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

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Received 9 January 2006/Accepted 4 April 2006

We show that the nitrogen regulatory protein GlnR of *Lactococcus lactis* represses transcription of the *amtR-glnK, glnRA*, and *glnPQ* operons. This likely occurs through a conserved DNA motif, 5′-TGTNA-7N-TNACAT-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) 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, argE, ornithine transaminase*), which are repressed by GlnR in other organisms (5, 6, 13) were weakly derepressed in IL1403 compared to MG1363. None of the other genes identified in 2% Casitone compared to 0.1% Casitone in our microarray experiments were also identified in the microarray experiments of Haren et al. (20), 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).

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<table>
<thead>
<tr>
<th>Gene name</th>
<th>Function</th>
<th>Expression ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ammB</em></td>
<td>Ammonium transporter</td>
<td>5.5</td>
<td>3.7e-12</td>
</tr>
<tr>
<td><em>glnK</em></td>
<td>Nitrogen sensory protein PII</td>
<td>3.7</td>
<td>3.5e-9</td>
</tr>
<tr>
<td><em>glnA</em></td>
<td>Glutamine synthetase</td>
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<td>2.2e-10</td>
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<tr>
<td><em>glnP</em></td>
<td>Glutamine ABC transport and substrate binding protein</td>
<td>1.6</td>
<td>1.9e-8</td>
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<tr>
<td><em>argC</em></td>
<td>Acetylglutamate semialdehyde dehydrogenase</td>
<td>-3.8</td>
<td>4.3e-9</td>
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<tr>
<td><em>gls</em></td>
<td>Arginine or glutamate transporter</td>
<td>-2.3</td>
<td>3.1e-8</td>
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<tr>
<td><em>arcC2</em></td>
<td>Carbamyl kinase</td>
<td>-1.8</td>
<td>2.5e-6</td>
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<tr>
<td><em>argG</em></td>
<td>Argininosuccinate synthetase</td>
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<td>3.4e-6</td>
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<td>Arginine deiminase</td>
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<td><em>arcD1</em></td>
<td>Arginine/ornithine antiporter</td>
<td>-1.4</td>
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Notes:

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and glnS) 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. 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.
sequences are available upon request) (17) revealed that the GlnR box upstream of the $\text{/H11002}$ region in the $\text{amtB-glnK}$ promoter is essential for efficient GlnR-mediated repression (Fig. 3). Promoter fragments without the entire upstream GlnR box ($\text{PamtB-2}$ and $\text{PamtB-3}$) still retained weak ($\approx 1.5$-fold) GlnR-mediated regulation, possibly originating from the remaining GlnR box that covers the $\text{/H11002}$ region of the core promoter (Fig. 3).

In addition to GlnR-mediated regulation of the $\text{amtB-glnK}$ 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, $\text{amtB}$ was shown to be twofold up-regulated in an $\text{L. lactis MG1363 codY}$ deletion mutant (11) grown in the nitrogen-rich medium GM17 (M17 with 0.5% glucose [23a]). A CodY operator is indeed present in the $\text{amtB-glnK}$ promoter (8), located downstream of the GlnR operator sites and the core promoter region (Fig. 2 and 3). In agreement, repression of the $\text{amtB-glnK}$ promoter in 2% Casitone was relieved in $\text{L. lactis MG/H9004 codY}$ (Fig. 3). This effect was also seen for the $\text{amtB-glnK}$ promoter fragment $\text{PamtB-3}$, in which GlnR-mediated repression was almost entirely abolished due to deletion of the first GlnR box (Fig. 3). Thus, CodY is able to override the GlnR-mediated control of the $\text{amtB-glnK}$ operon under nitrogen-rich conditions. The exact function of the $\text{amtB-glnK}$ gene pair, which is conserved among many bacterial species (26), remains to be established in $\text{L. lactis}$, 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 $\text{glnRA}$, $\text{amtB-glnK}$, and $\text{glnPQ}$ operons of $\text{L. lactis MG1363}$. 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.  

FIG. 2. Locations of predicted GlnR boxes in the promoter regions of the $\text{amtB-glnK}$, $\text{glnRA}$, and $\text{glnPQ}$ operons of $\text{L. lactis MG1363}$. 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.  

Glycine, a feedback inhibitor of $\text{B. subtilis}$ glutamine synthetase
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 /H1100235 and /H1100210 sequences shown as black rectangles. The extent of the promoter regions cloned upstream of lacZ in pILORI4 and the names of the respective promoter fragments are shown on the left below the map. The bars on the right below the map indicate the relative activities measured in L. lactis MG1363 (MG) and MG//H9004glnR (MG/glnR) grown to mid-exponential phase in CDM with 0.1% and 2% Casitone, as shown on the right. The bars on the left below the map indicate the relative activities measured in L. lactis MG//H9004glnR (MG/glnR) grown to mid-exponential phase in CDM with 0.1% and 2% Casitone, as shown on the right. The ratios of the activities of MG//H9004glnR over MG1363 and MG//H9004glnR over MG1363 are shown in italics. Standard deviations calculated from three independent biological replicates are given in parentheses.

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 MG/glnR (hatched bars). Specific /H9252-galactosidase activity (Spec. /H9252-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 (NH4+/H11001) as indicated below the graphs. Standard deviations (error bars) were calculated on the basis of three independent biological replicates.
(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 TrnA in B. subtilis (31). Thus, although there is similarity, L. lactis GlnR represents a mechanism of nitrogen control different from that of B. subtilis.

We thank Anne de Jong, Aldert Zomer and Sacha A. F. T. van Hijum for expert advice during execution and analysis of DNA microarray experiments.

REFERENCES