Roles of PucR, GlnR, and TnrA in Regulating Expression of the *Bacillus subtilis ure* P3 Promoter

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Expression of the P3 promoter of the *Bacillus subtilis ureABC* operon is activated during nitrogen-limited growth by PucR, the transcriptional regulator of the purine-degradative genes. Addition of allantoinic acid, a purine-degradative intermediate, to nitrogen-limited cells stimulated transcription of *ure* P3 twofold. Since urea is produced during purine degradation in *B. subtilis*, regulation of *ureABC* expression by PucR allows purines to be completely degraded to ammonia. The nitrogen transcription factor TnrA was found to indirectly regulate *ure* P3 expression by activating *pucR* expression. The two consensus GlnR/TnrA binding sites located in the *ure* P3 promoter region were shown to be required for negative regulation by GlnR. Mutational analysis indicates that a cooperative interaction occurs between GlnR dimers bound at these two sites. *B. subtilis* is the first example where urease expression is both nitrogen regulated and coordinately regulated with the enzymes involved in purine transport and degradation.

Urease degrades urea to two molecules of ammonia and carbon dioxide (14). In *Bacillus subtilis*, urease is encoded by the *ureABC* operon (7). Urease expression in *B. subtilis* is elevated during nitrogen-limited growth and is not induced by urea (1, 25). Although the *ureABC* operon has three promoters, this operon is primarily transcribed by the σ^A -dependent *ure* P3 promoter in exponentially growing cells (25). Expression of the *ure* P3 promoter has been shown to be regulated in response to nutrient availability by three transcriptional factors, GlnR, TnrA, and CodY (25). CodY activity is controlled by the levels of GTP, which serve as an indicator of the overall nutritional state of the cell (17). GlnR and TnrA are responsible for increased gene expression during nitrogen-limited growth in *B. subtilis* (10, 22, 26). TnrA and GlnR belong to the MerR family of DNA-binding proteins. The amino acid sequences of the N-terminal DNA-binding domain of TnrA and GlnR are nearly identical, and both proteins bind to sites with the same conserved DNA sequence, 5'-TGTNAN₇TNACA-3' (GlnR/TnrA site) (5, 22, 23, 26). GlnR and TnrA have little sequence similarity in their C-terminal signal transduction domains and are active under different nutritional conditions. GlnR represses gene expression during growth with excess nitrogen, while TnrA both activates and represses gene expression during nitrogen limitation. Although the mechanism regulating GlnR activity is not understood, the activity of TnrA is controlled by a reversible interaction with glutamine synthetase (28).

B. subtilis can utilize purines as a source of nitrogen (Fig. 1) (7, 15, 16, 20). The PucR transcription factor is a pathway-specific regulator of the genes belonging to the purine catabolic (*puc*) regulon (3, 24). PucR functions as both a transcrip-

tional activator and repressor. The regulation of gene expression by PucR is enhanced by growth in medium containing intermediates in purine catabolism, such as uric acid, allantoin, or allantoinic acid (3, 24). Mutational analysis of several *puc* promoters has identified a *cis*-acting sequence, 5'-WWWC NTTGGTTAA-3' (PucR box), which is required for PucR-dependent gene regulation (3). Interestingly, the expression of *pucR* is activated by TnrA (3).

PucR activates *ure* P3 expression during nitrogen-limited growth. Several observations suggested that the *ureABC* operon may be a member of the PucR regulon. First of all, urea is produced by the degradation of purines in *B. subtilis* (Fig. 1) (7, 24). Secondly, a nucleotide sequence that is highly similar to the PucR box consensus sequence was found to be located upstream of the *ure* P3 promoter (Fig. 2). In addition, microarray experiments revealed that high-level *ureABC* expression during nitrogen-limited growth was dependent upon PucR (H. Jarmer and H. H. Saxild, unpublished observations).

The role of PucR in regulating the expression of the *ure* P3 promoter was examined in cells containing a *ure* P3-*lacZ* transcriptional fusion (URE28). The strains used in this study are given in Table 1. Under nitrogen-limiting growth conditions, allantoinic acid was found to increase expression of the URE28 fusion twofold (Table 2). For unknown reasons, no induction was observed with allantoin (data not shown). β -Galactosidase levels from the URE28 fusion were 20-fold lower in a *pucR* mutant than in wild-type cells during nitrogen limitation, and allantoinic acid induction of *ure* P3 expression did not occur in the *pucR* mutant (Table 2). Finally, mutational inactivation of the PucR box in the *ure* P3 promoter (URE68 [Fig. 2]) reduced *ure* P3 expression in nitrogen-limited cells and abolished induction by allantoinic acid (Table 2). Taken together, these results indicate that *ure* P3 expression is activated by PucR.

TnrA indirectly regulates *ure* P3 expression. During growth with the nitrogen-limiting source glutamate, expression of the *ure* P3 promoter is 10-fold lower in the *tnrA* mutant than in

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FIG. 2. Nucleotide sequence of the *ure* P3 promoter. The transcriptional start site is indicated by an asterisk. The consensus sequences for the -10 and -35 regions of σ^A -dependent promoters, GlnR/TnrA sites, and PucR box are indicated above the nucleotide sequence. The nucleotide substitutions in URE63, URE68, and URE64 and the nucleotide deletions in URE65 and URE66 are indicated below the nucleotide sequence. The upstream endpoint of *ure* DNA in URE37 is indicated.

GlnR bound at the upstream and downstream GlnR sites in the *ure* P3 promoter region, these two GlnR sites must be appropriately spaced on the same side of the DNA helix. By analogy to other systems (21), GlnR-dependent regulation of *ure* P3 should be reduced when the distance between the upstream and downstream GlnR sites is changed by half-integral turns of DNA (5 bp). Moreover, deletion of a whole integral turn of DNA (10 bp) should restore the correct phasing between these two sites and increase regulation by GlnR. In the URE65 fusion, 5 bp of DNA was deleted between the upstream and downstream GlnR sites (Fig. 2). Expression of the URE65 fusion was regulated only eightfold by GlnR (Table 3). In contrast, expression of the URE66 fusion, which contains a

full-integral-turn 10-bp deletion between the two GlnR sites (Fig. 2), was regulated over 40-fold by GlnR (Table 3). The 10-bp deletion in the URE66 fusion moves a sequence containing 7 consecutive A's closer to the *ure* P3 promoter. The higher levels of expression seen with the URE66 fusion may result from this sequence acting as an upstream promoter element that enhances transcription from the *ure* P3 promoter (19).

The regulation of the *glnRA* operon by GlnR has previously been shown to involve cooperative interactions. GlnR represses *glnRA* transcription by binding to two adjacent operators that lie upstream and overlap the -35 region of the *glnRA* promoter (11, 23). Since GlnR binds simultaneously to both

TABLE 2. β -Galactosidase expression of various *ure* P3 promoter *lacZ* fusions in wild-type and mutant strains

Fusion ^a	Fusion genotype	Strain genotype ^b	β -Galactosidase sp act (U/mg of protein) in cells grown with ^c :		
			Glutamine	Glutamate	Glutamate + allantoinic acid
URE28	Wild type	Wild type	0.1	110	240
	Wild type	<i>glnR</i>	4.8	100	ND ^d
	Wild type	<i>tnrA</i>	0.1	11	ND
	Wild type	<i>pucR</i>	0.1	5.6	6.7
	Wild type	<i>glnR codY</i>	16	160	230
	Wild type	<i>glnR codY tnrA</i>	12	21	60
	Wild type	<i>pucR glnR codY</i>	11	7.9	8.4
URE63	Mutant upstream GlnR site	Wild type	2.1	130	220
URE64	Mutant downstream GlnR site	Wild type	5.9	270	350
URE68	Mutant PucR site	Wild type	0.1	6.4	7.7

^a The URE28 transcriptional *lacZ* fusion, which contains *ure* P3 DNA between -196 and $+118$, has been described previously (25). Derivatives of URE28 containing the mutations and internal deletions shown in Fig. 2 were constructed by oligonucleotide-directed mutagenesis. The URE37 fusion contains an *AluI-EcoRV* DNA fragment subclone of the *ure* P3 promoter.

^b All strains are 168 derivatives containing the indicated *ure* P3-*lacZ* fusion integrated as a single copy at the *amyE* locus. Strains were constructed by transforming mutant and wild-type cells to neomycin resistance with plasmid DNA. Transformants resulting from double-crossover events at the *amyE* locus were identified by screening for loss of amylase activity on tryptose blood agar-starch plates (12). The mutant alleles used in this study are shown in Table 1.

^c Cultures were grown in minimal medium containing glucose as the carbon source and the indicated nitrogen sources (2). Allantoinic acid was added to a final concentration of 0.48 mM. β -Galactosidase was assayed as previously described (2). Values are the average of 2 to 15 determinations, which did not vary by more than 20%.

^d ND, not determined.

TABLE 3. GlnR-dependent regulation of β -galactosidase expression from wild-type and mutant *ure P3-lacZ* fusions^a

Fusion ^b	Fusion genotype	β -Galactosidase sp act (U/mg of protein) in cells grown with ^c :	
		Glutamine	Glutamate
URE28	Wild type	0.1	16
URE37	Deletion of upstream GlnR site	1.4	11
URE63	Mutant upstream GlnR site	4.3	18
URE64	Mutant downstream GlnR site	12	14
URE65	5-bp deletion	1.4	11
URE66	10-bp deletion	2.2	86

^a GlnR-dependent regulation was examined by determining β -galactosidase levels in *pucR mraA codY* cells containing the indicated *ure P3-lacZ* fusions.

^b See Table 2, footnote b.

^c See Table 2, footnote c.

operators with in vitro and in vivo DNA footprinting experiments (5, 11), a cooperative interaction appears to occur between the GlnR dimers bound at these sites. This conclusion is supported by the observations that GlnR-dependent regulation of *glnR4* expression is completely relieved by either deletion of the upstream operator or insertion of a 5-bp DNA fragment between the two operators (23).

Although our analysis of the *ure P3* promoter indicates that a cooperative interaction occurs between the forms of GlnR bound at the two GlnR sites in the *ure P3* promoter region, GlnR still weakly regulates *ure P3-lacZ* fusions containing only the downstream GlnR site. It is possible that GlnR binds with sufficient affinity to the downstream GlnR site in the *ure P3* promoter to weakly regulate gene expression. Alternatively, this low level of regulation may result from GlnR binding to both the downstream GlnR site and a yet unrecognized sequence with poor homology to a GlnR site in the *ure P3* promoter region.

Significance of *ure P3* regulation by PucR. Urease is widely distributed in bacteria (14). The expression of urease is controlled by diverse regulatory mechanisms, suggesting that this enzyme has multiple physiological roles in bacteria. For example, urease expression in *Streptococcus salivarius* is induced by growth at acidic pH (6). Since the degradation of urea to carbon dioxide and two molecules of ammonia produces a net increase in pH, high levels of urease can facilitate alkalization and bacterial survival in acidic environments. Because ammonia produced during urea catabolism can provide nitrogen for cell growth, the expression of urease in some bacteria is regulated in response to nitrogen availability. During nitrogen-limited growth, urease expression is activated by the Ntr system in *Klebsiella aerogenes* (18).

B. subtilis urease is a novel example of an urease enzyme whose expression is controlled by both a global nitrogen transcription factor and a pathway-specific regulator that responds to purine availability. Purine degradation occurs during nitrogen-limited growth in *B. subtilis* (3, 15, 16, 24). Since urea is produced by the final step in purine catabolism (Fig. 1), activation of *ureABC* expression by PucR ensures that purines are completely degraded to ammonia under these growth conditions. Substrates for purine catabolism are likely obtained from the degradation of DNA present in the environment because *B. subtilis* synthesizes both extracellular nucleases and efficient

transport systems for DNA degradation products (8). It is also noteworthy that the expression of competence genes is induced during nitrogen-limited growth in *B. subtilis* (13). This observation raises the possibility that single DNA strands that are not incorporated into the chromosome during transformation may be degraded to provide nutrients for continued cell growth. Interestingly, the ability to utilize extracellular DNA as an energy source during stationary phase in *Escherichia coli* cells has been shown to be dependent upon homologs of proteins involved in transformation in *Haemophilus influenzae* and *Neisseria gonorrhoeae* (9).

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