biosynthesis and is transcribed independently of the polycistronic mRNA encoding the catabolic genes (*aguBDAC*). In response to agmatine, AguR acts as a transcriptional activator of the *aguB* promoter (P_{aguB}), which drives transcription of the *aguBDAC* operon. Inverted sequences required for P_{aguB} activity were identified by deletion analysis. Further work indicated AguR to be a transmembrane protein which might function as a one-component signal transduction system that senses the agmatine concentration of the medium and accordingly regulates the transcription of the *aguBDAC* operon through a LuxR_C_like cytoplasmic DNA binding domain.

INTRODUCTION

Lactococcus lactis is the lactic acid bacterium (LAB) most widely used as a primary starter in the dairy industry, especially in cheese manufacturing. Despite its 'qualified presumption of safety' (QPS) status (awarded by the European Food and Safety Authority [EFSA]) and its 'generally regarded as safe' (GRAS) status (awarded by the Food and Drug Administration [FDA]), some *L. lactis* strains possess enzymatic activities that produce undesirable flavors associated with food spoilage (1). Some even produce toxic compounds such as the biogenic amine (BA) putrescine (2). Putrescine - together with histamine and tyramine - is one of BAs in fermented dairy products most frequently encountered at potentially unsafe levels (3,4,5). It has a synergistic effect on the toxicity of other BAs, and can also react with nitrite to form carcinogenic nitrosamines (4,6). In addition, the metabolism of putrescine and of its derivatives (the polyamines spermine and spermidine) plays an important role in the promotion of colorectal tumorigenesis, via effects on cell proliferation and migration (7-10).

A number of putrescine-producing *L. lactis* strains of the subspecies *lactis* and *cremoris* isolated from artisanal cheeses have been shown to have a functional agmatine deiminase (AGDI) pathway. This catabolizes agmatine (a decarboxylated derivative of arginine) (11) into putrescine, yielding one molecule of ATP, one

73 molecule of CO_2 and two ammonium ions (2). AGDI pathway increases the growth
74 of *L. lactis* and causes the alkalinization of the culture medium, although it does not
75 seem to be an acid stress resistance mechanism (58). The AGDI cluster of *L. lactis*
76 is composed of five genes - *aguR, aguB*, *aguD*, *aguA* and *aguC* - the last four
77 being responsible for the conversion of agmatine to putrescine (2,12). Agmatine
78 enters the cell via AguD (an agmatine-putrescine antiporter encoded by *aguD*), and
79 is then hydrolyzed to *N*-carbamoylputrescine and an ammonium ion by AguA (an
80 agmatine deiminase encoded by *aguA*). AguB is a putrescine
81 carbamoyltransferase encoded by *aguB* that catalyzes the phosphorolysis of *N*-
82 carbamoylputrescine, yielding putrescine and carbamoylphosphate. Finally, a
83 phosphate group is transferred from carbamoylphosphate to ADP by AguC (a
84 carbamate kinase encoded by *aguC*) to generate ATP, CO_2 and a further
85 ammonium ion. Putrescine is then exchanged for agmatine via the antiporter AguD
86 (2). The protein encoded by *aguR* showed primary structure similarity to the AguR
87 of *Streptococcus mutans*, a transcriptional activator of the agmatine deiminase
88 system (13). The aim of the present work was to investigate whether *aguR* of
89 *Lactococcus lactis* is involved in the transcriptional regulation of the AGDI cluster.
90 The strain selected for study was *L. lactis* subsp. *cremoris* CECT8666 (formerly
91 GE2-14); originally isolated from a traditional cheese (2) this strain is a strong
92 putrescine-producer (12), and its genome has been completely sequenced (14).
93 Although previously demonstrated in a *L. lactis* subsp. *lactis* putrescine-producing
94 strain (2), it was first confirmed that the present strain's *aguR* was transcribed
95 independently of the catalytic genes, which are expressed as an operon
96 (*aguBDAC*). The construction of a Δ*aguR* knock-out mutant, and its subsequent

analysis, showed AguR to activate putrescine production. Transcriptomic studies, confirmed by independent transcriptional analysis of *aguR* and the *aguBDAC* operon, verified the involvement of AguR in the transcriptional activation of *aguBDAC.* Moreover, the transcriptional activation of *aguBDAC* was dependent on the agmatine concentration of the culture medium. *In silico* analysis of the topology of AguR, plus comparative studies of its structure, revealed the presence of a putative DNA binding domain at the C-terminus. It was also confirmed that AguR is located on the cell surface. Taking these results together, AguR would seem to act as a one-component signal transduction system that senses the agmatine concentration in the environment and accordingly regulates the transcription of the *aguBDAC* operon.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Table 1 shows the strains and plasmids used in this study. *Lactococcus lactis* strains were grown in M17 (Oxoid, Basingstoke, United Kingdom) supplemented with 30 mM glucose (GM17) or 60 mM galactose (GalM17) to prevent carbon catabolic repression (CCR) of the ADGI pathway (12). Where indicated, media were supplemented with agmatine (Sigma-Aldrich, Barcelona, Spain) at the specified concentration. *Escherichia coli* strains were grown in Luria Bertani (LB) medium at 37°C with aeration (15). When plasmid-containing clones were grown, the medium was supplemented with the appropriate antibiotics: for *L. lactis*, 5 μg ml^{-1} of chloramphenicol (Cm) and 2 μg ml^{-1} of erythromycin (Em); for *E. coli*, 150 μg ml^{-1} of Em.

Analysis of putrescine production by ultra high performance liquid chromatography

Cultures were grown in GM17 medium in the presence of 20 mM agmatine for 24 h. The cultures were then centrifuged at 8000 *g* and the supernatants collected. The putrescine concentration in the supernatants (100 µl) was assessed 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) (16).

DNA manipulation

L. lactis genomic DNA was obtained using Kirby lytic mix following a previously described protocol (17). Genetic constructs for *L. lactis* were produced using *L. lactis* NZ9000 as an intermediate host. Plasmid DNA from *L. lactis* was isolated and transformed as described previously (18). Genetic constructs for *E. coli* were produced using *E. coli* DH11S (Life technologies, Madrid, Spain) as an intermediate host. The *E. coli* plasmid DNA was isolated by the alkaline lysis method (15). Electroporation was performed in a Bio-Rad pulser apparatus (Bio-Rad, Barcelona, Spain) following the manufacturer's instructions. Restriction endonuclease digestions, alkaline phosphatase treatments, ligations and other DNA manipulation procedures were performed according to standard methods (15). PCR amplifications were performed in a MyCycler™ thermal cycler (Bio-Rad)

using Phusion High-Fidelity DNA polymerase (Thermo Scientific, Barcelona, Spain) according to the manufacturer's protocol. Table 2 shows the primers used for PCR amplifications. The primers used to amplify fragments of the *L. lactis* CECT8666 AGDI cluster were based on its nucleotide sequence (GenBank Accession No. HG317493.1). All plasmids constructed in this work were checked by nucleotide sequencing (performed by Macrogen Inc. Seoul, Republic of Korea).

Reverse Transcription PCR (RT-PCR)

Cells were grown in GM17 culture medium in the presence of 20 mM agmatine. Two milliliters of culture were collected at the end of the exponential phase of growth. Total RNA was extracted as previously described (19). cDNA was then synthesized from DNase-treated RNA samples using the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's recommendations. The *ycaC-aguR*, *aguR-aguB*, *aguB-aguC*, *aguC-aguD* and *aguD-aguA* intergenic regions (Fig. 2A) were analyzed by PCR amplification using cDNA as a template and specific pairs of primers (see Table 2). PCR reactions were performed using 2 µl of cDNA and 0.4 µM of each gene-specific primer. Amplifications were performed for 35 cycles (94°C for 30 s, 55°C for 45 s, and 72°C for 1 min); the resulting amplicons were separated on 1.5% agarose gels in TAE buffer. The absence of contaminating DNA was checked via omission of reverse transcriptase in PCR reaction; this was performed under the conditions described above, using the corresponding RNA as a template.

Construction of a *L. lactis* CECT 8666 Δ*aguR* deletion mutant

A *L. lactis* CECT8666 Δ*aguR* deletion mutant was constructed by homologous recombination using the selection/counter-selection vector pCS1966 (20). Table 2 shows the primers used to generate the Δ*aguR* knock-out. They were designed to include the following restriction recognition sites: *Spe*I (in primer KO-214AguR-AF2), *Pst*I (in KO-214AguR-AR), *Pst*I (in KO-214AguR-BF) and *Cla*I (in KO-214AguR-BR). A 826 bp PCR fragment containing a 610 bp fragment of the 3' end of the *ycaC* gene (upstream of *aguR*, GenBank Accession No. HG317493.1), the intergenic region between *ycaC* and *aguR*, and the sequence coding for the five first amino acids of the *aguR* gene of *L. lactis* CECT8666, was amplified using primers KO-214AguR-AF2 and KO-214AguR-AR. The resulting fragment was digested with *Spe*I and *Pst*I restriction enzymes and cloned into the pCS1966 vector, rendering the plasmid pIPLA1269. A second 1110 bp PCR fragment containing the last 18 bp of *aguR*, the intergenic region between *aguR* and *aguB*, and 856 bp of the beginning of the *aguB* gene, was PCR amplified using primers KO-214AguR-BF and KO-214AguR-BR. The resulting fragment was digested with *Pst*I and *Cla*I and cloned into the plasmid pIPLA1269, rendering the plasmid pIPLA1713. Plasmid pIPLA1713 was then transformed and integrated into *L. lactis* CECT8666 electrocompetent cells by homologous recombination. The methodology described by (20), based on 5-fluoroorotate sensitivity, was used to select for loss of the plasmid (second recombination step). The resulting mutants lacking *aguR* (*L. lactis* CECT 8666 Δ*aguR*) were confirmed by nucleotide sequence analysis of the amplicon obtained using primers KO-214AguR-AF2 and KO-

214AguR-BR, which rendered the expected 1936 bp fragment instead of the 2894 bp fragment corresponding to the wild type (WT) strain (data not shown).

DNA microarray experiments and data analysis

L. lactis CECT 8666 DNA microarrays (Agilent Technologies, Santa Clara, CA) were designed using the Agilent eArray v.5.0 program according to the manufacturers' recommendations (http://earray.chem.agilent.com/earray/). Each microarray (8x15 K) was designed to contain spots of two different 60-mer oligonucleotide probes (in duplicate) specific for each of the 2635 coding DNA sequences (CDS) representing the protein coding genes of the *L. lactis* CECT8666 genome (GenBank Accession No AZSI00000000.1) (14).

Total RNA was isolated from 10 ml of *L. lactis* CECT8666 and from Δ*aguR* mutants, both grown to late exponential phase in GalM17 supplemented with 20 mM agmatine. cDNA synthesis was performed using the SuperScript® III Reverse Transcriptase Kit (Life Technologies, Bleiswijk, Netherlands), following the manufacturer's instructions. Twenty micrograms of cDNA were then labeled with Cy-3/Cy-5 dyes using the DyLight® Amine-Reactive Dyes Kit (Thermo Scientific, Amsterdam, Netherlands) following the manufacturer's protocol. Nine hundred nanograms of both Cy3- and Cy5-labeled cDNA were then mixed and hybridized for 17 h at 60°C in the *L. lactis* CECT8666 DNA microarray using the *In situ* Hybridization Kit Plus (Agilent Technologies) following the manufacturer's instructions. Slides were scanned using a GenePix 4200A Microarray Scanner

(Molecular Devices, Sunnyvale, CA) and the images analyzed using GenePix Pro v.6.0 software. Background subtraction and LOWESS normalization were performed using the standard routines provided by GENOME2D software available at http://server.molgenrug.nl/index.php/dna-microarrays. DNA microarray data were obtained from three independent biological replicates and two technical replicates (including a dye swap). Expression ratios were calculated from the comparison of four spots per gene per microarray (total of 20 measurements per gene). A gene was considered differentially expressed when a *p* value of at least <0.05 was obtained and the expression fold-change was at least >|0.5|. The microarray data were deposited in Gene Expression Omnibus (GEO) database under the Accession No. GSE59514.

Quantification of gene expression by reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from cultures collected at the end of the exponential phase of growth and cDNA synthesized by retro-transcription as described above. cDNA samples were analyzed by quantitative real-time PCR (qPCR) using an ABI Prism Fast 7500 sequence detection system (Applied Biosystems, Carlsbad, CA). Reactions were performed as previously described (19) in a 25 µl reaction volume, which included 9 µM of each primer and Power SYBR® Green PCR Master Mix (which contains ROX as a passive reference) (Applied Biosystems). Cycling was performed under the Applied Biosystems default settings. Amplifications were performed with previously described specific primers (12) (Table 2); primers

specific for the thermo-unstable elongation factor (*tuf*) (12) and RNA polymerase alpha-subunit (*rpoA*) (21) genes were used as references. The linearity and amplification efficiency of the reactions were tested for each primer pair at five points in a 10-fold dilution series of *L. lactis* subsp. *cremoris* CECT8666 genomic DNA. Samples with no template were included in each run as negative controls. Relative gene expression was calculated using the $\Delta\Delta$Ct comparative method as previously described (22). For each condition, RT-qPCR analysis was performed on RNA purified from three independently grown cultures. Statistical comparisons were made using the Student *t* test; significance was set at $p<0.05$.

Generation of fusions with the *gfp* reporter gene

A transcriptional fusion of the *aguR* promoter (P_{aguR}) attached to the *gfp* reporter gene (which codes for green fluorescent protein [GFP]) was generated (P_{aguR}-*gfp*). For this, the P_{aguR} fragment was PCR-amplified using AgurNco and AguRBglII primers (Table 2) and cloned into the *Bgl*II-*Nco*I sites of plasmid pNZ8048 (23,24). The *gfp* gene was then PCR-amplified from plasmid pNZcGFP (25) using primers Gffor and Gfrev, and cloned into the resulting vector as a *Nco*I-*Sph*I fragment, yielding the plasmid pAG1.

Similarly, a transcriptional fusion of the promoter of *aguB* (P_{aguB}) attached to the *gfp* reporter gene was generated (P_{aguB}-*gfp*). For this, the P_{aguB} fragment was PCR-amplified using primers PtcNco and PtcBglII (Table 2) and cloned into the *Bgl*II-

*Nco*I sites of plasmid pNZ8048. The *gfp* gene was then PCR-amplified from plasmid pNZcGFP using primers Gffor and Gfrev, and inserted into the resulting vector as a *Nco*I-*Sph*I fragment, yielding the pAG2 plasmid.

Finally, for the cellular localization of AguR, a translational fusion between *aguR* and *gfp* under the control of the nisin-inducible promoter (P_{nisA}) was generated. For this, *aguR* was PCR-amplified using the Agurlicf and Agurlicr primers (Table 2) and cloned into the *Swa*I restriction site of the pNZcLIC-GFP expression vector (25,26), yielding the plasmid pAG3. All constructs were checked by nucleotide sequencing (performed by Macrogen Inc.).

Generation of *aguB* promoter deletion constructions

Plasmids pAGDIΔ1, pAGDIΔ2, pAGDIΔ3 and pAGDIΔ4 bearing deleted versions of P_{aguB} were all derived from previously constructed pAGDI (12). Plasmid pAGDI carries the cassette P_{aguR}-*aguR*-P_{aguB} fused to the *gfp* reporter gene. For each construct, pAGDI was first methylated with Dam methylase and S-adenosyl methionine (New England Biolabs, Hertfordshire, UK) following the manufacturer's instructions. The whole pAGDI plasmid was amplified using divergent primers (Table 2) flanking the region of P_{aguB} to delete. An *Eco*RI target site was included in the primers so that the obtained amplicons could be digested with *Eco*RI and self-ligated. The ligation mixture was digested with *Dpn*I (in order to digest the original pAGDI plasmid used as a Dam-methylated template) before transformation in *L. lactis* NZ9000.

282 **Whole-cell fluorescence measurements**

283 For whole-cell fluorescence measurements, equal quantities of cells were
284 harvested, washed, and subsequently resuspended in 50 mM KPi, pH 7.2 as
285 previously described (25). GFP emission was measured in a volume of 250 µl of
286 cells, using a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto,
287 CA) (excitation wavelength 485 nm; emission wavelength 530 nm). For direct
288 comparison, all GFP fluorescence data were normalized to the same OD_{600}.
289 Background fluorescence levels were assessed by measuring non-fluorescent
290 control cells; these values were subtracted. Statistical comparisons were made
291 using the Student *t* test; significance was set at $p<0.05$.

292

293 **Fluorescence microscopy**

294 *L. lactis* NZ9000 cells containing the pAG3 plasmid carrying the P_{nisA}-*aguR-gfp*
295 translational fusion (Table 2) were grown in GM17 supplemented with
296 chloramphenicol (5 µg ml^{-1}) at 30°C until an OD_{600} of 0.6. The expression of *aguR-*
297 *gfp* was then induced by the addition of 0.5 nM nisin for 2 h. Fluorescence was
298 analyzed using a Nikon Eclipse 90i (Nikon UK, Kingston, UK) microscope running
299 iControl software and ACT-2U camera control software, employing a X100
300 objective and the B2A Nikon filter (excitation filter 450-490 nm, dichroic mirror 505
301 nm, emission filter 520 nm). A minimum of 15 random fields of view were observed
302 for each sample. Each experiment was performed in triplicate.

303

304 ***In silico* analysis of inverted sequences of the *aguB* promoter**

In silico analysis of the nucleotide sequence of the putative *aguB* promoter (GenBank Accession No. HG317493.1, nucleotides 3518 to 3726) was performed using Clone Manager V.7 software (Scientific & Educational Software, Cary, NC).

In silico analysis of AguR

The NCBI BLASTP program (http://blast.st-va.ncbi.nlm.nih.gov) was used to determine the similarity of the deduced amino acid sequence for AguR to sequences present in databases. Functional domains in AguR were analyzed using the Pfam database (http://pfam.xfam.org/) (27). The topology of AguR was predicted using computer-based algorithms available on the SOSUI server (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html) (28). Homology modeling was performed by searching for the most suitable template protein structure using the SWISS MODEL workspace (http://swissmodel.expasy.org) (29). Model refinement and editing were performed using Swiss-PdbViewer software v.4.0.4 (30).

RESULTS

aguR is transcribed independently of the *aguBDAC* operon

The transcriptional profiles of *aguR* and of the genes encoding the putrescine biosynthetic pathway (*aguB*, *aguD*, *aguA* and *aguC*) were determined. Total RNA was isolated from *L. lactis* CECT8666 cells grown in GM17 supplemented with 20 mM agmatine, and was used in RT-PCR analysis involving five sets of primers (referred to as 1 to 5 in Fig. 1 A) designed to amplify the regions spanning the gene

junctions (Table 2, Fig. 1A). The *ycaC-aguR* and *aguR-aguB* intergenic regions rendered no RT-PCR product (Fig. 1B, lanes 1 and 2, respectively), indicating that neither *ycaC-aguR* nor *aguR-aguB* are co-transcribed. In fact, a potential transcription terminator was found in each intergenic region (ΔG = -9.4 and ΔG = −10.3 kcal/mol respectively). In contrast, RT-PCR amplifications of the *aguB-aguD*, *aguD-aguA* and *aguA-aguC* intergenic regions rendered DNA fragments of the expected size (Fig. 1B, lanes 3, 4 and 5 respectively), showing that *aguB*, *aguD*, *aguA* and *aguC* are co-transcribed. The RT-PCR reactions for the negative controls failed to yield any amplification product. DNA template controls (to ensure PCR fidelity for each primer pair) uniformly yielded the PCR product of the expected size. Overall, these results indicate that the *aguR* gene is transcribed from its own promoter (P_{aguR}) as a monocistronic mRNA, and that its transcription is independent of both the *ycaC* gene located upstream of the AGDI cluster and the *aguB* gene. In addition, the results indicate *aguB, aguD, aguA* and *aguC* genes to be co-transcribed as a polycistronic mRNA (*aguBDAC* operon) from the P_{aguB} promoter.

AguR is essential for putrescine biosynthesis

To investigate the involvement of AguR in putrescine production, a *L. lactis* CECT8666 Δ*aguR* mutant (KO) was constructed as described in Materials and Methods (section 2.4). Both the WT and KO strains were grown in GM17 supplemented with increasing agmatine concentrations (0, 0.05, 0.1, 0.25, 0.5, 1, 5, 10, and 20 mM) for 24 h. Samples were collected at the end of fermentation and

putrescine production determined by UHPLC (Fig. 2). Putrescine production by the WT strain correlated strictly with the initial concentration of agmatine in the medium. However, the deletion of *aguR* completely abolished the conversion of agmatine to putrescine; no putrescine was produced at any of the agmatine concentrations tested.

Effect of *aguR* deletion on the transcriptomic profile

To determine the effect of the deletion of *aguR* on the transcriptomic profile of *L. lactis* CECT8666, DNA microarray analysis was performed involving the WT and the KO strains grown in the presence of 20 mM agmatine. Genes differentially expressed by the KO and WT that fulfilled the criteria of at least a threefold change and a *p* value of <0.001 - as well as the results for *aguR* - are shown in Table 3. The four catalytic genes (*aguB*, *aguD*, *aguA* and *aguC*) coding for the proteins needed for the biosynthesis of putrescine were clearly downregulated in the Δ*aguR* strain (fold changes -27.44, -26.27, -28.50 and -28.55 respectively). However, although statistically significant (p=0.02), the downregulation of *aguR* in the KO was much less (-0.73). That is, the expression of *aguR* in the WT strain is only slightly higher than in the KO, suggesting that *aguR* expression must be very low in the WT strain.

Transcriptional regulation of *aguR* and *aguBDAC* by agmatine

The effect of the environmental agmatine concentration on *aguR* and *aguBDAC* expression was investigated by RT-qPCR. The expression profile of *aguB* (the first

gene of the *aguBDAC* operon) was analyzed as representative of the whole *aguBDAC* polycistronic mRNA. Total RNA was isolated from *L. lactis* CECT8666 cells grown in GM17 as well as GM17 supplemented with increasing concentrations of agmatine (0, 0.05, 0.1, 0.25, 0.5, 1, 5, 10, and 20 mM). Figure 3 shows the relative *aguR* and *aguB* gene expression levels (normalized against the *rpoA* reference gene). *aguR* expression was not affected by the increase in the agmatine concentration (Fig. 3A), whereas *aguB* expression was upregulated by concentrations of ≥0.25 mM, the strongest overexpression (1700-fold change) occurring in the presence of 5 mM agmatine (Fig. 3B). Agmatine concentrations of over 5 mM right up to 20 mM did not increase the *aguB* expression compared to that observed with 5 mM agmatine.

In response to agmatine, AguR acts as a transcriptional activator of P_{aguB}

To study the activity of the P_{aguR} and P_{aguB} promoters, P_{aguR}-*gfp* and P_{aguB}-*gfp* fusions were constructed by substituting the *aguR* or *aguBDAC* genes for the *gfp* reporter gene, and comparing with the P_{aguR}-*aguR*-P_{aguB}-*gfp* fusion (pAGDI, Table 3). Constructs were assayed in *L. lactis* NZ9000 -a strain without the AGDI cluster- grown in GM17 in the presence (20 mM) or absence of agmatine, measuring whole-cell fluorescence (see Fig. 4). Interestingly, when fused independently, neither P_{aguR} nor P_{aguB} was associated with any detectable activity. However, P_{aguB} activity was recorded in assays involving the P_{aguR}-*aguR*-P_{aguB}-*gfp* construct, but, strikingly, only under the agmatine-supplementation conditions. The fact that P_{aguB} only showed activity when *aguR* (driven by its own promoter) was included in the genetic cassette supports AguR to be a transcriptional activator of P_{aguB}.

399 To further determine the dose-dependent activator effect of agmatine on the
400 promoter activity, the *gfp*-fusion constructs were assayed in *L. lactis* NZ9000 grown
401 in GM17 supplemented with increasing amounts of agmatine. Figure 5 shows the
402 whole-cell fluorescence results obtained. Once again no activity was detected for
403 the P_{aguR}-*gfp* nor P_{aguB}-*gfp* construct (at any agmatine concentration tested) (Fig.
404 5A and 5B respectively), while a dose-dependent activation of the P_{aguR}-*aguR*-
405 P_{aguB}-*gfp* fusion was seen, with maximum activity recorded for 0.1 mM agmatine (6
406 fluorescent a.u.). Agmatine concentrations above 0.1 mM did not significantly
407 increase the intensity of fluorescence compared to 0.1 mM agmatine (Fig. 5C).
408
409 **Functional analysis of inverted sequences of the *aguB* promoter region**
410 Clone software analysis of the nucleotide sequence upstream of the putative -35
411 region of the *aguB* promoter revealed the presence of one direct and three
412 reversed sequences (Fig. 6A). To determine whether these inverted sequences are
413 necessary for transcriptional activity, a series of deletions in the pAGDI plasmid
414 (from -209 to -179 nucleotides [pAGDIΔ1 plasmid]; from -179 to -147 nucleotides
415 [pAGDIΔ2 plasmid]; from -147 to -119 nucleotides [pAGDIΔ3 plasmid]; and from -
416 119 to -92 nucleotides [pAGDIΔ4 plasmid]; Fig. 6B) were generated. *gfp* was used
417 as the reporter gene and *L. lactis* NZ9000 as the host. Plasmid pAGDI containing
418 the complete *aguB* promoter was used as a control. NZ9000 cells were
419 transformed with either pAGDI, pAGDIΔ1, pAGDIΔ2, pAGDIΔ3 or pAGDIΔ4 and
420 grown in GM17 supplemented with 20 mM agmatine. At the end of the exponential
421 phase, cells were collected and the activity of the promoters examined via whole-

cell fluorescence. The obtained transcriptional activities were expressed as percentages relative to pAGDI (100% activity) (Fig. 6B). The deletion of fragment -209 to -179 did not affect the activity of the promoter, which was equal to that shown by the control. However, the deletion of the fragments located downstream of this region did prevent expression. This indicates that these sequences are required for P_{aguB} activity.

AguR is a transmembrane protein

As described above, the P_{aguR}-*aguR*-P_{aguB}-*gfp* fusion became active in response to the extracellular agmatine concentration in *L. lactis* NZ9000, a strain lacking the AGDI cluster, in which *aguD* codes for the agmatine/putrescine antiporter. Database checks were made to confirm that the genome of the *L. lactis* NZ9000 strain (GenBank: CP002094.1) (31) is defective in predicted agmatine transporters. The ability of *L. lactis* NZ9000 to internalize agmatine *in vivo* was therefore assessed. The strain was grown in GM17 plus 20 mM agmatine, but after 24 h the concentration of extracellular agmatine in the supernatant was the same (20 mM), indicating that *L. lactis* NZ9000 likely lacks a system for agmatine internalization (data not shown). Thus, agmatine in the extracellular medium might trigger the induction of *aguBDAC* transcription.

To gain insight into the mechanism of response to the extracellular agmatine concentration, the sub-cellular localization of the AguR protein was predicted by *in silico* topology-analysis using the computer-based algorithms provided by the SOSUI server. The topology revealed a membrane protein secondary structure with seven predicted transmembrane-spanning segments, the N-terminal outside

446 the cell and a long (105 amino acid residues) C-terminal domain inside (Fig. 7A).
447 The localization of AguR was also examined experimentally using the P_{nis}-*aguR*-
448 *gfp* translational fusion (pAG3 plasmid) in which the *aguR* gene fused to the *gfp*
449 gene is under the control of the *nisA* promoter. This fusion was assayed in *L. lactis*
450 NZ9000 with induction by nisin, and the cells examined by fluorescence
451 microscopy. As shown in Figure 7-B1, the AguR-GFP fusion protein was evenly
452 distributed on the periphery of the cell, confirming the predicted trans-membrane
453 nature of AguR in *L. lactis*. A control with a cytoplasmic GFP showing contrast with
454 the fluorescent pattern of the AguR-GFP product was carried in parallel (Figure 7-
455 B2).
456
457 **AguR has a LuxR_C_like domain**
458 A C-terminal DNA-binding domain typically found in LuxR-like proteins
459 (LuxR_C_like domain), and which contains a helix-turn-helix (HTH) DNA-binding
460 functional motif (13, 32), was found in AguR (C-terminus 265 to 313 residues, Fig.
461 7A). Structure-based multiple alignment was performed between the predicted
462 LuxR_C_like domain sequence of AguR and the orthologous domains of LuxR
463 member proteins with known structures: DosR from *Mycobacterium tuberculosis*
464 (Wisedchaisri et al., 2005), GerE from *Bacillus subtilis* (33), StyR from
465 *Pseudomonas fluorescens* (34), CviR from *Chromobacterium violaceum* (35), and
466 VraR from *Staphylococcus aureus* (36) (Fig. 8A). Remarkably strong similarity was
467 found between the accepted four alpha-helix motif distribution model of the solved
468 LuxR domains and the predicted alpha-helix motifs within the AguR LuxR_C_like
469 domain (Fig. 9A). Half of the total residues involved within the LuxR_C_like domain

were conserved or conservatively substituted across all the compared structures, indicating strong sequence similarity. Moreover, 9 out of the 13 residues described to act as DNA-binding residues in the LuxR_C_like domain of the DosR regulator of *Mycobacterium tuberculosis* were conserved in AguR (Fig. 8A). Since the LuxR_C_like domain of DosR binds to DNA as a homodimer, it is remarkable that when we replaced in the solved DosR model (37) the LuxR_C_like domain of the chain A of AguR showed a perfect fit match (Fig. 8B). Further, the largest inverted repeated sequence found in the *aguR* promoter region (Fig. 6) showed a 13 bp match with the 20 bp palindromic consensus sequence of DosR binding sites (38).

DISCUSSION

Food safety is a major social concern in developed countries, in part stemming from the world-wide recorded incidence of food-borne illnesses. A great deal of effort has therefore been invested in the development of processing methods and techniques that avoid contaminants such as BAs entering foodstuffs. Fermented foods, and particularly cheese, are of special concern in this respect (39-41). Putrescine is one of the most commonly detected BAs in dairy products (2,3,42,43). Prompted by the increasing awareness of the risks associated with the dietary intake of high BA loads, and the importance of *Lactococcus lactis* as a primary starter in the dairy industry, the aim of this work was to decipher the genetic regulation of the putrescine biosynthesis cluster of *L. lactis* subsp. *cremoris* CECT8666.

The performed transcriptional studies detected the presence of a mRNA spanning the intergenic regions of *aguB*, *aguD*, *aguA* and *aguC*, thereby confirming that

494 these genes are co-transcribed from the P_{aguB} promoter as a single *aguBDAC*
495 polycistronic mRNA. In fact, no terminator-like sequences were identified in the
496 *aguBDAC* intergenic regions. The transcription of the catabolic genes of the AGDI
497 cluster as a single mRNA molecule has previously been reported for *Pseudomonas*
498 *aeruginosa* PAO1 (44), *Streptococcus mutans* UA159 (45), *Lactococcus lactis*
499 subsp. *lactis* CHCC7244 (2), and *Enterococcus faecalis* JH2-2 (46). The similar
500 degree of downregulation presently seen in the DNA microarray comparisons
501 between the Δ*aguR* mutant and WT strains (Table 3) supports the idea that these
502 genes are co-transcribed. In addition, the present data reveal the expression of the
503 adjacent upstream *aguR* gene occurs via an independent mRNA transcribed from
504 the P_{aguR} promoter.
505 The role of *aguR* in the AGDI operon has been described as a positive regulator in
506 *Streptococcus mutans* (13), *Enterococcus faecalis* JH2-2 (46) and *Enterococcus*
507 *faecalis* V583 (47), and as a TetR-family repressor in *Pseudomonas aeruginosa*
508 (44). In the present work, the deletion of *aguR* in putrescine-producing *L. lactis*
509 CECT8666 fully impaired expression of the catalytic AGDI genes and thereby the
510 catabolism of agmatine to putrescine (Fig. 2), indicating that this gene behaves as
511 a positive regulator. Moreover, when fused to *gfp*, the promoter regions of *aguR*
512 and *aguBDAC* showed no activity in NZ9000 cells (without AGDI cluster)
513 regardless of the agmatine concentration (Figs. 4-5). In contrast, when *aguR* was
514 present in the *gfp* fusion, an agmatine-specific induction of P_{aguB} activity was
515 observed. Together, these data reveal the dual role of AguR in *Lactoccocus lactis*:
516 not only is it required for sensing the agmatine concentration, it is involved in the
517 transcriptional activation of putrescine biosynthesis. However, the transcription of

518 *aguR* was revealed as completely independent of the agmatine concentration,
519 suggesting that AguR is constantly present in the cells, although its expression
520 must be very low-level since very small differences in *aguR* transcription were
521 observed between the WT and KO strains in transcriptomic analyses.
522 As previously reported, the transcription of the *aguBDAC* operon is regulated by
523 carbon catabolic repression (CCR) mediated by the catabolite control protein
524 CcpA. However, the expression level of *aguR* is independent of the glucose
525 concentration (12). CcpA would control the expression of the *aguBDAC* operon -
526 which promoter P_{aguB} has in fact a *cre* site (12)- and would not control *aguR*
527 expression. Therefore, our data suggest that CCR and AguR activation would work
528 as two independent systems exerting a parallel control on P_{aguB} promoter.
529 The *in silico* analysis of the amino acidic sequence of AguR revealed the presence
530 of seven transmembrane domains, a short extracytoplasmic N-terminus, and a
531 longer cytoplasmic C-terminus (Fig. 7A). A membrane localization of AguR has
532 also been predicted in its orthologous protein present in *S. mutants* (13), although
533 the authors of the latter work proposed a four-transmembrane-domain model and
534 determined the N-terminus to lie in the cytosol. In the present work, fluorescence
535 microscopy analysis of NZ9000 cells expressing *aguR* fused to *gfp* showed AguR
536 to localize at the bacterial surface where it is evenly distributed (Fig. 7B).
537 Moreover, agmatine concentration sensing was maintained when *aguR* was
538 coexpressed with P_{aguB}-*gfp* in *L. lactis* NZ9000, an AGDI-defective strain (lacking
539 *aguR*) unable to internalize agmatine. This confirms that extracellular agmatine
540 activates the AGDI system without being internalized. However, NZ9000 cells
541 containing the P_{aguB}-*gfp* construct, but lacking AguR, were unable to transduce the

agmatine signal to the inside of the cell and activate P_{aguB}. These results strongly suggest that AguR is a transmembrane protein that behaves both as a sensor of the extracellular agmatine concentration and as a signal transducer demanding the transcription of the *aguBDAC* genes be initiated. It should be noted that the non-AGDI cluster genes which, in the present transcriptomic studies, showed different degrees of expression in the KO and WT strains (Table 3), are not present in the genome of *L. lactis* NZ9000 (except for the glycosyltransferase and transposase genes). They do not, therefore, seem to be required by the proposed regulation model.

Blast analysis of the amino acid sequence of AguR showed this protein to belong to the transcriptional regulators of the LuxR family, as described for its orthologs in *L. lactis* subsp. *lactis* CHCC7244 (2), *S. mutants* UA159 (13,45) and *E. faecalis* JH2-2 (46). The LuxR family of DNA-binding proteins are transcription factors involved in quorum sensing via the detection of autoinducers such as oligopeptide signaling molecules (in Gram-positive bacteria) (48,49) or acylated homoserine lactones (in Gram-negative bacteria) (50). These proteins have two functional domains: an amino-terminal domain involved in the binding of the signaling molecule, and a LuxR-C-like transcription regulation domain at the C-terminus of the protein which includes a helix-turn-helix (HTH) DNA-binding motif (32). LuxR transcription factors can therefore behave as regulators (transcriptional activators) by binding a cognate extracellular inducer and targeting specific gene promoters (51). A high degree of structural homology was noted when the AguR intracellular C-terminal LuxR_C_like domain was compared to those of LuxR family members with solved structures (Fig. 8); indeed, the characteristic four alpha-helix secondary

566 structure for this domain was shared (52). Moreover, half of the amino acid
567 residues of the LuxR_C_like domain (34 of 62 residues) were strongly conserved
568 across all the compared structures, indicating high sequence conservation. In fact,
569 9 out of 13 DNA-binding residues in the LuxR_C_like domain of DosR in *M.*
570 *tuberculosis* (37) were conserved in the AguR LuxR_C_like domain, as were the
571 three residues involved in dimerization within the α10 helix of DosR monomers
572 (37). The similarity between the two proteins is such that a DosR chain could be
573 perfectly replaced by one from AguR. Since the LuxR_C_like domain of DosR
574 binds to DNA as a homodimer, the LuxR_C_like domain of AguR should be able to
575 bind to DNA, probably with a dimeric structure.
576 The target DNA-binding sites (lux-type boxes) of many LuxR-type proteins have a
577 dyad symmetry structure (50) and are often located just upstream of the -35 region
578 of the regulated promoters. Such is the case of a direct repeat element essential
579 for transcription from P_{aguB} promoter (Fig. 6A), which shows a 13 bp-match with the
580 20 bp DosR binding site consensus sequence (38). This similarity between the
581 proteins and their DNA-binding sites is even more remarkable considering the
582 taxonomic distance between the GC content of *M. tuberculosis* (65%) and *L. lactis*
583 (35%).
584 The results reveal the role of the regulatory protein AguR as both agmatine sensor
585 and transcriptional activator of the AGDI genes (*aguB*, *aguD*, *aguA*, and *aguC*). In
586 other lactic acid bacteria with the AGDI pathway as *S. mutants* (13,45) and *E.*
587 *faecalis* (46) the role of AguR would be the same. However, the system seems to
588 be slightly different in *Lb. brevis*, since the AGDI cluster does not contain an *aguR*
589 gene. *Lb. brevis* has a putative transcription regulator gene adjacent to the AGDI

cluster that belongs to the RpiR family, which is distantly related to AguR (57) and lacks transmembrane domains. A similar mechanism to the one proposed here for AguR has been described in *E. coli* for the biosynthesis operon of cadaverin, another BA: the transmembrane protein CadC binds lysine outside the cell, and the signal is then transduced to the N-terminal cytoplasmic portion of the protein, which contains the HTH domain (53, 54, 55, 56). Nevertheless, further analyses are needed to determine the precise mechanism by which AguR senses the agmatine concentration and transduces the activation signal to the promoter of the *aguBDAC* genes.

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TABLES

Table 1. Strains and plasmids.

Strain /Plasmid		Characteristics	Source
Strains			
L. lactis subsp. cremoris NZ9000		*L. lactis* subsp. *cremoris* MG1363. containing *nisRK* genes, non-putrescine producer	(24)
L lactis subsp. cremoris CECT8666		Isolated from artisanal cheese, putrescine producer	(2)
E. coli DH11S			Life technologies, Spain
L lactis subsp. cremoris CECT8666 Δ*aguR*		CECT8666 strain lacking the *aguR* gene	This work
Plasmids			
pNZ8048	Lactococal plasmid, Cm^r		(24)
pCS1966	Selection/counterselection vector, Em^r		(20)
pNZcLIC-GFP	pNZ8048 derivative harboring *gfp*, Cm^r		(25)
pNZcGFP	pNZ8048 derivative harboring *gfp*, Cm^r		(25)
pAGDI	pNZ8048 derivative bearing P_{aguR}-*aguR*-P_{aguB}-*gfp* fusion, Cm^r		(12)
pIPLA1269	pCS1966 derivative bearing a fragment of CECT8666 *ycaC-aguR* genes, Em^r		This work
pIPLA1713	pIPLA1269 derivative bearing a fragment of CECT8666 *ycaC-aguR* genes and a fragment of *aguR-aguB* genes, Em^r		This work
pAG1	pNZ8048 derivative bearing P_{aguR}-*gfp* fusion, Cm^r		This work
pAG2	pNZ8048 derivative bearing P_{aguB}-*gfp* fusion, Cm^r		This work
pAG3	pNZcLIC-GFP derivative bearing P_{nisA}-AguR-*gfp* fusion, Cm^r		This work
pAGDIΔ1	pAGDI derivative; deletion from -209 to -179 of P_{aguB}, Cm^r		This work
pAGDIΔ2	pAGDI derivative; deletion from -179 to -146 of P_{aguB}, Cm^r		This work
pAGDIΔ3	pAGDI derivative; deletion from -146 to -119 of P_{aguB}, Cm^r		This work
pAGDIΔ4	pAGDI derivative; deletion from -119 to -82 of P_{aguB}, Cm^r		This work

Cm^r: chloramphenicol resistance; Em^r: erythromycin resistance; P_{aguR}: *aguR* promoter; P_{aguB}: *aguB* promoter; P_{nisA}: *nisA* gene promoter

653 Table 2. Primers used.

654

Primer	Function	Nucleotide sequence (5´ to 3´)	Source
AguF	Intergenic region *ycaC-aguR* (F)	CGAACAGACAGCGTCCCTGA	This work
AgurNco	Intergenic region *ycaC-aguR* (R)	CCCCATGGGATTAGACCTACTTATCATATTATCA	This work
PtcBglII	Intergenic region *aguR-aguB* (F)	CCCCAGATCTAAGCATATGAAAAATCAGAACTTAG	This work
PTC3C	Intergenic region *aguR-aguB* (R)	CTTTAGTGTAATCTTCTGTTGTGATG	This work
RT1	Intergenic region *aguB-aguD* (F)	CCTTATGATTTGAAAGCACAAG	This work
RT2	Intergenic region *aguB-aguD* (R)	GAAAGAATAGCACTAAATAGAC	This work
RT3	Intergenic region *aguD-aguA* (F)	TTATTGGAGAACTGATTATTAAAG	This work
AgDI3Cre	Intergenic region *aguD-aguA* (R)	CTTGAGCTTCAAATTCACCGGGC	This work
RT4	Intergenic region *aguA-aguC* (F)	GGCGGTGGAAATATTCACTG	This work
RT5	Intergenic region *aguA-aguC* (R)	TTGTGCTGTAGGGTCACTGC	This work
qAguR-F	*aguR* expression analysis (F)	CTATCGACAGGTTAAGCAAAGCAGTT	(12)
qAguR-R	*aguR* expression analysis (R)	TCCAAAGATGATGGCCATTATGC	(12)
qPTC-F	*aguB* expression analysis (F)	ACTTGGTGGACATGAAACAATAGAAGAT	(12)
qPTC-R	*aguB* expression analysis (R)	GTCAACACGTGCCATTATGATATCG	(12)
rpoA-F	*rpoA* reference gene (F)	CACGGGCAGGTTCAACTTG	(21)
rpoA-R	*rpoA* reference gen (R)	TTCCGGCTGACGAAAATAAAG	(21)
qtufF	EF-Tu reference gene (F)	TCTTCATCATCAACAAGGTCTGCTT	(12)
qtufR	EF-Tu reference gene (R)	GAACACATCTTGCTTTCACGTCAA	(12)
AguRBglII	Cloning of P_{aguR} (F)	CCCCCCAGATCTGACAAGTTTGGCTCAGATTGCTTG	This work
PtcNco	Cloning of P_{aguB} (R)	CCCCCCATGGTGTTTATTCCTCCTGAATAAAATAG	This work
AgurlicF	Cloning of *aguR* (F)	ATGGGTGGTGGATTTGCTATGTTAAATTATATTTATACTACTTTTT	This work
AgurlicR	Cloning of *aguR* (R)	TTGGAAGTATAAATTTTCTTGACTAAGTTCTGATTTTTCATATG	This work
Gffor	Cloning of gfp (F)	GGCCATGGGTGGTGGATTTGCTCAATTC	This work
Gfrev	Cloning of gfp (R)	CCGCATGCCTGCATTAATGATGGTG	This work
KO1F	Generation of pAGDIΔ1 (F)	CACACGAATTCGAAAAAAGCACTAAACCCTCC	This work
KO1R	Generation of pAGDIΔ1 (R)	CACACGAATTCTCCATTCAAAAAATGGAGCT	This work
KO2F	Generation of pAGDIΔ2 (F)	CACACGAATTCCTCAACCCCTTGGTAGCAAAGG	This work
KO2R	Generation of pAGDIΔ2 (R)	CACACGAATTCAAACGCTTTCTTTTTATAAATAAA	This work
KO3F	Generation of pAGDIΔ3 (F)	CACACGAATTCTTGCTTTTTAAAAAGATTAAATCCT	This work
KO3R	Generation of pAGDIΔ3 (R)	CACACGAATTCGTCAAAAGGTTTAGGAGGGTTTAG	This work
KO4F	Generation of pAGDIΔ4 (F)	CACACGAATTCAGTTGATTGTTTTAAGAAATCAACC	This work
KO4R	Generation of pAGDIΔ4 (R)	CACACGAATTCAAAACCCTTTGCTACCAAGGG	This work
KO-214AguR-AF2	Generation of pIPLA1269 (F)	CACATGACTAGTTTAGAACCTAGAAACCCAGAAAC	This work
KO-214AguR-AR	Generation of pIPLA1269 (R)	AACTGCAGATTTAACATCATCGGATTAGACCTAC	This work
KO-214AguR-BF	Generation of pIPLA1713 (F)	AACTGCAGTCAGAACTTAGTCAATAATTTAAAAG	This work
KO-214AguR-BR	Generation of pIPLA1713 (R)	CCATCGATAACCGCATCAACAACTTC	This work

655 F: forward, R: reverse, P_{aguR}: *aguR* promoter, P_{aguB}: *aguB* promoter

656

657

Table 3. Genes differentially expressed in *L. lactis* subsp. *cremoris* CECT8666 Δ*aguR* and the WT for which the criteria of at least a threefold difference and a *p* value of <0.001 were met (*aguR* is also included although these criteria were not entirely met).

Locus tag[a] (gene name)	Description	Fold change[b]	*p* value[c]
Down-regulated			
U725_01346 (*aguR*)	Transcriptional regulator	-0.73	2.02E-2
U725_01347 (*aguB*)	Putrescine carbamoyl transferase	-27.44	1.00E-4
U725_01348 (*aguD*)	Agmatine/putrescine antiporter	-26.27	1.50E-4
U725_01349 (*aguA*)	Agmatine deiminase	-28.50	8.00E-5
U725_01350 (*aguC*)	Carbamate kinase	-28.55	8.00E-5
U725_00022	hypothetical protein	-4.39	8.60E-4
U725_00023	Glycosyltransferase	-6.00	5.70E-4
Up-regulated			
U725_02522	Transposase	5.00	8.00E-5
U725_02523	EpsR	16.26	2.30E-4
U725_02524	EpsX	16.00	7.00E-5
U725_02525	EpsA	21.20	1.20E-4
U725_02526	EpsB kinase	30.41	6.00E-5
U725_02527	EpsC	16.33	4.20E-4
U725_02528	Undecaprenyl-phosphate galactose phosphotransferase	18.43	7.00E-5
U725_02529	putative transposase	6.20	6.00E-5
U725_01694	Transposase	6.50	5.00E-5
U725_02271	putative replication protein repA	3.83	8.30E-4
U725_02289	Transposase	3.70	4.10E-4
U725_02472	Transposase	6.87	3.00E-5
U725_02477	EpsN protein	25.29	5.00E-5

[a] Locus tags refer to GenBank Accession No AZSI00000000.1

690 **FIGURE LEGENDS**

691

692 **Figure 1**. Transcriptional analysis of the AGDI operon of *L. lactis* subsp. *cremoris*
693 CECT8666. **A)** Genetic organization of the AGDI cluster and surrounding regions.
694 The putative *aguR* promoter (P_{aguR}), the *aguB* promoter (P_{aguB}), and the termination
695 regions (⍑) are indicated. **B)** RT-PCR amplification of intergenic regions was
696 conducted using total RNA extracted from cells grown in the presence of 20 mM
697 agmatine. Five set of primers were designed to amplify the intergenic regions:
698 *ycaC-aguR* (primer-pair 1, lane 1), *aguR-aguB* (primer-pair 2, lane 2), *aguB-aguD*
699 (primer-pair 3, lane 3), *aguD-aguA* (primer-pair 4, lane 4), and *aguA-aguC* (primer-
700 pair 5, lane 5). Negative controls were run with the same RNA samples but without
701 reverse transcriptase. Positive controls were run with chromosomal DNA. M: DNA
702 molecular marker.

703

704 **Figure 2**. Production of putrescine by *L. lactis* CECT8666 (WT) and the Δ*aguR*
705 deletion mutant at different agmatine concentrations. Both strains were grown in
706 GM17 supplemented with 0, 0.05, 0.1, 0.25, 0.5, 1, 5, 10 or 20 mM agmatine for 24
707 h. Supernatants were analyzed by UHPLC to determine the putrescine
708 concentration in the extracellular medium.

709

710 **Figure 3**. Influence of agmatine concentration on the expression of *aguR* and
711 *aguBDAC* as determined by RT-qPCR. Cell cultures were supplemented with 0,
712 0.05, 0.1, 0.25, 0.5, 1, 5, 10 or 20 mM agmatine, and samples collected at the end
713 of the exponential phase of growth. The relative expression of *aguR* **(A)** and *aguB*

714 –representing the whole *aguBDAC* operon- **(B)** was calculated relative to the
715 transcript level for samples grown in the absence of agmatine. Data were
716 normalized to total RNA content using *rpoA* and *tuf* as reference genes. The values
717 shown are the means of three replicates; the standard deviations are indicated by
718 bars. *p<0.05 **p<0.001
719
720 **Figure 4**. Cloning and assay of P_{aguR} and P_{aguB} activity, reported as gfp
721 fluorescence, in the presence and absence of 20 mM agmatine. The genetic
722 fusions P_{aguR}-*gfp*, P_{aguB}-*gfp* and P_{aguR}-*aguR*-P_{aguB}-*gfp* were transformed in *L. lactis*
723 NZ9000 cells and promoter activity determined by measuring whole-cell
724 fluorescence (250 µl of cells) at similar OD_{600}. The values shown are the means of
725 three replicates; standard deviations are indicated by bars. a.u.: arbitrary units.
726
727 **Figure 5**. Effect of agmatine concentration on the transcriptional activity of the
728 AGDI cluster promoters, measured by whole-cell fluorescence. *L. lactis* NZ9000
729 cells harboring either the P_{aguR}-*gfp* **(A)**, the P_{aguB}-*gfp* **(B)** or the P_{aguR}-*aguR*-P_{aguB}-
730 *gfp* **(C)** genetic fusions were grown in GM17 supplemented with 0, 10^{-6}, 10^{-5}, 10^{-4},
731 10^{-3}, 10^{-2}, 0.1, 0.5, 1, 2, 5, 10 or 20 mM agmatine for 7 h, after which gfp
732 fluorescence was monitored. The values shown are the means of three replicates;
733 standard deviations are indicated by bars. a.u.: arbitrary units.
734
735 **Figure 6**. **A.** Sequence of the *aguB* promoter region. The putative -10 and -35
736 regions, the ribosome binding site (RBS), and the *aguB* start codon, are shown in
737 bold. Direct and reversed sequences are indicated by arrows. The deletions

generated in this study (Δ1, Δ2, Δ3, and Δ4) are indicated by dashed lines. Asterisks indicate matches with the palindromic consensus sequence of the DosR binding site. **B.** Effect of sequential deletions within the *aguB* promoter region. Plasmids pAGΔ1, pAGΔ2, pAGΔ3 and pAGΔ4 were constructed from pAGDI (P_{aguR}-*aguR*-P_{aguB}-*gfp* fusion). The dashed lines indicate the fragments deleted. The corresponding gfp fluorescence was measured in *L. lactis* NZ9000 grown in GM17 supplemented with 20 mM agmatine. The activities associated with the deletion constructs are expressed as percentages relative to pAGDI activity (100% activity). The values shown are the means of three replicates; standard deviations are indicated by bars (*p<0.05).

Figure 7. Cellular localization of AguR. **(A)** Predicted secondary structure and topology of AguR obtained via the analysis of the amino acid sequence (performed using the SOSUI server). Seven trans-membrane domains were predicted (grey shadowing). **(B1)** *In vivo* membrane localization of AguR in *L. lactis* NZ9000 cells overexpressing the AguR-GFP translational fusion protein imaged by fluorescence microscopy. **(B2)** Control image showing fluorescent pattern of the same cells overexpressing a cytoplasmic GFP.

Figure 8. *In silico* structural analysis of the C-terminal LuxR-C-like domain of AguR. **(A)** Structural alignment of the LuxR-C-like domain of AguR with homologue domains retrieved from PDB (Protein Data Bank). Residues identical in the majority of the proteins are indicated by capital letters in the consensus sequence, while ‘c’

761 indicates conservative substitutions. The shadowed residues are those involved in
762 alpha-helices within the domain. The alpha-helices in the AguR sequence are
763 derived from a structural alignment performed with the DosR LuxR_C_like domain
764 as a template. Arrows indicate those residues from the DosR domain that interact
765 with DNA, while asterisks indicate those involved in DosR dimerization. DosR from
766 *Mycobacterium tuberculosis* (PDB code 1ZLK), GerE from *Bacillus subtilis* (1FSE),
767 VraR from *Staphylococcus aureus* (2RNJ), StyR from *Pseudomonas fluorescens*
768 (1YIO), CviR from *Chromobacterium violaceum* (3QP6), and unknown protein from
769 *Bacteroides thetaiotaomicron* (3CLO). **(B)** Homology modeling analysis between
770 LuxR_C_like domains from DosR and AguR. A DosR dimer (chains A and B)
771 bound to DNA was used as template. Modeling was performed by substituting the
772 LuxR_C_like domain from DosR chain A for the LuxR_C_like domain from AguR
773 (red). DosR chain B is shown in yellow; the DNA helix is grey. The residues
774 involved in DosR dimerization within the α10 helix are shown in dark blue, the
775 AguR putative DNA-interacting residues in green, and the putative dimerization
776 residues of AguR in light blue.
777
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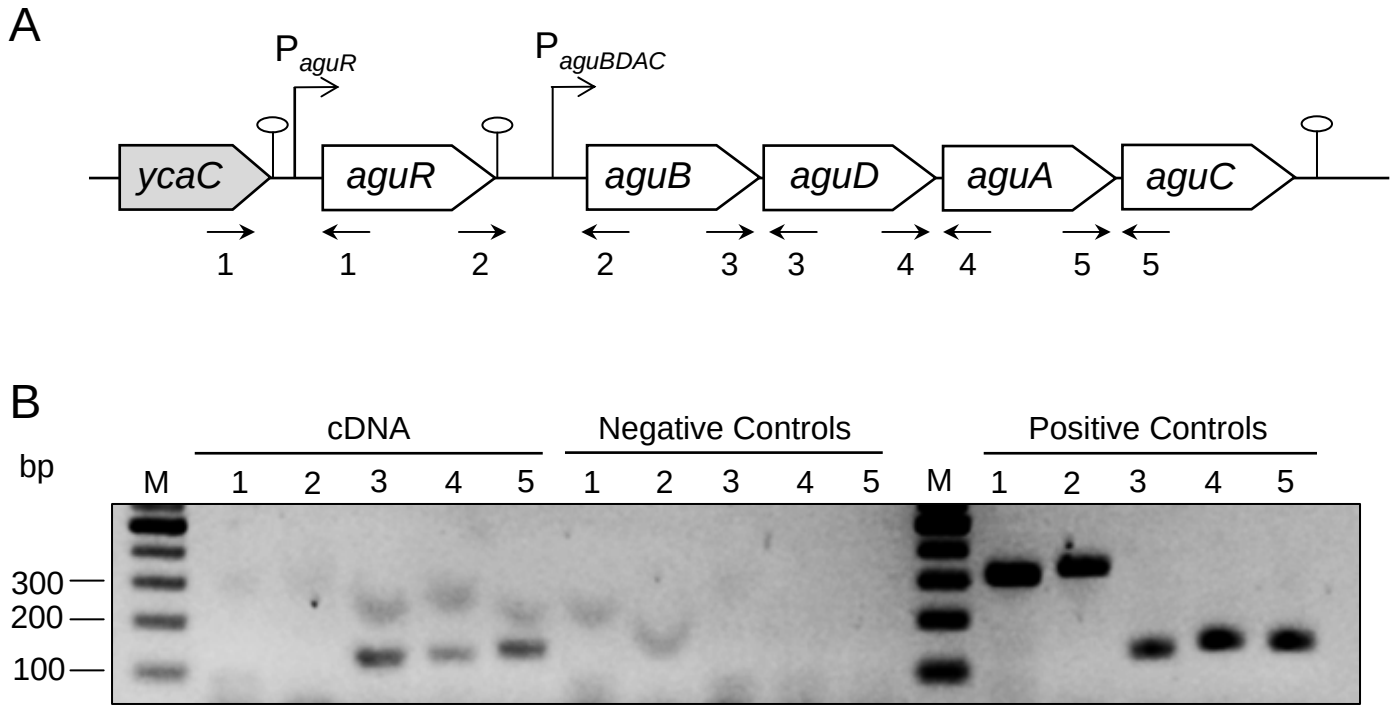

A
P aguR
P aguBDAC
ycaC
aguR
aguB
aguD
aguA
aguC
1 1 2 2 3 3 4 4 5 5
B
cDNA
Negative Controls
Positive Controls
bp
M 1 2 3 4 5 1 2 3 4 5 M 1 2 3 4 5
300
200
100

Figure 1

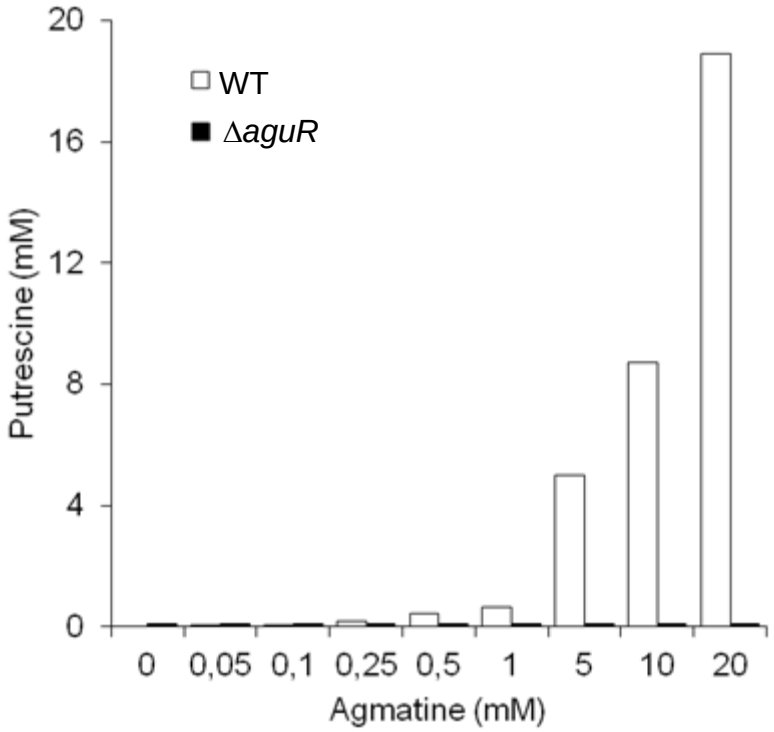

20
16
12
8
4
0
Putrescine (mM)
WT
ΔaguR
0
0,05
0,1
0,25
0,5
1
5
10
20
Agmatine (mM)

Figure 2

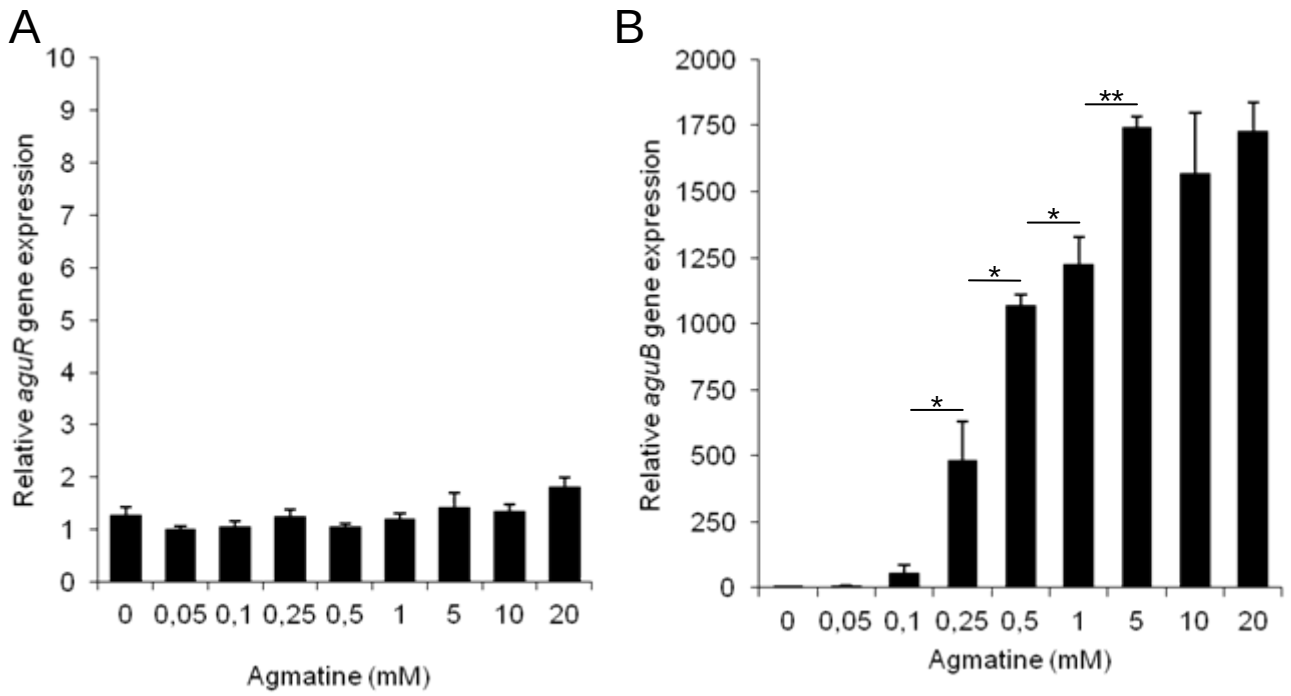

A
Relative aguR gene expression
10
9
8
7
6
5
4
3
2
1
0
0 0,05 0,1 0,25 0,5 1 5 10 20
Agmatine (mM)
B
Relative aguB gene expression
2000
1750
1500
1250
1000
750
500
250
0
*
*
*
**
0 0,05 0,1 0,25 0,5 1 5 10 20
Agmatine (mM)

Figure 3

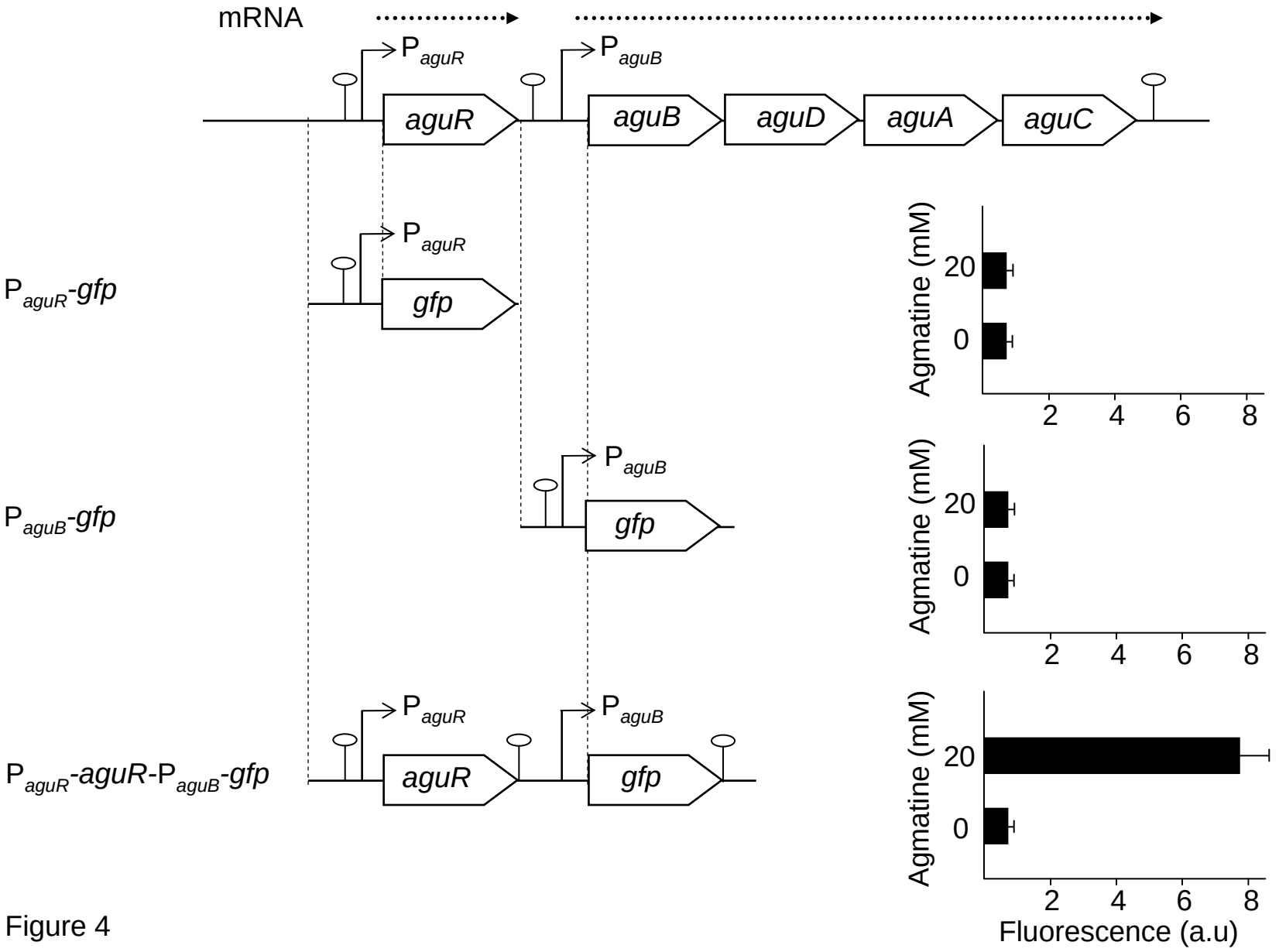

mRNA
P aguR
P aguB
aguR
aguB
aguD
aguA
aguC
P aguR-gfp
gfp
P aguB-gfp
P aguR-aguR-P aguB-gfp
Agmatine (mM)
20
0
2
4
6
8
Fluorescence (a.u)

Figure 4

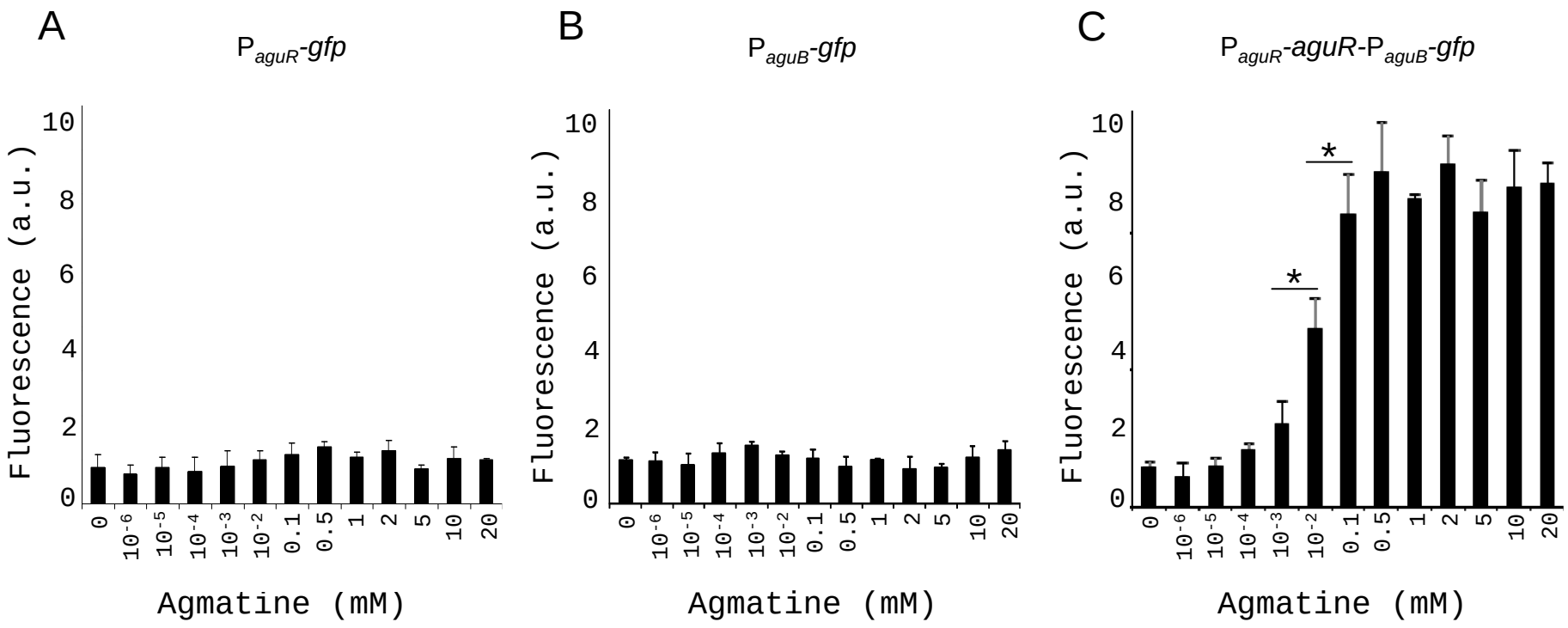
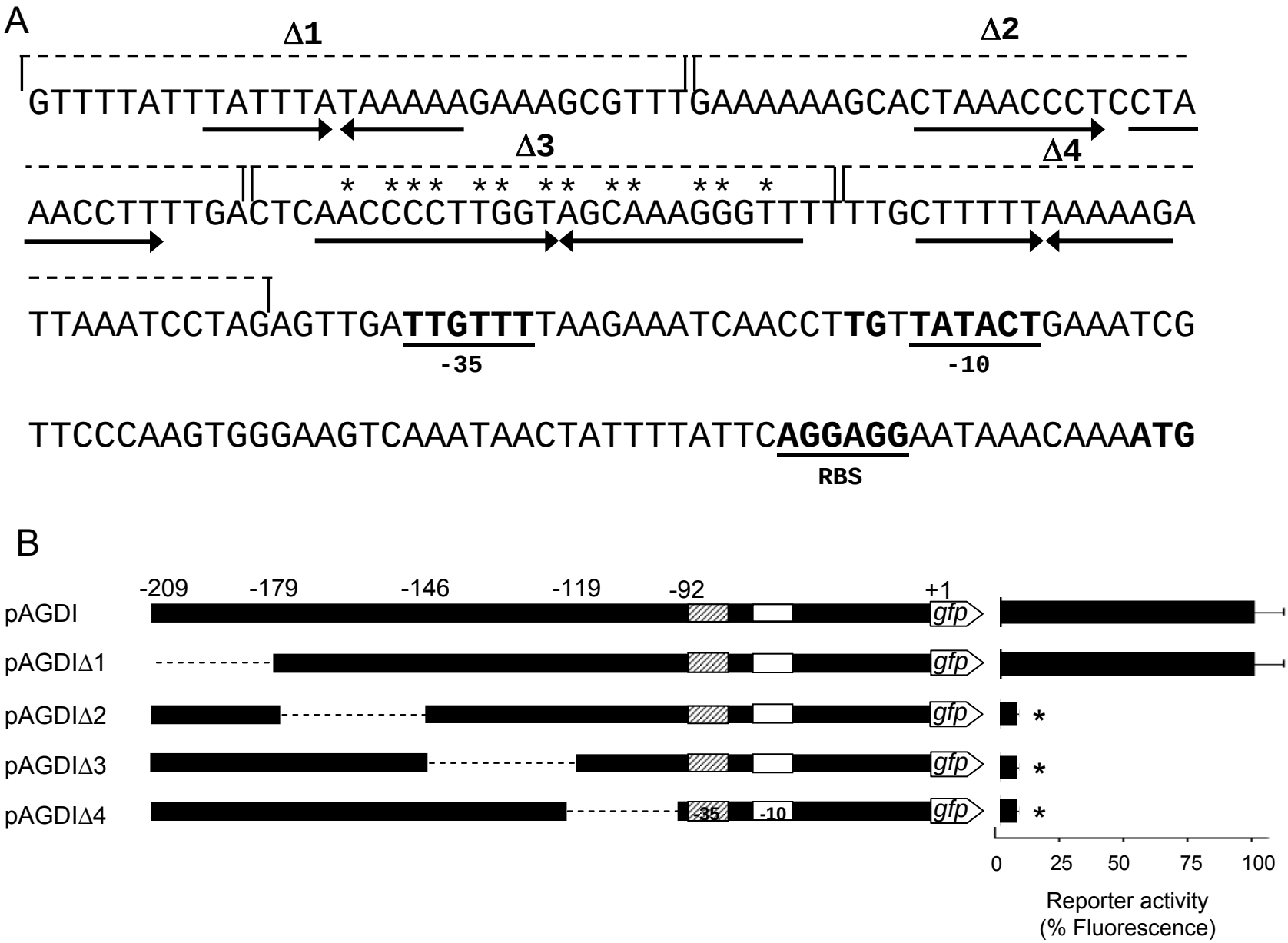

A
P aguR-gfp
B
P aguB-gfp
C
P aguR-aguR-P aguB-gfp
Fluorescence (a.u.)
0
2
4
6
8
10
0
10-6
10-5
10-4
10-3
10-2
0.1
0.5
1
2
5
10
20
Agmatine (mM)
*
*

Figure 5


A
Δ1
Δ2
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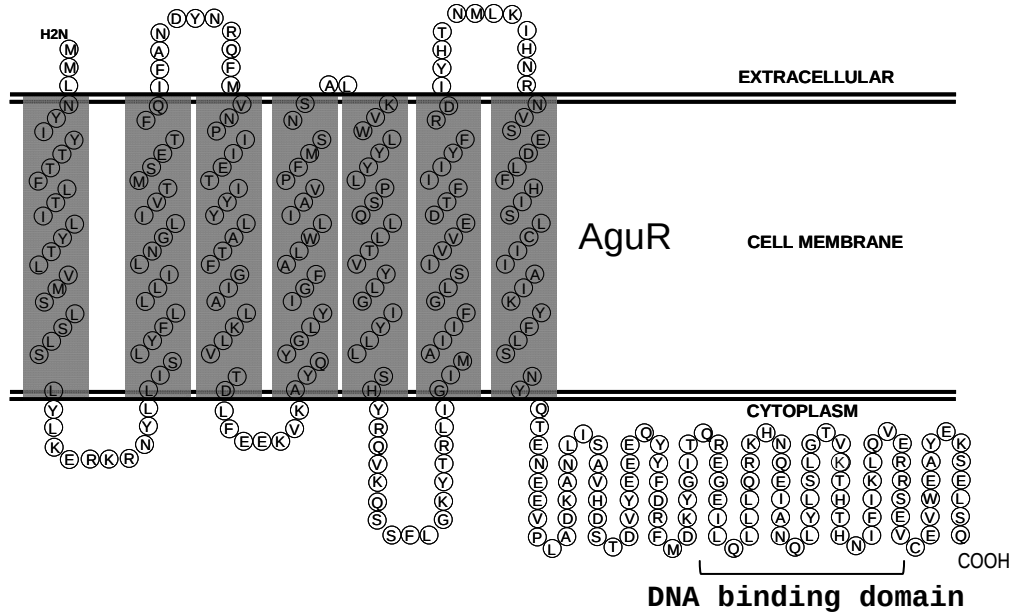
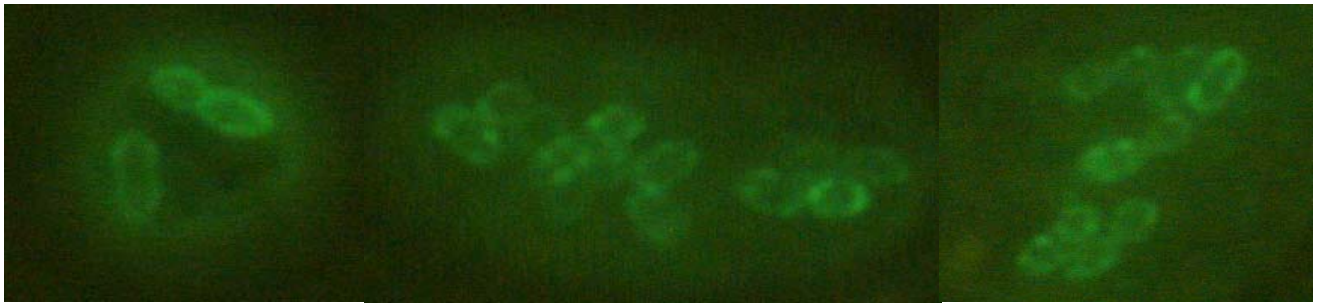
Δ3
Δ4
AACCTTTTGACTCAACCCCTTGGTAGCAAAGGGTTTTTTGCTTTTTAAAAAGA
TTAAATCCTAGAGTTGATTGTTTTAAGAAATCAACCTTGTTATACTGAAATCG
-35
-10
TTCCCAAGTGGGAAGTCAAATAACTATTTTATTCAGGAGGAATAAACAAAATG
RBS
B
-209
-179
-146
-119
-92
+1
pAGDI
pAGDIΔ1
pAGDIΔ2
pAGDIΔ3
pAGDIΔ4
gfp
-35
-10
0
25
50
75
100
Reporter activity
(% Fluorescence)

Figure 6

A



B1



B2

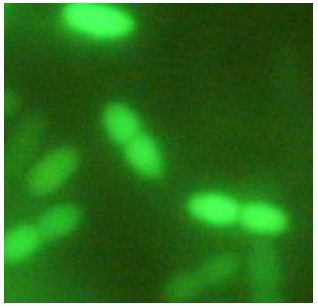
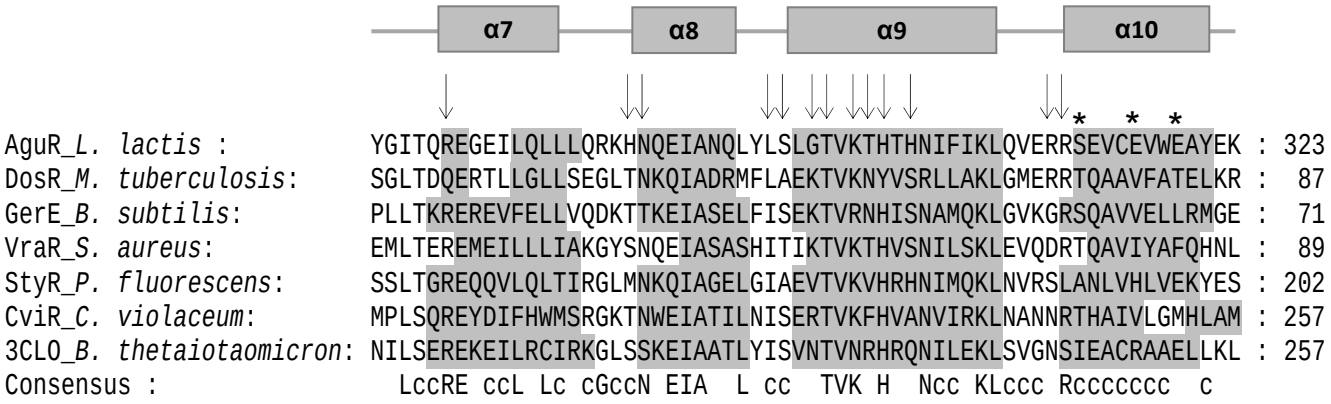
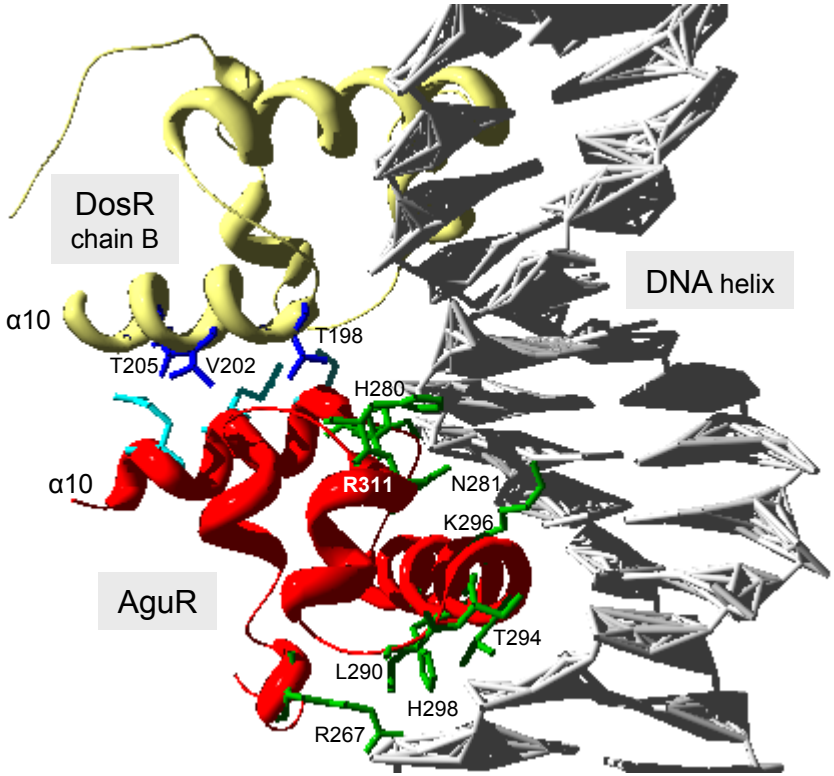


Figure 7

A


α7
α8
α9
α10
AguR_L. lactis : YGITQREGEILQLLLQRKHNQEIANQLYLSLGTVKTHTHNIFIKLQVERRSEVCEVWEAYEK : 323
DosR_M. tuberculosis: SGLTDQERTLLGLLSEGLTNKQIADRMFLAEKTVKNYVSRLLAKLGMERRTQAAVFATELKR : 87
GerE_B. subtilis: PLLTKREREVFELLVQDKTTKEIASELFISEKTVRNHISNAMQKLGVKGRSQAVVELLRMGE : 71
VraR_S. aureus: EMLTEREMEILLLIAKGYSNQEIASASHITIKTVKTHVSNILSKLEVQDRTQAVIYAFQHNL : 89
StyR_P. fluorescens: SSLTGREQQVLQLTIRGLMNKQIAGELGIAEVTVKVHRHNIMQKLNVRSLANLVHLVEKYES : 202
CviR_C. violaceum: MPLSQREYDIFHWMSRGKTNWEIATILNISERTVKFHVANVIRKLNANNRTHAIVLGMHLAM : 257
3CLO_B. thetaiotaomicron: NILSEREKEILRCIRKGLSSKEIAATLYISVNTVNRHRQNILEKLSVGNSIEACRAAELLKL : 257
Consensus : LccRE ccL Lc cGccN EIA L cc TVK H Ncc KLccc Rccccccc c

B


DosR
chain B
DNA helix
α10
T198
T205
V202
H280
α10
R311
N281
K296
AguR
T294
L290
H298
R267