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NOTES

GlnR-Mediated Regulation of Nitrogen Metabolism in *Lactococcus lactis*

Rasmus Larsen,†§ Tomas G. Kloosterman,† Jan Kok, and Oscar P. Kuipers*

Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

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We show that the nitrogen regulatory protein GlnR of *Lactococcus lactis* represses transcription of the *amtB-glnK*, *glnRA*, and *glnPQ* operons. This likely occurs through a conserved DNA motif, 5′-TGTNA-7N-TN ACAT-3′, and takes place in response to extracellular glutamine and ammonium. GlnR-independent repression of *amtB-glnK* is mediated by the pleiotropic nitrogen regulator CodY.

The lactic acid bacterium *Lactococcus lactis* has multiple amino acid auxotrophies (5, 6, 13). During growth in milk, it acquires free amino acids through degradation of extracellular proteins by a thoroughly characterized proteolytic system (15), which is controlled by the global regulator CodY (7, 8, 14). However, relatively little is known about central nitrogen regulation in lactic acid bacteria, which involves the amino acids glutamine and glutamate. In the gram-positive model organism *Bacillus subtilis*, the two transcriptional regulators TnrA and GlnR are important for the regulation of nitrogen metabolism (10). Although TnrA and GlnR recognize the same transcriptional operator sequence (TnrA/GlnR sites, 5′-T6TNA-7N-TNACAT-3′), TnrA acts both as an activator and a repressor of transcription when nitrogen is limiting (1, 10, 19, 22, 30, 31), whereas GlnR is active during conditions of nitrogen excess, repressing expression of the glutamine synthetase (*glnRA*) operon (4) and the urease (*ureABC*) operon (10, 29). The genome sequence of *L. lactis* subsp. *lactis* IL1403 does not encode a TnrA homologue, while GlnR is encoded in a putative *glnRA* operon (2). The presence of only one TnrA/GlnR homologue, as well as the different physiology and severe amino acid auxotrophy of *L. lactis* compared to *B. subtilis*, raises the question as to the role of GlnR in the nitrogen control of *L. lactis*.

To investigate the function of the putative transcriptional regulator GlnR in *L. lactis*, an in-frame marker-free deletion of *glnR* was constructed in strain MG1363 (12) essentially as described previously (18), yielding *L. lactis* MGΔ*glnR* (primer sequences are available upon request). By use of DNA microarrays, which were performed as described previously (8, 16, 27, 28), the transcription profile of this strain and MG1363 were compared in chemically defined medium (CDM) (20) with either a high (2%) or a low (0.1%) concentration of Casitone, a pancreatic digestion product of casein, as the nitrogen source. The most pronounced differences in gene expression between both strains were observed in 0.1% Casitone (Table 1). No additional differentially expressed genes were identified in 2% Casitone compared to 0.1% Casitone (data not shown).

Expression of *glnA*, encoding glutamine synthetase, and the putative ammonium transporter and sensor operon *amtB-glnK* were highly derepressed in *L. lactis* MGΔ*glnR* (Table 1). In addition, expression of the glutamine/glutamate ABC transporter gene *glnP* (23) was weakly yet significantly increased. Several genes involved in arginine biosynthesis (*argC*, *argG*,

TABLE 1. Transcriptome analysis comparing *L. lactis* MG1363 and MGΔ*glnR*[a]

Gene name[b]	Function[c]	Expression ratio[d]	*P* value[e]
Up-regulation			
amtB	Ammonium transporter	5.5	3.7e−12
glnK	Nitrogen sensory protein P_{II}	3.7	3.5e−9
glnA	Glutamine synthetase	3.2	2.2e−10
glnP	Glutamine ABC transport and substrate binding protein	1.6	1.9e−8
Down-regulation			
argC	Acetylglutamate semialdehyde dehydrogenase	−3.8	4.3e−9
gltS	Arginine or glutamate transporter	−2.3	3.1e−8
arcC2	Carbamate kinase	−1.8	2.5e−6
argG	Argininosuccinate synthetase	−1.6	3.4e−6
arcA	Arginine deiminase	−1.6	4.0e−5
arcD1	Arginine/ornithine antiporter	−1.4	5.7e−4

[a] *L. lactis* MG1363 and MGΔ*glnR* were grown to the mid-exponential phase of growth (optical density at 600 nm of 0.8) in CDM with 0.1% Casitone.
[b] Gene names as annotated for the genome sequence of *L. lactis* IL1403 (2), except for *glnK*, which was changed from *glnB*.
[c] Putative function as determined by BLAST searches.
[d] Expression in *L. lactis* MGΔ*glnR* divided by that in *L. lactis* MG1363.
[e] Genes were considered differentially expressed with a *P* of <0.001 and FDR (false discovery rate) of <0.01. IL1403 amplicon sequences and the microarray data are available online at http://molgen.biol.rug.nl/publication/glnr_data/.

* Corresponding author. Mailing address: Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands. Phone: 31-50-3632093. Fax: 31-50-3632348. E-mail: O.P.Kuipers@rug.nl.
† These authors contributed equally to this study.
§ Present address: Inflammation Group, Instituto Gulbenkian de Ciência, Apartado 14, 2781-901 Oeiras, Portugal.

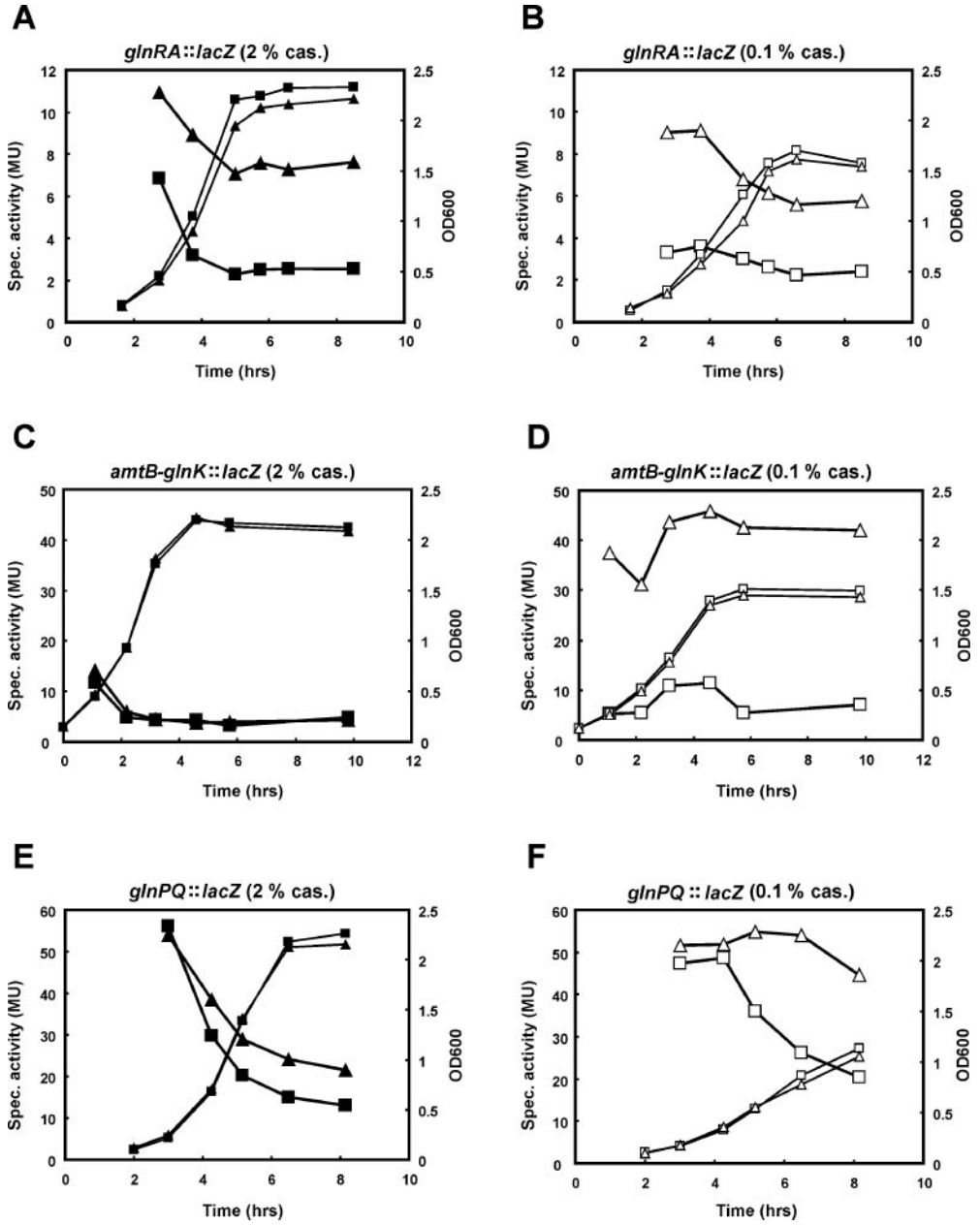

FIG. 1. Analysis of expression of *glnRA*, *amtB-glnK*, and *glnPQ* during growth of *L. lactis*. The wild-type strain MG1363 (squares) and its *glnR* derivative (triangles) carrying chromosomal *glnRA*::*lacZ*, *amtB-glnK*::*lacZ*, and *glnPQ*::*lacZ* fusions were grown in CDM containing 2% (A, C, E; closed symbols) and 0.1% (B, D, F; open symbols) Casitone (cas.). Growth (optical density at 600 nm [OD600]) is depicted with small symbols, and specific β-galactosidase activity (Spec. activity) (in Miller units [MU]) is depicted with large symbols. These are representative graphs of several repeats.

and *gltS*) and degradation (*arcC2*, *arcA*, and *arcD1*) were moderately down-regulated in the *glnR* mutant in 0.1% Casitone (Table 1). These changes in arginine metabolism could be caused by the disrupted metabolism of glutamine and glutamate, which serve as precursors of arginine synthesis.

Analysis of chromosomal transcriptional *lacZ* fusions to the *glnRA*, *amtB-glnK*, and *glnPQ* operons (primer sequences are available upon request), which were made using the integration plasmid pORI13 as described earlier (21), confirmed the DNA microarray results (Fig. 1). Interestingly, *amtB-glnK* expression was strongly derepressed only in 0.1% Casitone, indicating that this operon is also regulated independently of GlnR (Fig. 1).

Using the online tool MotifSampler (24, 25), two putative GlnR operator sites that showed high similarity to the GlnR operator of *B. subtilis* were identified in the *amtB-glnK* promoter (Fig. 2). In the *glnRA* promoter, a single putative GlnR box was found, and in the *glnPQ* promoter, a possible GlnR box is present at the start of *glnP* (Fig. 2). Promoter subcloning in the low-copy-number expression vector pILORI4 (primer

FIG. 2. Locations of predicted GlnR boxes in the promoter regions of the *amtB-glnK*, *glnRA*, and *glnPQ* operons of *L. lactis* MG1363. GlnR box sequences are boxed, and the predicted CodY box is shaded. Numbers to the left of the sequences indicate positions relative to the first base of the translation start codon. −35, −10, and extended (ex) −10 sequences are shown on a black background. Ribosomal binding sites (RBS) are underlined. Italicized, bold nucleotides are parts of open reading frames. Stop codons of upstream genes are indicated by three asterisks.

sequences are available upon request) (17) revealed that the GlnR box upstream of the −35 region in the *amtB-glnK* promoter is essential for efficient GlnR-mediated repression (Fig. 3). Promoter fragments without the entire upstream GlnR box (P*amtB*-2 and P*amtB*-3) still retained weak (~1.5-fold) GlnR-mediated regulation, possibly originating from the remaining GlnR box that covers the −10 region of the core promoter (Fig. 3).

In addition to GlnR-mediated regulation of the *amtB-glnK* operon, strong Casitone-dependent regulation of this operon that was independent of GlnR was seen (Fig. 1C and D and 3). In a recent transcriptome analysis, *amtB* was shown to be twofold up-regulated in an *L. lactis* MG1363 *codY* deletion mutant (11) grown in the nitrogen-rich medium GM17 (M17 with 0.5% glucose [23a]). A CodY operator is indeed present in the *amtB-glnK* promoter (8), located downstream of the GlnR operator sites and the core promoter region (Fig. 2 and 3). In agreement, repression of the *amtB-glnK* promoter in 2% Casitone was relieved in *L. lactis* MGΔ*codY* (Fig. 3). This effect was also seen for the *amtB-glnK* promoter fragment P*amtB*-3, in which GlnR-mediated repression was almost entirely abolished due to deletion of the first GlnR box (Fig. 3). Thus, CodY is able to overrule the GlnR-mediated control of the *amtB-glnK* operon under nitrogen-rich conditions. The exact function of the *amtB-glnK* gene pair, which is conserved among many bacterial species (26), remains to be established in *L. lactis*, but the fact that it is regulated by both GlnR and CodY suggests that it has an important role in nitrogen control in this organism.

Glutamine synthetase enzymatically converts glutamate and ammonium into glutamine. Therefore, the effects of these compounds on the expression of *glnRA*, *amtB-glnK*, and *glnPQ* were investigated. Instead of using Casitone as the nitrogen source, a chemically defined medium that contained all amino acids except either glutamine or glutamate was used. To be able to examine the effect of ammonium in the medium, ammonium citrate, which is normally present in CDM, was replaced by sodium citrate. In CDM with a low concentration of glutamate, expression of *glnRA* was the same in *L. lactis* MG1363 and MGΔ*glnR*. However, expression was repressed approximately three- and fivefold in response to high extracellular concentrations of ammonium and glutamine, respectively, in a GlnR-dependent manner (Fig. 4A). Expression of *amtB-glnK* was likewise repressed by glutamine (5.7-fold) via GlnR (Fig. 4B), but ammonium had the strongest (9-fold) repressive effect (Fig. 4B). Remarkably, significant repression (3.5-fold) was still measured in strain MGΔ*glnR* (Fig. 4B), indicating that the *amtB-glnK* operon is also repressed by extracellular ammonium independently of GlnR. Since CodY was shown to repress expression of *amtB-glnK* depending on the Casitone concentration (Fig. 3), it may well be possible that CodY is responsible for this ammonium-induced repression, possibly via an ammonium-induced increase in the intracellular level of branched-chain amino acids, which are effectors of *L. lactis* CodY (7). Glutamine had no regulatory effect on *glnPQ* expression, but both ammonium and glutamate had an approximately twofold repressive effect (Fig. 4C). However, whereas the ammonium effect seemed to be mediated by GlnR, glutamate still repressed *glnPQ* expression in MGΔ*glnR* (Fig. 4C). Glycine, a feedback inhibitor of *B. subtilis* glutamine synthetase

FIG. 4. Effects of glutamine, ammonium, and glutamate on the expression of (A) *glnRA*, (B) *amtB-glnK*, and (C) *glnPQ* as determined by single-copy, chromosomal *lacZ* fusions in *L. lactis* MG1363 (wild type) (black bars) and MGΔ*glnR* (hatched bars). Specific β-galactosidase activity (Spec. β-galactosidase act.) (in Miller units [MU]) is shown on the *y* axes. Cells were grown to mid-exponential phase in CDM with various concentrations (in milligrams/milliliter) (shown in parentheses) of glutamine (Gln), glutamate (Glu), and ammonium (NH_4^+) as indicated below the graphs. Standard deviations (error bars) were calculated on the basis of three independent biological replicates.

	0.1 % casitone					2 % casitone				
	MG	Δ*glnR*	Δ*codY*	Δ*glnR*/MG	Δ*codY*/MG	MG	Δ*glnR*	Δ*codY*	Δ*glnR*/MG	Δ*codY*/MG
P*amtB-1*	22 (2)	105 (5)	50 (4)	*4.8*	*2.0*	6 (1)	12 (1)	37 (1)	*2.0*	*5.9*
P*amtB-2*	72 (3)	99 (23)	98 (8)	*1.4*	*1.6*	13 (1)	14 (3)	62 (1)	*1.1*	*4.7*
P*amtB-3*	62 (2)	100 (12)	116 (2)	*1.6*	*2.2*	12 (0)	14 (2)	72 (2)	*1.2*	*5.9*

FIG. 3. Deletion analysis of the *amtB-glnK* promoter of *L. lactis*. The promoter region of *amtB-glnK* is drawn schematically, with GlnR boxes shown as white oval boxes, the CodY box shown as a gray oval, and −35 and −10 sequences shown as black rectangles. The extent of the promoter regions cloned upstream of *lacZ* in pILOR14 and the names of the respective promoter fragments are shown on the left below the map. β-Galactosidase activities specified by the three constructs, measured in *L. lactis* MG1363 (MG) and MGΔ*glnR* (Δ*glnR*) grown to mid-exponential phase in CDM with 0.1% and 2% Casitone, are shown on the right. The ratios of the activities of MGΔ*glnR* over MG1363 and MGΔ*codY* (Δ*codY*) over MG1363 are shown in italics. Standard deviations calculated from three independent biological replicates are given in parentheses.

(9), had no measurable effect on GlnR-mediated regulation when added in a concentration of 5 mg/ml (data not shown), demonstrating that the effects seen with glutamate, ammonium, and glutamine are specific.

This work presents the first investigation into the transcriptional regulation by GlnR of central nitrogen metabolism in the low-G+C-content gram-positive model organism *L. lactis*. The limited number of targets of GlnR in both *L. lactis* and *B. subtilis* may suggest a functional similarity. The only common GlnR target in both organisms is the *glnRA* operon. The *ureABC* genes, which are regulated by GlnR in *B. subtilis* (3, 29) are not present in *L. lactis*, while the *amtB-glnK* operon and *glnPQ* genes (in the *glnQHPM* operon) are regulated by TnrA in *B. subtilis* (31). Thus, although there is similarity, *L. lactis* GlnR represents a mechanism of nitrogen control different from that of *B. subtilis*.

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