Transcriptional regulation of central amino acid metabolism in Lactococcus lactis

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Chapter 3

ArgR and AhrC are both required for regulation of arginine metabolism in *Lactococcus lactis*

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Summary

The DNA binding proteins, ArgR and AhrC, are essential for regulation of arginine metabolism in *Escherichia coli* and *Bacillus subtilis*, respectively. A unique property of these regulators is that they form hexameric protein complexes, mediating repression of arginine biosynthetic pathways as well as activation of arginine catabolic pathways. The *gltS-argE* operon of *L. lactis* encodes a putative glutamate or arginine transport protein and acetylornithine deacetylase, which catalyses an important step in the arginine biosynthesis pathway. By random integration knockout screening we found that derepression mutants had IS*S1* integrations in, among others, *argR* and *ahrC*. Single as well as double regulator deletion mutants were constructed in *L. lactis* subsp. *cremoris* MG1363. The three arginine biosynthetic operons *argCJDBF*, *argGH* and *gltSargE* were shown to be repressed by the products of *argR* and *ahrC*. Furthermore, the arginine catabolic *arcABD1C1C2TD2* operon was activated by the product of *ahrC* but not by *argR*. Expression from the promoter of the *argCJDBF* operon reached similar levels in the single mutants and in the double mutant, suggesting that the regulators are interdependent and not able to complement each other. At the same time they also appear to have different functions as only AhrC is involved in activation of arginine catabolism. This is the first study where two homologous arginine regulators are shown to be involved in arginine regulation in a prokaryote, representing an unusual mechanism of regulation.

Introduction

Arginine, a non-essential amino acid in the lactic acid bacterium *Lactococcus lactis*, is synthesised *de novo* from glutamate in eight enzymatic steps (see Fig. 3.1). The recent publication of the *L. lactis* genome sequence (24) has revealed that the putative arginine biosynthesis genes are encoded by the three operons *argCJDBF*, *gltS-argE* and *argGH*. The products of these genes all show homology to known arginine biosynthetic enzymes, except for that of *gltS*, which has been annotated as a putative glutamate or arginine ABC transporter (24). While the biosynthetic genes have been shown to be regulated by the presence of arginine in other organisms, this has not been investigated in lactic acid bacteria (LAB). The activity of the biosynthetic enzymes have been shown to be repressed by arginine in *Lactobacillus plantarum* (28), but regulatory studies on the transcriptional level have not been performed in LAB.

Mechanisms for arginine catabolism vary between organisms (1). In *L. lactis,* complete degradation of arginine into ornithine, ammonium and carbon dioxide takes place via the arginine deiminase (ADI) pathway in three enzymatic steps catalysed by arginine deiminase (ArcA), ornithine carbamoyltransferase (ArcB) and carbamate kinase (ArcC) (Fig. 3.1). The genes *arcA*, *arcB*, *arcC1* and *arcC2* encoding these enzymes are located in the *arcABD1C1C2TD2* gene cluster. *L. lactis* harbours an extra *arcC* homologue, called *arcC3*, which is located distant from the remainder of the arginine related genes in the chromosome. The genes *arcD1* and *arcD2* encode antiporter proteins, allowing ATP-independent 1:1 arginine/ornithine exchange (197), while *arcT* specifies an aminotransferase.

It has long been known that carbon metabolism and arginine catabolism are closely connected in *L. lactis* (49). However, the presence of arginine has a higher regulatory effect than the available carbon source (197). The ADI pathway enzymes and amino acid transport systems are more stable during starvation than enzymes of glycolysis (139). Thus, the ADI pathway plays an important role in supplying the cells with energy during recovery from starvation without energy expenditure. Additionally, glycolysis enzymes are more sensitive for low pH than the ADI enzymes. Consequently, the ADI pathway represents an additional source of ATP production, combats acid stress by production of ammonium and finally supplies

carbamoyl phosphate, which is essential for *de novo* synthesis of pyrimidines. The identification of two putative *cre* (catabolite recognition element) sites in the *arcA* promoter of *Lactobacillus sake* (271) strongly suggests that carbon source-dependent regulation of the arginine catabolic genes is mediated by the major carbon catabolite repressor CcpA in this organism.

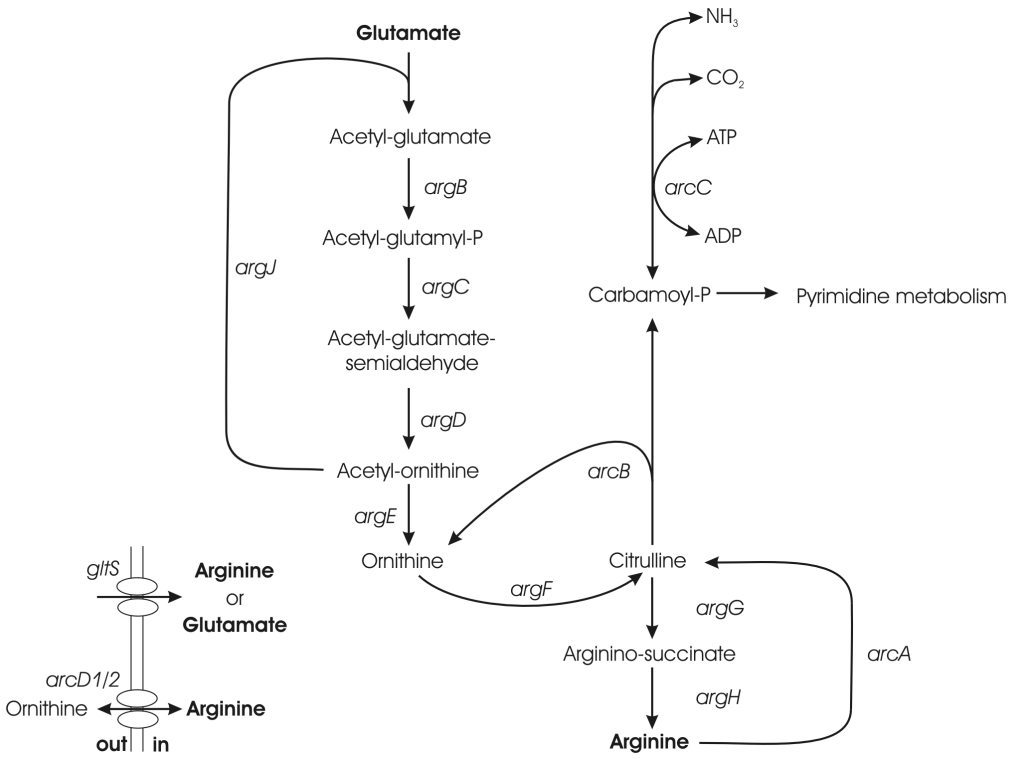

Figure 3.1. Schematic representation of arginine metabolism in *L. lactis*. Written genes encode the following enzymes: *argB*, *N*-acetylglutamate 5-phosphotransferase; *argC*, *N*-acetylglutamate 5-semialdehyde dehydrogenase; *argD*, N^2-acetylornithine 5-aminotransferase; *argJ*, ornithine acetyltransferase; *argE*, acetylornithine acetyltransferase; *argF*, ornithine carbamoyltransferase; *argG*, argininosuccinate synthetase; *argH*, argininosuccinase; *arcA*, arginine deiminase; *arcB*, ornithine carbamoyltransferase; *arcC*, carbamate kinase; *gltS*, arginine or glutamate transporter.

Arginine metabolism has been shown to be regulated by a transcriptional regulator called ArgR or AhrC in several diverse organisms (51, 53, 148, 183, 216). In this respect arginine regulation deviates from the 'rule' of attenuation regulation of amino acid metabolism in prokaryotes (44, 206, 268). Regulation of amino acid metabolism in LAB via the direct action of a DNA binding protein has only been

observed in the case of CmbR, which activates expression of the sulfur related *metCcysK* operon in response to acetylserine in *L. lactis* (77).

Several characteristic features of ArgR/AhrC-type regulators have been described: (i) they form hexa-oligomeric complexes (53, 148), (ii) they have a winged helix-turn-helix (wH-T-H) DNA binding domain (236) and (iii) ArgR plays a role as an accessory factor in multimer resolution of colE1 plasmids in *E. coli* (113, 233). ArgR and AhrC repress their own expression (148) and activate the transcription of arginine catabolic genes by interacting with other regulation factors, such as ANR and RocR of *E. coli* and *B. subtilis*, respectively (91, 160, 255).

ArgR and AhrC monomers consist of two domains, an N-terminal DNA binding domain containing the wH-T-H structure and a C-terminal domain involved in arginine binding and subunit-multimerization (236). Investigation of the hexameric structure by crystallization has shown that six arginine molecules bind in the interphase between the C-terminal domains of two trimers (250) and that arginine thereby functions as co-repressors.

ArgR and AhrC homo-hexamers bind to operator sites (called ARG boxes) in regions of biosynthetic and catabolic arginine promoters. The ARG box is an 18-bp imperfect palindromic sequence, the consensus of which varies slightly between organisms (52, 147, 163, 176). The number of boxes was shown to correlate with the observed regulation. Thus, repression is stronger when two or three ARG boxes are present, as seen in the *E. coli* biosynthetic promoters, than when only a single box is present, as in the *argR* promoter of *E. coli* (51).

The publication of the entire *L. lactis* subsp. *lactis* IL1403 genome (24) has led to the identification of two ArgR/AhrC orthologues. Multiple putative arginine regulators have also been found in the genomes of other bacteria (14) but the function of these and the reason for the presence of more than one regulator in one organism remains to be established.

In this paper we show that *L. lactis* subsp. *cremoris* MG1363 harbours two functional arginine regulators. They cooperate in the repression of arginine biosynthesis, but have different functions in the activation of arginine catabolism.

Results

gltSargE derepression mutations in *L. lactis* target to two ArgR/AhrC-type regulators

A pTn*Nuc* integration library of *L. lactis* MG1614, an isogenic *L. lactis* ssp *cremoris* MG1363 derivative (201), was plated on GM17 plates containing erythromycin and X-gal. Colonies were screened by replica-plating onto CDM plates containing erythromycin, X-gal and 4% or 0.1% casitone. An *L. lactis* MG1614 strain called C17, showing casitone-dependent β-galactosidase activity, had *lacZ* of Tn*Nuc* integrated in the C-terminal part of *argE*, the second gene of the arginine biosynthetic *gltSargE* operon. Expression of *gltSargE* was high on the 0.1% casitone medium and low on medium with 4% casitone. Random IS*S1* transposon integration screening using pGh8::IS*S1* (162) was performed in *L. lactis* C17, to identify genes involved in casitone-dependent regulation of *gltSargE*. Approximately 14.000 colonies were screened and 18 integrants (called *gdm* for *gltSargE* derepression mutation) that were clearly derepressed on a rich medium containing X-gal were isolated. Chromosomal IS*S1* integration sites were determined for 9 of the integrants by sequencing of inverse PCR products. The resultant target genes of 7 of these are presented in Table 3.1.

The chromosomally integrated copy of pGh8::IS*S1* was cured from the C17(*gdm*) strains by growing them in GM17 without tetracyclin selection (162). In this way only the IS*S1* element was left at the chromosomal integration site (strains denoted "*gdm-ex*"), allowing for a direct comparison between the cured strains and the parental strain *L. lactis* C17 under the same culturing conditions. Excision of pGh8::IS*S1* was confirmed by Southern blotting, PCR on chromosomal DNA and by testing for tetracyclin sensitivity.

In strains C17(*gdm8*) and C17(*gdm29*) the integration sites could be located to an open reading frame (ORF) with high homology to *arcD2* of *L. lactis* IL1403. This gene encodes a putative arginine/ornithine antiporter and is the last gene of the arginine catabolic pathway operon *arcABD1C1C2TD2*. The chromosomal IS*S1* targets of the six remaining C17(*gdm*) strains all located in or upstream of either of two ORFs of which the products show high homology to ArgR/AhrC-type DNA binding proteins in other organisms. Growth of the integrants was found to be strongly reduced in the absence of arginine and experiments in the following sections

were all performed with cells grown in the presence of (different concentrations of) arginine.

Table 3.1. Characterization of *L. lactis gdm-ex* mutants.

Strain	IS*S1* target gene (insertion site relative to start of gene)	*gltS-argE* expression determined as specific β-galactosidase activity (Miller Units), at the indicated arginine concentrations[a]			Repression ratio (0.1mM/10mM)[b]
		0.1 mM	1 mM	10 mM	
C17	None	1.1	0.5	0.3	3.7
C17(*gdm24ex*)	*argR* (231 bp)	116.6	96.9	88.3	1.3
C17(*gdm25ex*)	*argR* (411 bp)	85.5	14.3	3.6	23.8
C17(*gdm28ex*)	*argR* (59 bp)	91.1	63.9	64.9	1.4
C17(*gdm1ex*)	*ahrC* (105 bp)	87.3	79.1	73.2	1.2
C17(*gdm26ex*)	*ahrC* (28 bp)	88.2	84.8	80.3	1.1
C17(*gdm8ex*)	*arcD2*[c]	17.5	10.0	0.6	29.2
C17(*gdm29ex*)	*arcD2*	14.8	8.9	0.8	18.5

[a] Activity was measured in cells from two independent cultures in CDM15 harvested during exponential growth phase.
[b] Specific β-galactosidase activity in CDM15 with 0.1mM L-arginine divided by that in CDM15 with 10mM L-arginine.
[c] Exact location in *arcD2* not determined.

The *gltS-argE* operon of *L. lactis* is strongly derepressed in both *argR* and *ahrC* mutants

IS*S1* of pGh8::IS*S1* had integrated in the very N-terminal part of *ahrC* in strains C17(*gdm1ex*) and C17(*gdm26ex*), resulting in strong derepression of *gltS-argE* expression. Surprisingly, the expression of *gltS-argE* in strains C17(*gdm1ex*) and C17(*gdm26ex*) was much higher than observed for strain C17 even at very low arginine concentrations (see Table 3.1). Strain C17(*gdm27ex*) showed the same derepression phenotype as strains C17(*gdm1ex*) and C17(*gdm26ex*), but IS*S1* insertion had occurred in the *yiiB* gene located just upstream of *ahrC* (data not shown). The genes *yiiB* and *ahrC* overlap by 4 bps suggesting that they are transcriptionally coupled. Homology searches predict YiiB to be a 23S rRNA methyl-transferase, with some homology to an S4 RNA binding domain and to an FtsJ-like methyl-transferase (E-values of 5.9e-3 and 1.8e-5, respectively), not known to have any influence on arginine metabolism. The observed derepression in strain C17(*gdm27ex*) is probably caused by a polar effect on *ahrC* expression rather than inactivation of the *yiiB* gene product. The fact that derepression reached the same

levels as measured for the other *ahrC* integration knockouts is in accordance with this hypothesis (data not shown). The *ahrC* gene is followed by a terminator structure with a calculated free energy of –13.0 kcal. A *recN* homologue is present downstream of *ahrC*, with an intergenic spacing of 180 bps. A weak putative promoter (TTGTGC-18N-TATAAT) and ribosomal binding site (AGAAAGGAAAT) precede *recN*. Considering the genetic structure of the *ahrC* region, disruption of *ahrC* expression alone is expected to cause the derepression of *gltS-argE* expression in strains C17(*gdm1ex*) and C17(*gdm26ex*), and possibly also in strain C17(*gdm27ex*).

The C17(*gdm24ex*), C17(*gdm25ex*) and C17(*gdm28ex*) strains all carry IS*S1* in a 459-bp gene annotated as *argR* in *L. lactis* IL1403. The strains differed with respect to the extent to which *gltS-argE* was derepressed. Strain C17(*gdm28ex*), in which *argR* is disrupted in the start of the gene, showed a complete *gltS-argE* derepression phenotype similar to the *ahrC* knockout strains C17(*gdm1ex*) and C17(*gdm26ex*) (Table 3.1). In strain C17(*gdm24ex*) the insertion had taken place in the centre of *argR* (Table 3.1). Interestingly, disruption of *argR* in this region resulted in a drastic growth inhibition, with growth rates of $0.39h^{-1}$ in CDM15 with 0.1mM arginine to $0.31h^{-1}$ in CDM15 with 10mM arginine, compared to growth rates between $0.5h^{-1}$ and $0.63h^{-1}$ for the other strains. Finally, with IS*S1* insertion at the very end of *argR*, strain C17(*gdm25ex*) showed maximum derepression to a level comparable to that in strain C17(*gdm28ex*), but differing in that it had maintained the ability to sense and respond to arginine availability (see Table 3.1). Two transcriptional terminator structures with calculated free energies of –12.5 and –14.4 kcal, respectively, are located in the *argR-murC* intergenic region. The *argR* gene is located in a divergent orientation with *argS* (encoding arginyl-tRNA synthetase) and is separated from this gene by a putative promoter region of only 67 bp. A consensus extended –10 box (TGGTATAAT) is located upstream of *argR* but no clear ribosome binding site could be identified. As *argR* is in opposite orientation with respect to the neighbouring *argS* and *murC* genes, disruption of *argR* is expected to be the sole cause of *gltS-argE* derepression in the strains C17(*gdm24ex*), C17(*gdm25ex*) and C17(*gdm28ex*).

Regulation of the arginine biosynthesis *argCJDBF* operon in *L. lactis*

A fragment of 296 bp containing the entire *argC* promoter (P*argC*) was cloned upstream of *lacZ* in the promoter expression vector pILORI4. This expression

construct was introduced in the wild-type strain *L. lactis* MG1363, as well as its single isogenic regulator mutants *L. lactis* MGΔ*argR* and MGΔ*ahrC*, and the double regulator mutant *L. lactis* MGΔ*argRahrC*. Expression of *lacZ* from this promoter was investigated during growth on chemically defined media (CDM15) containing different concentrations of arginine. Clear arginine-dependent repression was observed in the wild-type strain MG1363 (Fig. 3.2). In each of the single regulator mutants, arginine repression was no longer seen and β-galactosidase expression reached the same levels as that in the double regulator mutant (Fig. 3.2). As was observed for the expression of the *gltS-argE* operon in the *argR*::IS*S1* or *ahrC*::IS*S1* knock-out strains, disruption of a single regulator gene resulted in complete derepression of expression from P*argC*. Thus, it appears that the two regulators, ArgR and AhrC, have a co-repressing effect rather than a cumulative effect on repression of the *argCJDBF* arginine biosynthetic operon in *L. lactis*.

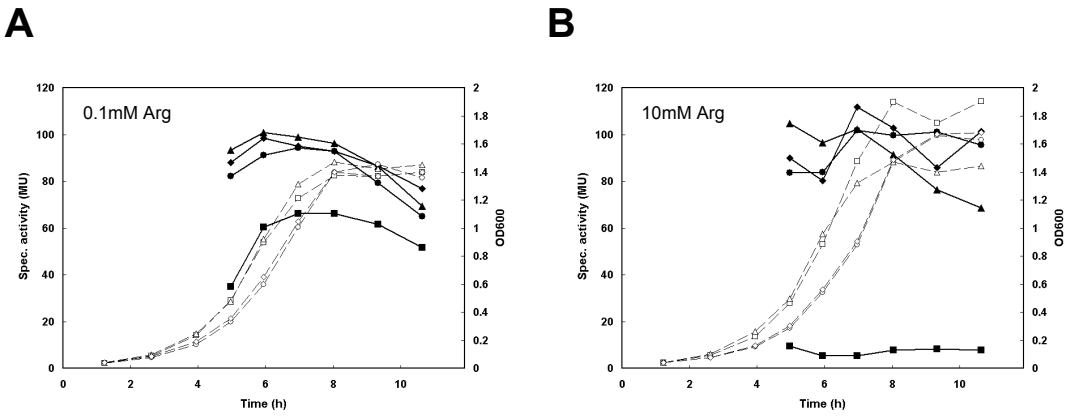

Figure 3.2. Growth (- - -) and β-galactosidase activities (—) of *L. lactis* MG1363 (□■), MGΔ*argR* (○●), MGΔ*ahrC* (Δ▲) and MGΔ*argR*Δ*ahrC* (◊♦), all harbouring p4::P*argC*, in CDM15 with (A) 0.1mM L-arginine, and (B) 10mM L-arginine.

ArgR and AhrC have different roles in regulation of the arginine catabolism operon of *L. lactis*

In the light of the regulation of the arginine biosynthetic *gltS-argE* and *argCJDBF* operons, we decided to examine the role of the regulators in the expression of the arginine catabolic *arc* gene cluster. To that end, the *arcA* promoter

region (P*arcA*) up to 260 bp upstream of the *arcA* start codon (same construct as P*arcA*-1 in Fig. 3.4) was cloned in the expression vector pILORI4, which was then introduced in *L. lactis* MG1363 and its isogenic regulator deletion mutants. As shown in Fig. 3.3, clear arginine-dependent regulation was observed in *L. lactis* MG1363, with expression from P*arcA* increasing with an increase in the arginine concentration. Deletion of *ahrC* resulted in no or only low expression from P*arcA* even at a high arginine concentration in the medium. In contrast, expression was constitutively high in the *argR* mutant and in the *argR ahrC* double deletion strain. This would suggest that activation of arginine catabolism in *L. lactis* MG1363 is mediated by AhrC, and that a repressing effect is exerted by ArgR. Alternatively, the high P*arcA* expression in the *argR* deletion mutants could be caused by a constant high intracellular level of arginine resulting from the derepression of arginine biosynthesis. However, in the double mutant the function of AhrC seems to be 'overruled' by the removal of ArgR.

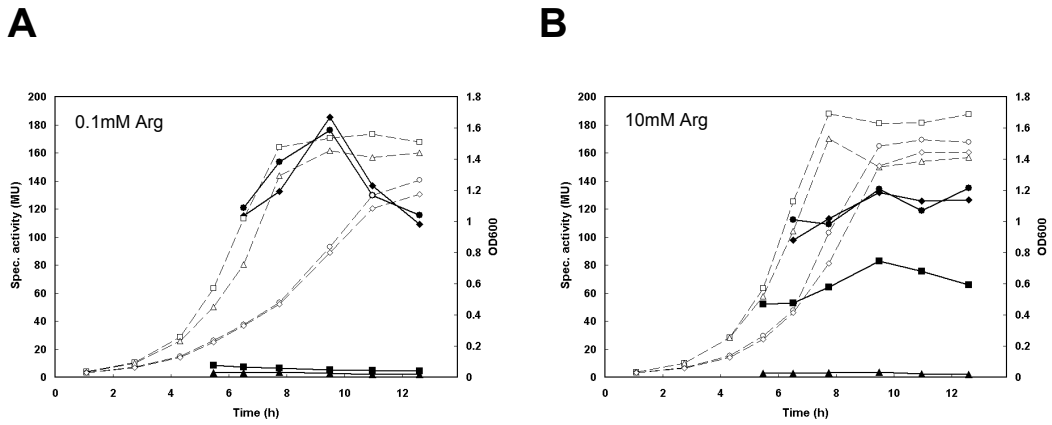

Figure 3.3. Growth (- - -) and β-galactosidase activities (—) of *L. lactis* MG1363 (□■), MGΔ*argR* (○●), MGΔ*ahrC* (Δ▲) and MGΔ*argR*Δ*ahrC* (◊♦), all harbouring p4::P*arcA*, in CDM15 with (A) 0.1mM L-arginine, and (B) 10mM L-arginine.

The *argS-arcA* intergenic region contains several features (see Fig. 3.4): a putative transcription terminator with a calculated free energy of -9.1 kcals composed of a dyad symmetry followed by a stretch of thymidine residues, starting 19 bp downstream of *argS*; two core promoter structures, P1 (5'-TTGACA-17N-TATAAT) and P2 (5'-TTGTCA-17N-TATAAA) are located at 56 to 84 bp and at 118 to 146 bp, respectively, from the start of *arcA*; a characteristic ribosomal binding site (5-

AAAGGA) 9 bp upstream of *arcA*. In order to identify possible operator sites involved in the observed regulation, deletion derivatives of the *arcA* promoter region were transcriptionally fused to *lacZ* in pILORI4 (Fig. 3.4). β-galactosidase activities of these promoter fragments were measured in the wild-type strain MG1363 grown in CDM15 with 0.1mM or 10mM arginine (Table 3.2).

Removing the –35 box of P1 (Fig. 3.4, compare P*arcA*-7 to P*arcA*-8) resulted in a severe decrease of expression of *lacZ* (Table 3.2). Fragment P*arcA*-8 gave arginine independent expression, defining P1 as the minimal promoter, lacking operators involved in arginine regulation. Partial arginine-dependent activation, compared to P*arcA*-1 containing the entire promoter region, took place in P*arcA*-5 and P*arcA*-6, suggesting the presence of operator(s) of arginine-regulation in this region. Including the entire putative promoter P2 (the region up to –156 upstream of *arcA*) did not result in increased β-galactosidase activity, questioning the functionality of P2. However, the region included in P*arcA*-4 just upstream of the P2 structure had a dramatic effect on expression, resulting in high arginine-independent expression. Regulation was only restored by including sequences further upstream, 223 to 260 bp from *arcA*, with maximal arginine-dependent regulation taking place with the largest fragment, P*arcA*-1.

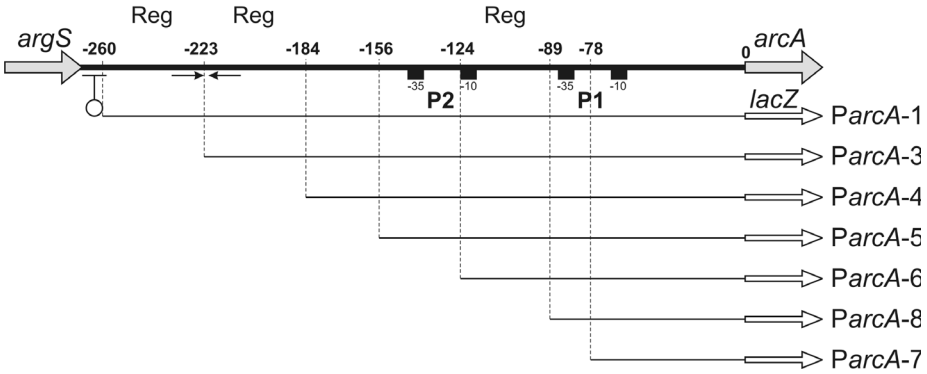

Figure 3.4. Schematic representation of the *argS-arcA* intergenic region. Numbers on the top line refer to the positions relative to the AUG start codon of *arcA* (0). The indicated fragments were cloned in the pILORI4 promoter expression vector in transcriptional fusion with *lacZ*. Fragment names are shown on the right. The putative *argS* transcriptional terminator is indicated by a 'lollipop', –10 and –35 boxes of the putative promoters P1 and P2 are shown with boxes and a putative regulatory palindromic structure is shown with arrows. 'Reg' denotes regions involved in arginine-dependent regulation (see text for details).

Table 3.2. Expression of P*arcA* subclones.

P*arcA* promoter construct	Specific β-galactosidase activity (Miller Units), at the indicated arginine concentrations[a]		Fold regulation (10mM/0.1mM)[b]
	0.1 mM	10 mM	
pILORI4::P*arcA*-1	7.0 (± 0.7)	51.0 (± 15.8)	7.3
pILORI4::P*arcA*-3	32.6 (± 5.3)	97.5 (± 1.1)	3.0
pILORI4::P*arcA*-4	84.1 (± 16.6)	67.0 (± 8.0)	0.8
pILORI4::P*arcA*-5	11.9 (± 2.2)	35.1 (± 8.7)	2.9
pILORI4::P*arcA*-6	16.7 (± 1.0)	37.5 (± 8.2)	2.2
pILORI4::P*arcA*-8	11.8 (± 2.3)	9.6 (± 3.1)	0.8
pILORI4::P*arcA*-7	1.0 (± 0.6)	0.4 (± 0.1)	0.4

[a] Activity was measured in cells from three independent cultures in CDM15 harvested during the transition phase of growth. Standard deviations are shown in parenthesis.
[b] Specific β-galactosidase activity in CDM15 with 10mM arginine divided by that in CDM15 with 0.1mM arginine.

The lactococcal arginine regulators lack conserved amino acid residues

The *argR* gene of *L. lactis* subsp. *cremoris* MG1363 (which is isogenic to strain MG1614, (93)) encodes a putative protein of 152 amino acids, called ArgR*Ll* in the following, while *ahrC* specifies a putative protein of 148 amino acids, named AhrC*Ll*. The two regulators show mutual identity for 50 amino acid residues (32%), and are homologous to well known arginine regulators, like ArgR of *E. coli*, AhrC of *B. subtilis* and ArgR of *B. stearothermophilus* (Fig. 3.5). All these proteins contain an N-terminal DNA binding domain, a central hinge region and a C-terminal arginine sensing and subunit-multimerization domain (see Fig. 3.5). Mutagenesis studies of the arginine regulators of *E.* coli (ArgR*Ec*) (33, 157, 245) and *B. stearothermophilus* (ArgR*Bst*) (126) have identified amino acid residues that are essential for regulator functionality. Of these residues, Ser47 and Arg48 of ArgR*Ec* conserved are in both lactococcal regulators (Fig. 3.5). However, other residues known to play a role in operator-regulator interaction have changed in ArgR*Ll* and AhrC*Ll*. Ser44 of ArgR*Ec* has changed to Ala, Thr51 is replaced by Lys or Arg, and Arg57 has changed to Lys in the regulators of the aligned Gram-positive organisms in Fig. 3.5. A range of residues in the N-terminal part of the arginine regulators of the Gram-positive bacteria are highly conserved, but less so in the Gram-negative ArgR*Ec*, e.g. amino acid residues 36 to 45 of AhrC*Ll* shows a highly conserved VTQATVSRDI motif. In the

C-terminal domains of the proteins there appears to be higher similarity between the Gram-negative *E. coli* regulator and the Gram-positive regulators and, in most cases, residues known to be essential for subunit-multimerization and arginine binding have been conserved. However, it is noteworthy that of the conserved GTI-X-GDDT motif (residue 123-130 of ArgR*Ec*), only the Ile and the double Asp residues are maintained in AhrC*Ll*. Whereas most of these residues are preserved in ArgR*Ll*, it should be noted that Asp128, which is essential for arginine-binding in ArgR*Ec*, is replaced by an Ala residue. The possible significance of these changes will be discussed below.

Discussion

In this work we have investigated the regulation of arginine metabolic genes in *Lactococcus lactis* and have shown that two ArgR/AhrC-type regulators are required for repression of the arginine biosynthetic *gltS-argE* operon. Chromosomal *argR* and *ahrC* deletion mutants of *L. lactis* MG1363 were made to confirm that repression of the central arginine biosynthesis operon *argCJDBF* is also dependent on the presence of both regulators. Arginine-dependent regulation of the catabolic *arcABD1C1C2TD2* gene cluster was also abolished in the regulator mutants. However, in this case the mutations had different effects, as the lack of ArgR resulted in high and arginine-independent expression while lack of AhrC resulted in constitutive low expression. Until now, the function of arginine regulators has only been investigated in organisms carrying a single arginine regulator (*e.g.* ArgR in *E. coli* and AhrC in *B. subtilis*). What has mainly caught our interest is the fact that two functional, homologous regulators are involved in and necessary for arginine-dependent gene regulation in *L. lactis*.

The presence of two homologous regulators suggests that (i) the regulators are paralogs, able to perform the same function(s) and to complement each other, or that (ii) they have different functions, *e.g.* one regulating arginine biosynthesis and the other arginine catabolism, as proposed by (100). Neither supposition holds true for the arginine regulators of *L. lactis*. The results on the regulation of the *gltSargE* and *argCJDBF* biosynthetic operons clearly demonstrate that the two regulators are not complementary. Not only did the IS*S1* integration knockout screening allow identification of both regulators, which would not be the case could any one of them perform the action of the other, but also arginine-dependent regulation was abolished

in either of the single regulator deletion mutants. Both regulators have different functions with respect to regulation of the arginine catabolic pathway, but neither of the single regulators could be shown to be responsible for the arginine-dependent regulation of arginine catabolism observed in the wild-type strain. Another surprising observation was that expression of *gltS-argE* in the wild-type strain, although regulated in dependence of arginine availability, was much lower than that in either of the regulator knockout strains. A similar observation was made in the study of ArgR in two different *E. coli* strains, K12 and B (244). Only a single amino acid substitution differentiates ArgR of *E. coli* K12, which showed strong arginine-dependent regulation, from ArgR of *E. coli* B, which only mediated weak arginine-dependent regulation resulting in so-called super-repression of arginine biosynthesis (232, 244). Both ways to regulate arginine metabolism are effective, and a mechanism of super-repression as observed for ArgR of *E. coli* B might be utilized by *L. lactis*. This putative super-repression in the wildtype *L. lactis* MG1363 was not observed in the promoter expression studies, but this may be explained by the possibly low levels of ArgR*Ll* and AhrC*Ll* in the cell: the multicopy vector situation may, to some extent, dilute the regulator proteins relative to the plasmid-located operators, despite pILORI4 being a low-copy number vector. Alternatively, the difference in the level of regulation between the *argC* and *gltS* promoters could be explained by the presence of only one ARG box upstream of the *gltS* operon as opposed to two in the *argC* operon (see below), as the number of ARG boxes is known to correlate with the level of regulation in *E. coli* (51).

The three different IS*S1* integration sites in *argR* yielded entirely different growth characteristics or *gltSargE* expression patterns, which allows to confirm the functions of the ArgR*Ll* subdomains. Integration in the putative hinge region of ArgR, disrupting the C-terminal part, not only caused arginine-independent derepression but also a considerable growth inhibition (Table 3.1). As seen for ArgR*Ec* and AhrC*Bs*, this suggests that the C-terminus of ArgR*Ll* is essential for arginine sensing. Additionally, the N-terminal part may have some intrinsic DNA-binding capacity, disturbing other metabolic functions of the cell. The reappearance of arginine sensing when disruption takes place in the very C-terminal region of the regulator confirms the sensory function of this domain. The more pronounced derepression of *gltSargE* caused by the latter mutation compared to the wild-type is most likely the result of incorrect arginine sensing.

The *arcD1* and *arcD2* genes most likely encode the arginine/ornithine antiporter described by (197). The gene *arcD2* is the last gene in the catabolic *arc* operon and, therefore, the only gene of which the expression was affected by the IS*S1* insertion in strains *gdm8ex* and *gdm29ex*. The observed effect on gene regulation is probably indirect: derepression of *gltS-argE* expression as a result of *arcD2* disruption is probably caused by low arginine uptake rates, leading to endogenous arginine deficiency with subsequent increased expression of the arginine biosynthetic genes. In these integrants (*gdm8ex* and *gdm29ex*) *gltSargE* was still regulated as a function of arginine availability, presumably via the ArgR and AhrC proteins that are present in these strains. However, only in the highest extracellular concentration of arginine tested was *gltS-argE* expression restored to wild-type level.

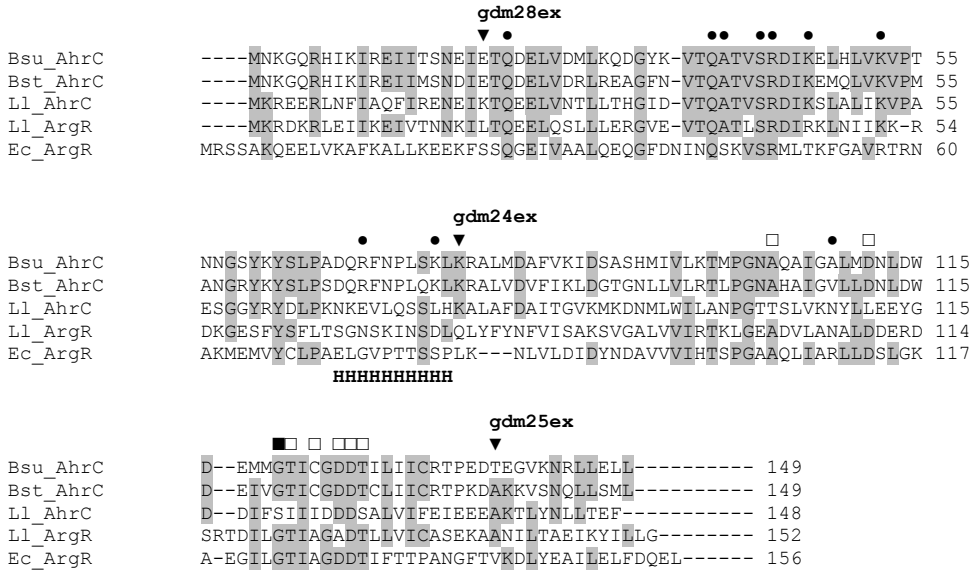

Figure 3.5. ClustalW-aligned sequences of arginine regulators from *B. subtilis* 168 (Bsu_AhrC), *B. stearothermophilus* (Bst_ArgR), *L. lactis* MG1363 (Ll_ArgR and Ll_AhrC) and *E. coli* K12 (Ec_ArgR). Shaded residues are identical in more than 50% of the sequences. 'H' indicates the hinge region residues connecting the C- and N-terminal domains, as determined from the *B. stearothermophius* ArgR crystal structure (Ni *et al.*, 1999). Functions of specific residues are specified as follows: Involved in operator recognition and binding (●); involved in subunit-multimerization (■); involved in arginine binding (□). IS*S1* integration sites in ArgR*Ll* and integrant strain names are indicated with (▼).

Regulation mediated by ArgR/AhrC-type regulators hints to the presence of ARG box operators. Indeed, operators similar to ARG boxes of *E. coli* and *B. subtilis*,

5'-WNTGAATWWWWATTCANW (157) and 5'-CATGAATAAAAATKCAAK (173), respectively, are present in the promoter regions of the *argCJDBF* and *gltS-argE* operons: $gltS_O$ 5'-AATGTATAATTATACTTA (at –43 to –26 from the start of *gltS*), $argC_{O1}$ 5'-AAAGTATAATAATACATA (at –82 to –65 from *argC*) and $argC_{O2}$ 5'-AGTGTATAAAAATACATA (at –32 to –15 from *argC*), where positions identical to the *E. coli* ARG box are underlined. $gltS_O$ and $argC_{O2}$ are both located in the putative core promoters of *gltS* and *argC*, respectively. The 32-bp spacing of $argC_{O1}$ and $argC_{O2}$ is unusual as double ARG boxes are generally only 3 bp apart (157). Still, this organization would be in accordance with repression of these promoters taking place via direct interaction between the arginine regulators and the ARG box operators. This possibility is further supported by the fact that the N-terminal DNA-binding domains of both lactococcal arginine regulators show high mutual similarity and to those of ArgR*Ec*, ArgR*Bst* and AhrC*Bsu* (Fig. 3.5).

A catabolite responsive element (*cre* site) overlaps the core promoter of *arcA*, which is in agreement with the previously described carbon source-dependent regulation of arginine degradation in *L. lactis* (49). Subcloning of the *arcA* promoter allowed to locate regions involved in the observed arginine-dependent regulation. However, in none of these regions could consensus ARG boxes be identified. Regions of regulatory importance located to three different parts of the *argS-arcA* intergenic region (Fig. 3.4). The region just upstream of P1 partially restored arginine-dependent regulation of *arcA*, suggestive of an element activating expression from P1. The high arginine independent expression observed by including the region upstream of P2 could be the result of activation via an upstream operator lacking regulatory capacity and inducing expression from P1 or P2 or both. That the regulatory capacity was restored by including the entire promoter region points to operators involved in arginine-dependent control by a repressing mechanism. This pattern of regulation is intriguing and reveals a rather complex regulatory scheme, involving activation as well as repression. An A/T-rich palindromic structure (5'-TCTTTTTTAAAATATTTTGTAAAATA, 206 to 231 bps upstream of the start of *arcA*; nucleotides of the palindrome are underlined) that lacks features of a typical transcriptional terminator is present in the region upstream of P2 (see Fig. 3.4). Approximately half of the structure is included in P*arcA*-3 and the complete structure is present in P*arcA*-1. Whether this structure in reality is involved in regulation of P*arcA* remains to be verified. The fact that the arginine degradative pathway is

involved in a range of diverse cellular functions such as energy production, acid stress resistance and pyrimidine biosynthesis, could explain the presence of such a complex regulatory circuit. Interestingly, (191) have reported on an essential two component system that is involved in activation of arginine degradation. Whether, and how, this system is responsible for some of the effects described above, remains to be elucidated.

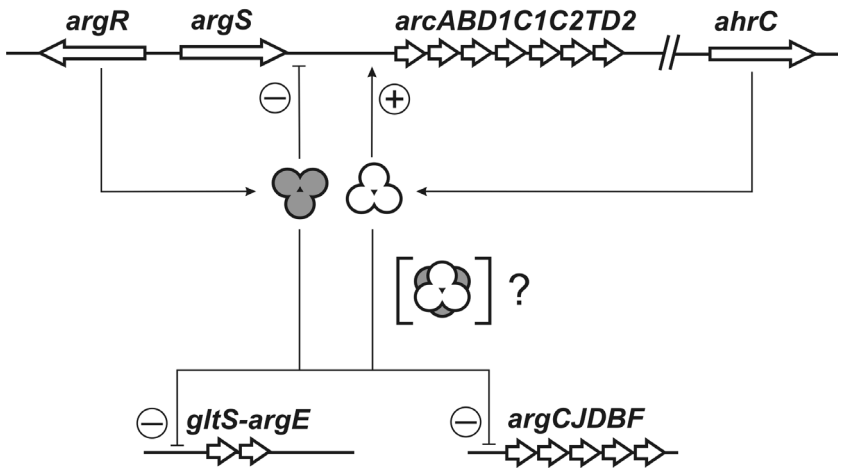

Figure 3.6. Working model of the possible regulatory mechanism exerted by ArgR and AhrC of *L. lactis.* Encircled plus and minus signs at promoter regions indicate positive and negative regulation, respectively. For details, see text.

Whereas the N-termini of ArgR*Ll* and AhrC*Ll* are highly similar, larger divergence is seen between the C-terminal domains, in particular between AhrC*Ll* and the other regulators aligned in Fig. 3.5. The lack of conservation is especially intriguing for those residues with known functions, in the *B. stearothermophilus*, *B. subtilis* and *E. coli* regulators (33, 126, 186, 245): whereas *e.g.* ArgR*Ll* lacks one of the C-terminal Asp residues directly involved in arginine binding (183), AhrC*Ll* harbours an extra Asp residue at the equivalent location (Fig. 3.5).

The fact that both regulators are essential for regulation and that the missing conserved arginine-binding Asp residue of ArgR*Ll* seems to be complemented in AhrC*Ll* has led us to postulate a working hypothesis in which both proteins are thought to interact to form hetero-hexameric complexes, consisting of one ArgR*Ll* trimer interacting with one AhrC*Ll* trimer (see Fig. 3.6).

The presence of two arginine regulator homologues in *Enterococcus faecalis* has recently been described (11). Only a single Asp residue, as is the case for ArgR*Ll*, is present in the putative arginine-binding region of both *E. faecalis* homologues, leading to the suggestion that these regulators may bind metabolites other than arginine. However, the functionality of the *E. faecalis* gene products remains to be investigated.

A gene regulatory mechanism of the type we have described in this paper is, to our knowledge, unprecedented in prokaryotes and is the focus of ongoing research.

Experimental procedures

Bacterial strains and media

Strains of *L. lactis* used in this study are listed in Table 3.3. *L. lactis* was grown at 30°C or 37°C in M17 medium (238) with 0.5% glucose as carbon source (GM17). A chemically defined medium (CDM) was made as described earlier (172) with casitone (Difco, West Molesey, UK) in concentrations of 0.1% or 4%. CDM buffer containing 15 free amino acids (CDM15) was made as described previously (138), omitting arginine unless stated otherwise. Arginine stock solutions were made in destilled H_20; pH was set to 7.0 with HCl. For solid media, agar was added to a concentration of 15 g x l^{-1}. The following components were added when needed: Erythromycin (Ery), 4 μg x ml^{-1} for selection of plasmids or 1 μg x ml^{-1} for maintaining Tn*Nuc* integrations; Tetracyclin (Tet), 2 μg x ml^{-1}; Chloramphenicol (Cam), 4 μg x ml^{-1}; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 40 μg x ml^{-1}. Antibiotics were purchased from Sigma Chemical Co. (St. Louis, USA) and X-gal from Roche Molecular Biochemicals (Manheim, Germany).

Table 3.3. Bacterial strains and plasmids.

Strain/plasmid	Description	Source / Reference
Strains		
MG1363	*L. lactis* subsp. *cremoris*, plasmid free derivative of NCDO 712	(93)
MG1614	MG1363, Str^R, Rif^R	(93)
C17	MG1614, chromosomal pTn*Nuc* insertion in *glS-argE*	This work
gdm strains	C17, pGh8::IS*S1* random integration mutants	This work
gdm-ex strains	*gdm*, pGh8::IS*S1* excised from chromosome	This work
MGΔ*argR*	MG1363; Chromosomal deletion of *argR*	This work
MGΔ*ahrC*	MG1363; Chromosomal deletion of *ahrC*	This work
MGΔ*argRahrC*	MG1363; Chromosomal deletions of *argR* and *ahrC*	This work
Plasmids		
pTn*Nuc*	Cam^R, Ery^R, TetR, AmpR, contains promoterless *S. aureus nuc* gene, transcriptionally fused to *lacZ*	(201)
pGh8::IS*S1*	Tet^R, *ori*Ts, random integration vector	(162)
pORI13	Ery^R, ori^+ $repA^-$, promoterless *lacZ*	(214)
pORI280	Ery^R, ori^+ *rep*A^-, *lacZ* expressed constitutively via promoter P32	(146)
pIL252	Ery^R, Low copy number cloning vector	(227)
pVE6007	Cam^R, *ori*(Ts)	(161)
p280Δ*argR*	Ery^R, pORI280 containing *argR* deletion construct	This work
p280Δ*ahrC*	Ery^R, pORI280 containing *ahrC* deletion construct	This work
pORI13P32	Ery^R, P32 cloned upstream of *lacZ* in pORI13	This work
pILORI4	Ery^R, pIL252 carrying the MCS and promoterless *lacZ* of pORI13	This work
pILORI4::P*argC*	Ery^R, pILORI4 carrying *argC-1*/*argC-2* PCR fragment	This work
pILORI4::P*argA-1*	Ery^R, pILORI4 carrying *arcA-1*/*arcA-2* PCR fragment	This work
pILORI4::P*argA-3*	Ery^R, pILORI4 carrying *arcA-2*/*arcA-3* PCR fragment	This work
pILORI4::P*argA-4*	Ery^R, pILORI4 carrying *arcA-2*/*arcA-4* PCR fragment	This work
pILORI4::P*argA-5*	Ery^R, pILORI4 carrying *arcA-2*/*arcA-5* PCR fragment	This work
pILORI4::P*argA-6*	Ery^R, pILORI4 carrying *arcA-2*/*arcA-6* PCR fragment	This work
pILORI4::P*argA-7*	Ery^R, pILORI4 carrying *arcA-2*/*arcA-7* PCR fragment	This work
pILORI4::P*argA-8*	Ery^R, pILORI4 carrying *arcA-2*/*arcA-8* PCR fragment	This work

DNA isolation and manipulations

Chromosomal and plasmid DNAs were isolated from *L. lactis* according to (124) and (22), respectively. DNA was manipulated essentially as described by (212) and lactococcal strains were transformed with plasmid DNA by electroporation (114). Chromosomal deletion mutants were made using pVE6007 (161) as helper plasmid for single crossover integration of p280Δ*argR* and p280Δ*ahrC* in *L. lactis* MG1363 grown at 37°C. Excision of pORI280, leaving the deletion constructs in the

chromosome of strain MG1363, was performed at 37°C without antibiotic selection. Excissants grown on solid medium were screened by Polymerase Chain Reaction (PCR), and mutants were confirmed with Southern blotting. Probe labelling, hybridization and detection were performed using the ECL direct nucleic acid labelling system according to the specifications of the manufacturer (Amersham Pharmacia Biotech, Little Chalfont, UK). Restriction enzymes were purchased from New England BioLabs (Beverly, USA). DNA was amplified using specific primers as listed in Table 3.4. PCR products were purified with the High Pure PCR Product Purification Kit (Roche Molecular Biochemicals). *Taq* DNA polymerase (Roche Molecular Biochemicals) was used for colony PCR, and PWO DNA polymerase (Roche Molecular Biochemicals) was used for DNA constructs.

Nucleotide sequencing reactions were performed on a DNA Labstation 625 (Vistra DNA System) using the Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Pharmacia). Fragments were separated and detected with the ALFexpress II gel system (Amersham Pharmacia).

Construction of *lacZ* expression plasmids

The constitutive lactococcal promoter P32 was amplified using primers P32-1 and P32-2, and cloned in pORI13 resulting in pORI13P32. With this plasmid as template, a PCR product containing the multiple cloning site (MCS) and *lacZ* of pORI13 was obtained using the PORI13m2 and PORI13s2 primers. The PCR fragment was inserted as an *Mfe*I/*Spe*I restriction fragment in the *Eco*RI/*Xba*I sites of pIL252, yielding plasmid pILORI4. Only very low intrinsic β-galactosidase activity could be measured in cells carrying the empty pILORI4 vector.

Promoter fragment to be analysed for expression was amplified from chromosomal DNA of *L. lactis* MG1363 by PCR using the primers listed in Table 3.4 and cloned in the low-copy expression vector pILORI4.

Isolation of mutants derepressed in arginine metabolism

L. lactis C17 (*gltSargE*::*lacZ*) was transformed with pGh8::ISS1 (162) and submitted to random integration screening on CDM containing erythromycin, tetracycline, X-gal and 4% casitone at the non-permissive temperature (37°C). Integrants showing a clear *gltSargE*::*lacZ* derepression phenotype were isolated for

further characterization. pGh8::IS*S1* was cured from the strains by repeated 1000-fold dilution and growth in GM17 plus erythromycin at the permissive temperature (28°C).

Table 3.4. Oligonucleotides used in this study.

Name	Sequence	Purpose
pORI13m2	GGCAATTGAAGGCAGCTGATCTCAAC	Construction of pILORI4.
pORI13s2	GGACTAGTAGATCTAATCGATGCATGC	As above.
P32-1	GCTCTAGACTTGTTTTTCGTGTGC	Construction of pORI13P32
P32-2	GCTCTAGACATTTCAAAATTCCTCCG	As above.
ISS1-For	ATTGTAAAACGACGGCCAGTGTTCATTGATAT ATCCTCGCTGTC	Inverse PCR of MG1614::IS*S1* integrants.
ISS1-T7	ACCTAATACGACTCACTATAGGGCTACTGAGA TTAAGGTCTTAATGGG	As above.
argR-1	GAAGATCTAATCTTCTTTAGCTTCCG	Construction of MG1363 *argR* deletion mutant.
argR-2	CGGAATTCTTCTAATCTTTTATCTCT	As above
argR-3	CGGAATTCGCAAATATTTTGACAGC	As above
argR-4	GCTCTAGAGATATGACAGATGTTGC	As above
ahrC-1	GAAGATCTTAGAAAAAGCGCTCAAAG	Construction of MG1363 *ahrC* deletion mutant.
ahrC-2	CGGAATTCCTTTTCATAGTTCTTCGC	As above
ahrC-3	CGGAATTCAGAGTTTTAAATTTACTG	As above
ahrC-4	GCTCTAGATTGACTGTCATGTTGACC	As above
argC-1	CGGAATTCTGGAACATAATAAAGCG	Cloning of *argC* promoter in pILORI4.
argC-2	GCTCTAGATATAACCTCTAATTCCG	As above
arcA-1	CGGAATTCATTCTTGCTGATGAGAG	Cloning of *arcA* promoter fragments.
arcA-2	GCTCTAGAATTTCCCAATTTCTGAG	As above
arcA-3	CGGAATTCAAATATTTTGTAAAATAAG	As above
arcA-4	CGGAATTCGAATCCCATGATAAGC	As above
arcA-5	CGGAATTCAACGTGAAATTGTCAG	As above
arcA-6	CGGAATTCTATAAATGAATAAACC	As above
arcA-7	CGGAATTCAAAATATGCATAGATG	As above
arcA-8	CGGAATTCGCTTGACAAAAAATATGC	As above

Enzyme assays

β-galactosidase activity assays were performed on cell suspensions that were permeabilized by chloroform as described previously (117).

Data analysis

The ClustalW program was used for protein sequence alignments (242). Clone Manager 6.0 was used for free energy calculations of palindromic DNA structures.

Nucleotide sequence accession numbers

The new sequences generated in this work have been given the accession numbers AY518512 (*argR*), AY518513 (*ahrC*), AY518514 (P*argC*), and AY518515 (P*arcA*).

Acknowledgements

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