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Expression of the *Bacillus subtilis ureABC* Operon Is Controlled by Multiple Regulatory Factors Including CodY, GlnR, TnrA, and Spo0H

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Expression of urease, which is encoded by the *ureABC* operon, is regulated in response to nitrogen availability in *Bacillus subtilis*. Three *ureABC* promoters were identified in primer extension experiments and by examination of β-galactosidase expression from *ure-lacZ* fusions. P1, a low-level constitutive promoter, lies immediately upstream of *ureA*. The P2 promoter is transcribed by the Eσ^H form of RNA polymerase and initiates transcription 270 bp upstream of the *ureA* start codon. The transcriptional start site for the σ^A-dependent P3 promoter is located 839 bp upstream of the *ureA* start codon. To identify transcription factors that control *ureABC* expression, regulation of the P2 and P3 promoters was examined in wild-type and mutant strains. During rapid growth in minimal medium containing glucose and amino acids, CodY represses expression of the P2 and P3 promoters 30- and 60-fold, respectively. TnrA activates expression of the P3 promoter 10-fold in nitrogen-limited cells, while GlnR represses transcription from the P3 promoter 55-fold during growth on excess nitrogen. Expression of the *ureABC* operon increases 10-fold at the end of exponential growth in nutrient sporulation medium. This elevation in expression results from the relief of CodY-mediated repression during exponential growth and increased σ^H-dependent transcription during stationary phase.

Microbial ureases are multisubunit metalloenzymes that hydrolyze urea to form carbonic acid and two molecules of ammonia (24). The carbonic acid then dissociates, and the ammonia molecules protonate to form ammonium, causing the pH to increase. Thus, the degradation of urea provides ammonium for incorporation into intracellular metabolites and facilitates survival in acidic environments (7, 24). The structural genes encoding both the urease subunits, *ureA*, *ureB*, and *ureC*, and the accessory proteins required for assembly of the urease nickel metallocenter are typically clustered at a single locus (24).

Different patterns of urease expression have been observed in various bacteria. Expression of the *Proteus mirabilis* chromosomal *ure* genes and the plasmid-encoded *ure* genes of *Escherichia coli* and *Providencia stuartii* is induced by urea and requires UreR, a member of the AraC family of positive regulatory proteins (12, 13). In contrast, constitutive synthesis of urease has been observed in *Bacillus pasteuri*, *Morganella morgnii*, *Sporosarcina urea*, and *Anabaena variabilis* (24). In *Yersinia enterocolitica*, urease is preferentially expressed during stationary-phase growth in cells grown in the absence of urea (11).

Nitrogen-limited growth causes high-level expression of urease in *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Rhizobium meliloti*, *Klebsiella aerogenes*, and *Klebsiella pneumoniae* (1, 8, 18, 22, 23). In *K. aerogenes* and *K. pneumoniae*, urease expression is activated during nitrogen-restricted growth by the Ntr regulatory cascade (8, 18, 28). The phosphorylated form of NtrC activates expression of the LysR homolog Nac, and Nac activates *ure* transcription.

All available evidence indicates that the regulation of gene expression in response to nitrogen availability in *B. subtilis* does not involve an Ntr-like regulatory system (29, 41). Instead, the transcription of genes involved in nitrogen metabolism is controlled by multiple factors, each of which regulates gene expression in response to different nutritional signals. The *B. subtilis* homolog of the enteric σ^{54} RNA polymerase subunit, σ^L, is required for the utilization of arginine, ornithine, isoleucine, and valine as nitrogen sources (10). RocR, an NtrC homolog, activates transcription of the arginine and ornithine catabolic genes from σ^L-dependent promoters (17).

During nitrogen-limiting growth conditions, the TnrA protein activates the expression of the γ-aminobutyric acid permease, the nitrate utilization genes, urease, and the *nrgAB* operon (14, 41). GlnR, a TnrA homolog, represses the expression of the *glnRA* operon during growth on good nitrogen sources (29, 30). In *B. subtilis glnA* mutants, which are deficient in glutamine synthetase (GS), TnrA- and GlnR-regulated genes are expressed constitutively (1, 25, 30, 41). Because high-level expression of TnrA-regulated genes does not occur in *tnrA glnA* mutants (41), GS is involved in the transduction of the signal for nitrogen availability to the TnrA protein. It has also been proposed that GS transmits the nitrogen signal to GlnR (30). In *B. subtilis* cells growing rapidly in medium containing amino acids, another regulatory protein, CodY, represses the expression of the dipeptide permease operon, the histidine utilization operon, and the γ-aminobutyric acid permease as well as the expression of two genes required for the development of competence (14, 15, 32, 33, 35, 36).

In *B. subtilis*, high-level urease expression occurs only during nitrogen-limited growth and is not induced by urea (1). Here we report that the *B. subtilis ureABC* operon, which encodes the three subunits of urease (9), is transcribed from three promoters and regulated by CodY, TnrA, and GlnR.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* strains used in this study are listed in Table 1. Derivatives of strain 168 containing the *ure-lacZ* fusions were constructed as previously described (42). When *ure-lacZ* plasmid DNA was used to transform Δ*glnR* strains containing the *glnRA-lacZ cat* fusion, loss of chloram-

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TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype[a]	Reference, source, and/or derivation[b]
168	*trpC2*	This laboratory
SF168U	*ureC*::*spc trpC2*	9
BH1	Δ*spo0H*::*cat trpC2*	20; A. L. Sonenshein
SF62	*tnrA62*::Tn*917 trpC2*	41
PS37	*unkU*::*spc* Δ*codY trpC2*	P. Serror
HJS31	Δ*glnR57*	30; A. L. Sonenshein
KH566	*spo0H-lacZ cat trpC2 pheA1*	40; A. Grossman
SF168SH	*spo0H-lacZ cat trpC2*	168 × KH566 DNA
SF168A	Δ*glnA14*::*spc trpC2*	41; 168 × pGLN14 DNA
SF168G	Δ*glnR57 trpC2*	SF168A × HJS31 DNA
G21	*amyE*::[(*glnRA-lacZ*)*21 cat*] *trpC2*	41; 168 × pGLN21 DNA
G21G	*amyE*::[(*glnRA-lacZ*)*21 cat*] Δ*glnR57 trpC2*	SF168G × pGLN21 DNA
G21YG	*amyE*::[(*glnRA-lacZ*)*21 cat*] Δ*glnR57 unkU*::*spc* Δ*codY trpC2*	G21G × PS37 DNA
G21TG	*amyE*::[(*glnRA-lacZ*)*21 cat*] Δ*glnR57 tnrA62*::Tn*917 trpC2*	G21G × SF62 DNA
G21TYG	*amyE*::[(*glnRA-lacZ*)*21 cat*] Δ*glnR57 unkU*::*spc* Δ*codY tnrA62*::Tn*917 trpC2*	G21YG × SF62 DNA
U24	*amyE*::[(*ureA-lacZ*)*24 neo*] *trpC2*	168 × pURE24 DNA
U24U	*amyE*::[(*ureA-lacZ*)*24 neo*] *ureC*::*spc trpC2*	U24 × pURE4 DNA
U24A	*amyE*::[(*ureA-lacZ*)*24 neo*] Δ*glnA14*::*spc trpC2*	U24 × SF168A DNA
U24Y	*amyE*::[(*ureA-lacZ*)*24 neo*] *unkU*::*spc* Δ*codY trpC2*	U24 × PS37 DNA
U24T	*amyE*::[(*ureA-lacZ*)*24 neo*] *tnrA62*::Tn*917 trpC2*	U24 × SF62 DNA
U24G	*amyE*::[(*ureA-lacZ*)*24 neo*] Δ*glnR57 trpC2*	G21G × pURE24 DNA
U24SH	*amyE*::[(*ureA-lacZ*)*24 neo*] Δ*spo0H*::*cat trpC2*	U24 × BH1 DNA
U23	*amyE*::[(*ureA-lacZ*)*23 neo*] *trpC2*	168 × pURE23 DNA
U26	*amyE*::[(*ureA-lacZ*)*26 neo*] *trpC2*	168 × pURE26 DNA
U29	*amyE*::[(*ureA-lacZ*)*29 neo*] *trpC2*	168 × pURE29 DNA
U30	*amyE*::[(*ureA-lacZ*)*30 neo*] *trpC2*	168 × pURE30 DNA
U22	*amyE*::[(*ureA-lacZ*)*22 neo*] *trpC2*	168 × pURE22 DNA
U31	*amyE*::[(*ureA-lacZ*)*31 neo*] *trpC2*	168 × pURE31 DNA
U31SH	*amyE*::[(*ureA-lacZ*)*31 neo*] Δ*spo0H*::*cat trpC2*	U31 × BH1 DNA
U31Y	*amyE*::[(*ureA-lacZ*)*31 neo*] *unkU*::*spc* Δ*codY trpC2*	U31 × PS37 DNA
U34	*amyE*::[(*ureA-lacZ*)*34 neo*] *trpC2*	168 × pURE34 DNA
U28	*amyE*::[(*ureA-lacZ*)*28 neo*] *trpC2*	168 × pURE28 DNA
U28A	*amyE*::[(*ureA-lacZ*)*28 neo*] Δ*glnA14*::*spc trpC2*	U28 × pGLN14 DNA
U28Y	*amyE*::[(*ureA-lacZ*)*28 neo*] *unkU*::*spc* Δ*codY trpC2*	U28 × PS37 DNA
U28T	*amyE*::[(*ureA-lacZ*)*28 neo*] *tnrA62*::Tn*917 trpC2*	U28 × FS62 DNA
U28G	*amyE*::[(*ureA-lacZ*)*28 neo*] Δ*glnR57 trpC2*	G21G × pURE28 DNA
U28TY	*amyE*::[(*ureA-lacZ*)*28 neo*] *unkU*::*spc* Δ*codY tnrA62*::Tn*917 trpC2*	U28Y × SF62 DNA
U28TG	*amyE*::[(*ureA-lacZ*)*28 neo*] *tnrA62*::Tn*917* Δ*glnR57 trpC2*	G21TG × pURE28 DNA
U28YG	*amyE*::[(*ureA-lacZ*)*28 neo*] *unkU*::*spc* Δ*codY* Δ*glnR57 trpC2*	G21YG × pURE28 DNA
U28TYG	*amyE*::[(*ureA-lacZ*)*28 neo*] *tnrA62*::Tn*917 unkU*::*spc* Δ*codY* Δ*glnR57 trpC2*	G21TYG × pURE28 DNA
U27	*amyE*::[(*ureA-lacZ*)*27 neo*] *trpC2*	168 × pURE27 DNA
U32	*amyE*::[(*ureA-lacZ*)*32 neo*] *trpC2*	168 × pURE32 DNA
U25	*amyE*::[(*ureA-lacZ*)*25 neo*] *trpC2*	168 × pURE25 DNA

[a] Genotype symbols are those of Biaudet et al. (4), with *tnrA* (41) and *ureABC* (9) genes added.
[b] Strains were derived by transforming the first strain listed with DNA from the second strain or the plasmid listed.

phenicol resistance was used to identify double-crossover events at the *amyE* locus.

The *lacZ*α-complementing *E. coli* DH12S (Life Technologies, Inc.) was used as the host for DNA cloning experiments with plasmid pMTL21P. *E. coli* MC1061, which contains a deletion of the chromosomal *lac* genes, was used for the construction of *lacZ* fusions.

Plasmid pSFL7 is a *lacZ* transcriptional fusion vector that integrates into the *B. subtilis* chromosomal *amyE* gene. pSFL7 was constructed by replacing the chloramphenicol resistance and hybrid *trp-lacZ* fusion genes of ptrpBG1 (34) with neomycin resistance (38) and promoterless *spoVG-lacZ* (27) genes, respectively. DNA restriction fragments from pλnarA4 (9) containing the *ure* promoters were cloned into the polylinker region of pMTL21P (6). The *Xba*I-*Hin*dIII DNA fragments from these clones were inserted into pSFL7 to construct transcriptional *ure-lacZ* fusions (see Fig. 1).

Cell growth, media, and enzyme assays. The methods used for bacterial cultivation in minimal medium and Difco nutrient sporulation medium (DSM) (37) have been previously described (2, 3). Cells grown in DSM medium and minimal medium containing glucose with arginine as the sole nitrogen source were inoculated with overnight cultures grown for four generations in fresh medium before they were used to inoculate the cultures harvested for enzyme assays. Liquid minimal cultures were grown in the minimal medium of Neidhardt et al. (26). Glucose was added at 0.5% to minimal medium. All other carbon and nitrogen sources were added at 0.2% to minimal medium except where otherwise noted. L-Glutamine was freshly prepared and filter sterilized for each experiment. The composition of the 16-amino-acid mixture was described previously (2).

Extracts for enzyme assays were prepared from cells harvested during exponential growth (70 to 90 Klett units) as previously described (2). β-Galactosidase was assayed in crude extracts as described previously (2). β-Galactosidase activity was always corrected for endogenous β-galactosidase activity present in *B. subtilis* 168 cells containing the promoterless *lacZ* gene from pSFL7 integrated at the *amyE* site.

Primer extensions. RNA was isolated from *B. subtilis* cells grown to mid-log growth phase (70 to 90 Klett units) by extraction with guanidine thiocyanate and CsCl centrifugation (2). Primer extensions were performed as described previously (16). The URE1 (5′-TCAGCAGAACGCCCCGCGCC) and URE2 (5′-TCGCTGTTTGGCTAATTCCC) oligodeoxynucleotide primers are complementary to the 5′ end of the *ureA* coding sequence. The ALP3 (5′-TTCAAGCT GGGGATCTAAGC) primer is complementary to a unique sequence in the pSFL7 vector that is located immediately downstream of the site for insertion of promoter-containing DNA fragments.

RESULTS

Deletion analysis of the *ureABC* regulatory region. To identify the promoter(s) that transcribe the *ureABC* operon, DNA

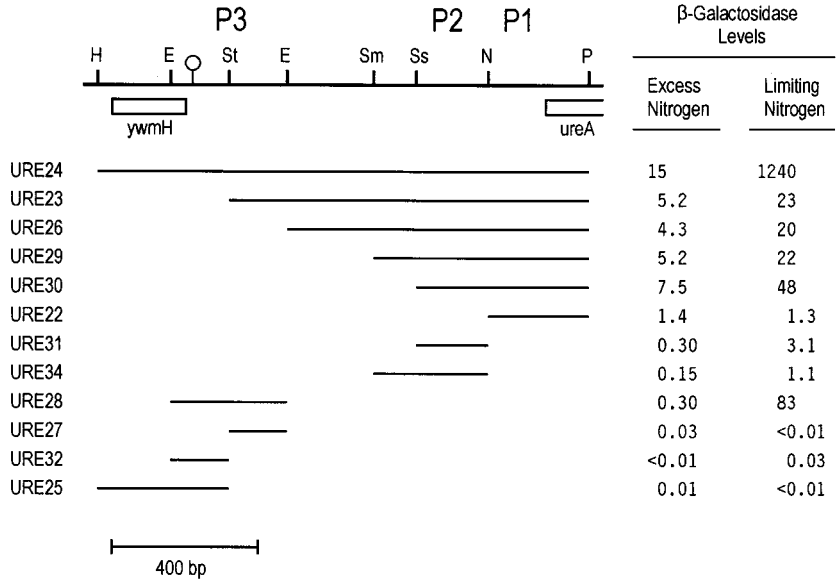

FIG. 1. Deletion analysis of the *ureABC* promoter region. The rectangles indicate coding sequences. The three *ureABC* promoters are indicated by P1, P2, and P3. The DNA fragments used to construct *ureA-lacZ* transcriptional fusions are diagrammed. β-Galactosidase levels determined in cells containing the various *ureA-lacZ* fusions grown with either excess nitrogen (glutamate plus ammonium) or limiting nitrogen (glutamate) are shown. The stem-loop structure indicates a putative transcriptional terminator. Restriction enzyme abbreviations: H, *Hpa*I; E, *Eco*RV; St, *Stu*I; Sm, *Sma*I; Ss, *Ssp*I; N, *Nco*I; P, *Pml*I. Other abbreviations: *ureA*, α subunit of urease; *ywmH*, putative coding sequence.

fragments containing sequences upstream of *ureA* were transcriptionally fused to *lacZ*, and the *ureA-lacZ* fusions were integrated into the *B. subtilis* chromosome as a single copy at the *amyE* locus. β-Galactosidase levels were determined in cells growing exponentially in minimal medium containing glucose and either excess nitrogen (glutamate plus ammonium) or limiting nitrogen (glutamate).

The (*ureA-lacZ*)*22* fusion, which contains DNA extending from the *Pml*I site in *ureA* to the upstream *Nco*I site, expressed β-galactosidase at low levels (Fig. 1). Moreover, β-galactosidase expression was not regulated in response to nitrogen availability. This result indicates that a low-level constitutive promoter, P1, is located immediately upstream of *ureA*.

β-Galactosidase expression increased four- to sixfold during nitrogen-limited growth in cells containing *ureA-lacZ* fusion *23*, *26*, *29*, or *30* (Fig. 1). The *ure* DNA in these fusions extends from the *Pml*I site in *ureA* to the upstream *Stu*I, *Eco*RV, *Sma*I, or *Ssp*I site. The (*ureA-lacZ*)*31* fusion, which contains the *Ssp*I-*Nco*I *ure* DNA fragment fused to *lacZ*, also exhibited nitrogen-regulated promoter activity (Fig. 1). This result indicates that a second *ure* promoter, P2, lies between the *Ssp*I and *Nco*I sites upstream of *ureA*. Interestingly, when the P2 *ure* promoter DNA contained the *ure* sequences immediately upstream of the *Ssp*I site, β-galactosidase expression was reduced two- to threefold [compare (*ureA-lacZ*)*31* with (*ureA-lacZ*)*34* and (*ureA-lacZ*)*30* with (*ureA-lacZ*)*29* in Fig. 1]. These results suggest that the DNA sequences immediately upstream of the *Ssp*I site weakly inhibit transcription from the P2 promoter.

β-Galactosidase levels in cells containing the (*ureA-lacZ*)*24* fusion, which contains 1,206 bp of DNA upstream of the *ureA* start codon, were 83-fold higher in nitrogen-limited cells than in cells grown with excess nitrogen (Fig. 1). In addition, cells containing the (*ureA-lacZ*)*24* fusion synthesized higher levels of β-galactosidase than did cells with the (*ureA-lacZ*)*23* fusion. These results are consistent with the (*ureA-lacZ*)*24* fusion containing a nitrogen-regulated *ureABC* promoter, P3, which is not present in the (*ureA-lacZ*)*23* fusion. Further analysis showed that short DNA fragments that contained only sequences upstream [(*ureA-lacZ*)*25* and *-32*] or downstream [(*ureA-lacZ*)*27*] of the *Stu*I site exhibited no promoter activity (Fig. 1). In contrast, expression of β-galactosidase from the (*ureA-lacZ*)*28* fusion, which contains DNA that spans the *Stu*I site, is regulated 280-fold by nitrogen availability (Fig. 1). These results indicate that the P3 promoter overlaps the *Stu*I site located upstream of *ureA*.

Identification of the *ureABC* transcriptional start sites. Primer extension analysis was used to identify the transcriptional start sites for the three *ureABC* promoters. In experiments using RNA isolated from cells grown in minimal medium containing glucose and a limiting nitrogen source (glutamate) and oligonucleotide primers complementary to the *ureA* coding sequence, the major primer extension products corresponded to apparent mRNAs with 5′ ends located 87 and 88 bp upstream of the *ureA* start codon (data not shown). Several longer extension products with lower intensities were also observed. The intensities of all of these primer extension products were significantly reduced in experiments that used RNA isolated from cells grown with excess nitrogen (glutamate plus ammonium). If the major primer extension products corresponded to the location of a promoter, then the (*ureA-lacZ*)*22* fusion would be expected to exhibit high-level nitrogen-regulated expression. Since this *lacZ* fusion has only low-level constitutive promoter activity (Fig. 1), the major primer extension products may represent the 5′ ends of RNA degradation products or result from the premature termination of reverse transcriptase elongation.

To circumvent this problem, RNA was isolated from separate strains containing *lacZ* fusions to the *ure* P1, P2, and P3 promoters. A 20-bp oligodeoxynucleotide complementary to sequences within the *lacZ* vector was used as the primer in the extension reactions so that only transcription initiating from the *ureA-lacZ* fusion would be examined. No primer extension products were detected when *Saccharomyces cerevisiae* RNA or *B. subtilis* RNA isolated from U22

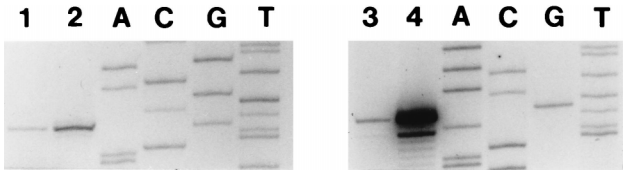

FIG. 2. *ureABC* primer extension analysis. *B. subtilis* RNA used in the primer extensions was isolated from strain U31 containing the (*ureA-lacZ*)*31* fusion (lanes 1 and 2) and from strain U28 containing the (*ureA-lacZ*)*28* fusion (lanes 3 and 4). The oligonucleotide primer ALP3 was used for dideoxynucleotide sequencing of pURE31 (lanes A, C, G, and T to the right of lanes 1 and 2) and pURE28 (lanes A, C, G, and T to the right of lanes 3 and 4) and for the primer extensions. RNA was isolated from cells grown in minimal medium containing glucose with either glutamate plus ammonium (lanes 1 and 3) or glutamate (lanes 2 and 4) as the nitrogen sources.

[(*ureA-lacZ*)*22*] cultures was used as the template (data not shown). These results suggest that the level of the (*ureA-lacZ*)*22* P1 transcript(s) is too low to be detected in primer extension experiments.

Higher levels of the (*ureA-lacZ*)*31* P2 transcript were present in nitrogen-limited U31 cells than in cells grown with excess nitrogen (Fig. 2, lanes 1 and 2). This result is in agreement with the observation that β-galactosidase expression from the (*ureA-lacZ*)*31* fusion is nitrogen regulated (Fig. 1). The transcriptional start site for (*ureA-lacZ*)*31* is such that transcription of the P2 promoter would initiate 270 bp upstream of the *ureA* start codon. Examination of the sequences upstream of the P2 transcriptional start site reveals that this promoter is most likely recognized by the Eσ^H form of *B. subtilis* RNA polymerase (Fig. 3). The −10 region of the P2 promoter has four of five matches to the σ^H −10 consensus sequence, while the −35 region of P2 is a perfect match for the σ^H −35 consensus sequence (19).

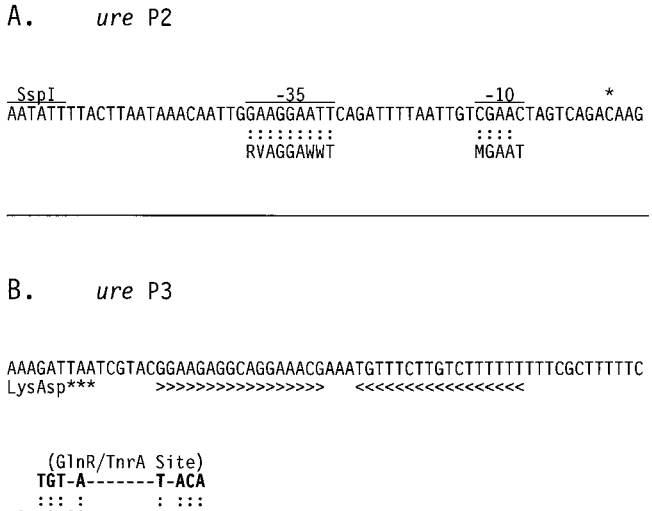

FIG. 3. Nucleotide sequences of the *ureABC* P2 and P3 promoters. (A) Nucleotide sequence of the *ure* P2 promoter. The consensus sequences for the −10 and −35 regions of σ^H-dependent promoters are indicated below the nucleotide sequence. Abbreviations for ambiguous nucleotides: R, A or G; V, A, G, or C; W, A or T; M, A or C. The transcriptional start site is indicated by the asterisk. (B) Nucleotide sequence of the *ure* P3 promoter. The transcriptional start sites are indicated by asterisks. The consensus sequences for the −10 and −35 regions of σ^A-dependent promoters are indicated below the nucleotide sequence. Two potential binding sites for GlnR and TnrA are aligned with the GlnR/TnrA consensus sequence (41). A potential transcriptional terminator is indicated by the converging arrowheads.

TABLE 2. β-Galactosidase levels in wild-type and UreC$^-$ strains containing the (*ureA-lacZ*)*24* fusion

Strain[a] (relevant genotype) and nitrogen source	β-Galactosidase sp act (U/mg of protein) in cells grown[b]:	
	Without urea	With urea
U24 (wild type)		
Glutamine + AA[c]	0.2	ND[d]
Glutamine	13	ND
Arginine	35	ND
Glutamate + ammonium	16	ND
Ammonium	17	19
Urea		215
Glutamate	1,206	253
Allantoin	1,168	ND
U24U (*ureC*::*spc*), glutamate	885	917

[a] All strains are derivatives of strain 168 with the (*ureA-lacZ*)*24* fusion, which contains all three *ureABC* promoters, integrated as a single copy at the *amyE* locus.

[b] Data are the averages of three to twenty determinations which did not vary by more than 30%. Cultures were grown in minimal medium containing glucose and the indicated additions.

[c] AA, the 16-amino-acid mixture (2).

[d] ND, not determined.

One major extension product and one minor extension product were observed when transcription from the (*ureA-lacZ*)*28* fusion was examined (Fig. 2, lanes 3 and 4). Since the 5′ ends of these two transcripts are separated by only a single nucleotide, they most likely originate from a single promoter. Alternatively, the minor transcript may be an incomplete extension product. The observation that higher levels of the (*ureA-lacZ*)*28* transcript were present in RNA isolated from nitrogen-limited cells than from cells grown with excess nitrogen (Fig. 2, lanes 3 and 4) is consistent with the results obtained in examining β-galactosidase expression of this *ureA-lacZ* fusion (Fig. 1). The major transcriptional start site for the P3 promoter is located 839 bp upstream of the *ureA* start codon. Sequences upstream of the transcriptional start site indicate that this promoter is likely to be a nonoptimal σ^A-dependent promoter (Fig. 3B). The −10 promoter region, which is a perfect match to the −10 consensus sequence, is separated by 16 bp from the −35 promoter region, which has four of six matches to the σ^A −35 consensus sequence (Fig. 3B). Seventeen base pairs separate the −35 and −10 promoter regions in the optimal σ^A consensus sequence (19).

Nutritional regulation of the (*ureA-lacZ*)*24* fusion. Previously published work showed that the expression of *B. subtilis* urease is activated during nitrogen limitation but is not induced by urea (1). Because urease activity could not be detected in cells grown with excess nitrogen in these studies, *ure* regulation was further examined by determining β-galactosidase levels in U24 [(*ureA-lacZ*)*24*] cultures grown with various nitrogen sources.

The highest levels of β-galactosidase expression were observed when strain U24 was grown in media containing the limiting nitrogen sources glutamate or allantoin (Table 2). β-Galactosidase levels were five- to sixfold lower in cells grown with either urea or glutamate plus urea than in glutamate-

TABLE 3. β-Galactosidase levels in wild-type and mutant strains containing the (*ureA-lacZ*)*24* fusion

Strain[a]	Relevant genotype	β-Galactosidase sp act (U/mg of protein) in cells grown on[b]:		
		Glutamine + AA[c]	Glutamine	Glutamate
U24	Wild type	0.2	8.3	1,365
U24Y	Δ*codY*	4.6	12	1,188
U24G	Δ*glnR57*	1.8	84	1,336
U24T	*tnrA62*	0.2	7.7	207
U24A	Δ*glnA14*	167	1,178	ND[d]
U24SH	Δ*spo0H*	0.1	3.6	510

[a] All strains are derivatives of strain 168 with the (*ureA-lacZ*)*24* fusion, which contains all three *ureABC* promoters, integrated as a single copy at the *amyE* locus.
[b] Data are the averages of three to five determinations which did not vary by more than 25%. Cultures were grown in minimal medium containing glucose and the indicated nitrogen sources.
[c] AA, the 16-amino-acid mixture (2).
[d] ND, not determined.

grown cells. These results agree with previously published data indicating that the degradation of urea in *B. subtilis* produces sufficient nitrogen catabolites to partially repress urease expression (1). This hypothesis is supported by the observation that strain U24U (*ureC*::*spc*), which is unable to catabolize urea, expresses the (*ureA-lacZ*)*24* fusion similar levels when grown with either glutamate or glutamate plus urea as nitrogen sources (Table 2).

When the growth medium contained a good nitrogen source such as ammonium, glutamine, or arginine, β-galactosidase levels were reduced 34- to 93-fold compared to the levels seen in glutamate-grown cells (Table 2). The addition of urea to medium containing the nitrogen source ammonium did not increase β-galactosidase expression (Table 2). In cultures grown with glutamine plus the 16-amino-acid mixture, β-galactosidase levels were 65-fold lower than in glutamine-grown cells (Table 2).

Expression of the (*ureA-lacZ*)*24* fusion in mutant strains. The roles of CodY, GlnR, TnrA, and GS in controlling the expression of the *ureABC* operon were examined by measuring β-galactosidase levels produced by the (*ureA-lacZ*)*24* fusion in wild-type and mutant strains grown with different nitrogen sources. Addition of the 16-amino-acid mixture to glutamine medium repressed β-galactosidase expression 41-fold in wild-type cultures but only 3-fold in *codY* mutant cultures (Table 3). In contrast, similar levels of β-galactosidase expression were seen in *codY* and wild-type cultures grown in medium containing either glutamine or glutamate as the sole nitrogen source (Table 3). These results indicate that CodY is required for repression of *ureABC* expression in rapidly growing *B. subtilis* cultures utilizing amino acids as the nitrogen source.

Compared to the levels seen in wild-type cultures, the *glnR* mutation caused a 9- to 10-fold increase in β-galactosidase levels in cultures grown with either glutamine plus the 16-amino-acid mixture or glutamine alone but did not affect β-galactosidase expression in nitrogen-limited cultures (Table 3). Thus, GlnR represses *ureABC* expression in cells grown with excess nitrogen. β-Galactosidase levels were 6.5-fold lower in glutamate-grown *tnrA* mutant cultures than in wild-type cultures (Table 3). No difference in (*ureA-lacZ*)*24* expression was seen when wild-type and *tnrA* mutant cultures were grown with excess nitrogen (Table 3). These results indicate that TnrA is required for high-level expression of *ureABC* expression during nitrogen-limited growth.

Previous work has shown that urease is synthesized at elevated levels in *glnA* mutants (1), presumably due to a defect in the nitrogen signal transduction pathway. In glutamine-grown cells, expression of the (*ureA-lacZ*)*24* fusion was completely derepressed in the Δ*glnA14* mutant (Table 3). In addition, the 16-amino-acid mixture repressed β-galactosidase expression 41-fold in the Gln^+ strain but only 7-fold in the Δ*glnA14* mutant strain (Table 3). Because the Δ*glnA14* mutant requires glutamine for growth, expression of the (*ureA-lacZ*)*24* fusion could not be examined in cultures with glutamate as the sole nitrogen source.

Regulation of the *ureABC* P2 promoter. The results of primer extension experiments suggested that the *ureABC* P2 promoter is transcribed by RNA polymerase containing σ^H. This was confirmed by showing that mutational inactivation of *spo0H*, which encodes σ^H, reduced expression of the (*ureA-lacZ*)*24* fusion 2- to 2.5-fold (Table 3) and abolished expression of the (*ureA-lacZ*)*31* fusion (Table 4). P2 expression is also regulated by CodY. The addition of amino acids to glutamine-containing minimal medium repressed the expression of the (*ureA-lacZ*)*31* fusion more than 30-fold in wild-type cultures but less than 2-fold in *codY* mutant cultures (Table 4).

In glutamate-grown cultures, the wild-type (U31) strain produced 11-fold-higher levels of β-galactosidase than in glutamine-grown cultures. It is possible that this increase in P2 expression during nitrogen-limited conditions results from higher levels of σ^H activity. Expression of the σ^H-encoding *spo0H* gene was examined by measuring β-galactosidase production from a *spo0H-lacZ* fusion (40) in wild-type cells grown with various nitrogen sources. β-Galactosidase expression from this *spo0H-lacZ* fusion was not elevated by nitrogen restriction (data not shown).

Regulation of the *ureABC* P3 promoter. Regulation of the *ureABC* P3 promoter was analyzed by examining β-galactosidase expression of the (*ureA-lacZ*)*28* fusion (Fig. 1). β-Galactosidase levels in U28 cultures grown with glutamine plus amino acids were 20-fold lower than those seen in glutamine-grown cultures (Table 5). When the growth medium contained the limiting nitrogen source glutamate, β-galactosidase levels increased 400-fold compared to the levels seen in cultures grown with glutamine (Table 5). These results demonstrate that the *ureABC* P3 promoter is highly regulated in response to the nitrogen source of the growth medium.

In the *codY* (U28Y) and *tnrA codY* (U28TY) mutants, the 16-amino-acid mixture repressed β-galactosidase expression only four- to fivefold (Table 5). These results indicate that CodY is the major contributor to the repression of P3 promoter activity observed in cells grown in the amino acid mixture. While the 16-amino-acid mixture repressed β-galactosidase expression 55-fold in the *glnR* mutant U28G and 60-fold

TABLE 4. β-Galactosidase levels in wild-type and mutant strains containing the (*ureA-lacZ*)*31* fusion

Strain[a]	Relevant genotype	β-Galactosidase sp act (U/mg of protein) in cells grown on[b]:		
		Glutamine + AA[c]	Glutamine	Glutamate
U31	Wild type	<0.01	0.30	3.5
U31SH	Δ*spo0H*	<0.01	0.01	<0.01
U31Y	Δ*codY*	0.30	0.50	3.3

[a] All strains are derivatives of strain 168 with the (*ureA-lacZ*)*31* fusion, which contains the *ureABC* P2 promoter, integrated as a single copy at the *amyE* locus.
[b] Data are the averages of three to five determinations which did not vary by more than 20%. Cultures were grown in minimal medium containing glucose and the indicated nitrogen sources.
[c] AA, the 16-amino-acid mixture (2).

TABLE 5. β-Galactosidase levels in wild-type and mutant strains containing the (*ureA-lacZ*)*28* fusion

Strain[a]	Relevant genotype	β-Galactosidase sp act (U/mg of protein) in cells grown on[b]:		
		Glutamine + AA[c]	Glutamine	Glutamate
U28	Wild type	<0.01	0.2	83
U28Y	Δ*codY*	0.05	0.2	87
U28G	Δ*glnR57*	0.09	5.0	90
U28YG	Δ*codY* Δ*glnR57*	11	11	108
U28T	*tnrA62*	<0.01	0.2	11
U28TY	*tnrA62* Δ*codY*	0.04	0.2	10
U28TG	*tnrA62* Δ*glnR57*	0.06	3.6	7.8
U28TYG	*tnrA62* Δ*codY* Δ*glnR57*	6.9	8.7	8.5
U28A	Δ*glnA14*	15	66	ND[d]

[a] All strains are derivatives of strain 168 with the (*ureA-lacZ*)*28* fusion, which contains the *ureABC* P3 promoter, integrated as a single copy at the *amyE* locus.
[b] Data are the averages of three to five determinations which did not vary by more than 30%. Cultures were grown in minimal medium containing glucose and the indicated additions.
[c] AA, the 16-amino-acid mixture (2).
[d] ND, not determined.

in the *tnrA glnR* mutant U28TG, amino acids did not alter the level of β-galactosidase expression in the *codY glnR* mutant U28YG (Table 5). Thus, both GlnR and CodY contribute to the reduced levels of β-galactosidase seen in cultures grown with glutamine plus amino acids as the nitrogen source.

In cultures grown with glutamine as the nitrogen source, the wild-type (U28), *codY* mutant (U28Y), and *tnrA* mutant (U28T) strains had identical levels of β-galactosidase (Table 5). In contrast, glutamine-grown cultures of the *glnR* mutant (U28G) had 25-fold-higher β-galactosidase levels than the wild-type strain (Table 5). This result indicates that GlnR is the predominant regulator of P3 expression during growth with the good nitrogen source glutamine. This result is consistent with the observation that in glutamine-grown cultures β-galactosidase levels are 18-fold higher in the *tnrA glnR* mutant (U28TG) than in the *tnrA* mutant (U28T) and 55-fold higher in the *codY glnR* mutant (U28YG) than in the *codY* mutant (U28Y) (Table 5).

During nitrogen-limited growth, the levels of β-galactosidase expression were 8- to 10-fold lower in *tnrA* mutant cultures than in the corresponding wild-type, *codY*, and *glnR* cultures (Table 5). These results indicate that TnrA is required for activation of (*ureA-lacZ*)*28* expression during nitrogen restriction. Because no significant regulation of (*ureA-lacZ*)*28* expression occurs in the *tnrA codY glnR* mutant (Table 5), TnrA, CodY, and GlnR appear to be the only regulatory factors that control P3 expression.

When expression of the (*ureA-lacZ*)*28* fusion was examined in a Δ*glnA14* mutant, the results were similar to those obtained with the (*ureA-lacZ*)*24* fusion. β-Galactosidase expression was derepressed in glutamine-grown Δ*glnA14* cells, and the addition of the amino acid mixture to glutamine medium repressed β-galactosidase expression an additional fourfold (Table 5).

Expression of the (*ureA-lacZ*)*24* fusion in nutrient broth sporulation medium. Expression of genes transcribed by the form of RNA polymerase containing σ^H is elevated during the initiation of sporulation in nutrient broth medium (19, 20, 40). Moreover, CodY-dependent repression is relieved at the onset of stationary growth phase in the same medium (36). To determine whether *ureABC* expression increases during stationary phase in nutrient sporulation medium, expression of the (*ureA-lacZ*)*24* fusion was examined in wild-type, *spo0H*, and *codY* strains during growth in this medium.

β-Galactosidase expression from the (*ureA-lacZ*)*24* fusion increased 10-fold immediately following the end of exponential growth of the wild-type strain in nutrient sporulation growth medium (Fig. 4). Since β-galactosidase levels were fivefold higher in exponentially growing *codY* cultures than in wild-type cultures, CodY represses *ureABC* expression during logarithmic growth (Fig. 4). The threefold increase in β-galactosidase levels which occurs at the onset of stationary growth phase in the *codY* cultures most likely results from σ^H-dependent transcription of *ureABC*. In the *spo0H* strain, β-galactosidase expression derepressed sevenfold at the onset of stationary phase but did not reach the levels observed with wild-type and *codY* strains (Fig. 4). These results suggest that increased σ^H activity is, in part, responsible for high-level expression of *ureABC* expression during stationary growth phase.

DISCUSSION

The predominant promoter controlling expression of the *ureABC* operon is the σ^A-dependent P3 promoter. Expression from this promoter is controlled by CodY, GlnR, and TnrA. The *ureABC* P3 promoter is novel in that it is the only *B. subtilis* promoter, other than the *glnRA* promoter, that is known to be regulated by GlnR (29). In addition, while CodY is the major factor in the repression of P3 expression in cells grown in medium containing amino acids, GlnR also contributes to this repression. Although the signal controlling GlnR activity is not known, the results presented here suggest that this signal is potentiated in amino acid-grown cells.

The amino acid sequences of the putative DNA binding domains within the GlnR and TnrA proteins are very similar (41). In addition, the GlnR and TnrA proteins both bind to DNA sequences that have a conserved consensus sequence of $TGTNAN_7TNACA$ (41). It is not known what nucleotides within these sites facilitate optimal binding by GlnR or TnrA. Two potential GlnR and/or TnrA sites are located within the P3 promoter region (Fig. 3). One site is centered 90 bp upstream of the *ureABC* transcriptional start site, while a second site is centered 17 bp downstream of the start site. The positions of the putative GlnR and/or TnrA binding sites in the P3

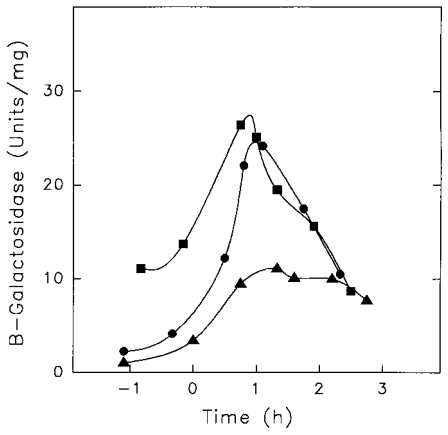

FIG. 4. Expression of the (*ureA-lacZ*)*24* fusion in strains U24 (wild type), U24SH (*spo0H*::*cat*), and U24Y (Δ*codY*) during growth in Difco sporulation medium. Samples were removed periodically, and β-galactosidase activity was determined in extracts of wild-type (circles), $Spo0H^-$ (triangles), or $CodY^-$ (squares) strains. Data from a typical experiment are shown. Time zero corresponds to the end of exponential growth.

promoter region are unique compared to the location of these binding sites in other promoters. In the *glnRA* promoter, GlnR binds at two adjacent operators which are centered 27 and 50 bp upstream of the transcriptional start site (5, 30, 31). The TnrA binding sites for the *nrgAB*, *nasB*, *nasA*, and *gabP* P2 promoters are centered 49 to 51 bp upstream of the start sites (14, 25, 41). Mutational inactivation of the putative GlnR and TnrA sites at the *ure* P3 promoter will be necessary to elucidate their role in regulating the expression of this promoter.

CodY reduces expression of the *ure* P2 and P3 promoters during growth in medium containing amino acids. Since CodY is a DNA binding protein (32, 33), we presume that CodY functions directly as a repressor to inhibit transcription from these promoters, but we cannot rule out the possibility that CodY only indirectly regulates expression of the P2 and P3 promoters. No consensus binding sequence has been identified for CodY. Serror and Sonenshein have proposed that CodY recognizes and binds to a three-dimensional structure formed by A+T-rich DNA regions (33). The *ure* P2 and P3 promoter regions are A+T rich and thus may contain CodY binding sites.

In a *glnA* mutant, expression of the (*ureA-lacZ*)*24* fusion, which contains the P1, P2, and P3 promoters, and the (*ureA-lacZ*)*28* fusion, which contains only the P3 promoter, is derepressed in glutamine-grown cells. These results agree well with previously published work showing that urease expression is derepressed in *glnA* mutants and that glutamine synthetase is required for regulated expression of promoters controlled by GlnR and TnrA (1, 25, 31, 41). Surprisingly, the expression of both *ure* fusions was only partially derepressed in amino acid-grown *glnA* mutants (Tables 3 and 5). During growth in the presence of amino acids, *ureABC* expression is repressed by CodY in response to an as yet unidentified signal. The CodY regulatory signal reflects the culture growth rate because the highest levels of CodY-dependent repression occur in fast-growing cultures, while little or no CodY-dependent regulation is seen in slow-growing cultures (15). In medium containing amino acids and glutamine as the nitrogen sources, the doubling time of *glnA* cultures (45 min) is slower than that of wild-type cultures (30 min). Thus, the reduced growth rate of the *glnA* mutant cultures in medium containing amino acids is most likely responsible for the partial defect in CodY-dependent repression seen in these strains.

Examination of the expression from *ure-lacZ* fusions in wild-type and *spo0H* strains showed that the σ^H-dependent P2 promoter plays a minor role in transcription of the *ureABC* operon in cells growing exponentially in minimal medium (Table 3). In contrast, P2 makes a more significant contribution to *ureABC* transcription during early stationary phase in nutrient sporulation medium (Fig. 4). This most likely reflects the fact that σ^H activity increases during the onset of sporulation in this medium (19, 20, 40). We were unable to identify the factor(s) responsible for the 10-fold elevation in P2 expression in nitrogen-limited cultures. The *ure* P2 promoter region does not contain any DNA sequences with significant similarity to the GlnR and TnrA binding site consensus sequence. Although expression of the σ^H-encoding *spo0H* gene is not regulated in response to nitrogen availability, it is still possible that nitrogen-limited cells contain higher levels of σ^H activity. Posttranscriptional regulatory mechanisms including increased stability of *spo0H* mRNA, decreased turnover of Spo0H protein, and competition with other sigma factors have been shown to contribute to the in vivo activity of σ^H (20, 21).

The regulation of the *B. subtilis ureABC* operon is complex, especially when compared to the regulation of *ure* genes seen in other bacteria. Urea, a mammalian waste product and soil fertilizer, is readily available as an external nitrogen source in the natural habitat of *B. subtilis*. In addition, urea is produced by the degradation of purines and arginine (39). The catabolism of these intracellular metabolites can provide energy and metabolic precursors during germination and the late stages of sporulation when *B. subtilis* cannot obtain nutrients from the environment. Thus, the multifactorial regulation of the *B. subtilis ureABC* genes may reflect the fact that urea is obtained from different sources during the life cycle of *B. subtilis.*

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