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Nitrogen Regulation of *nasA* and the *nasB* Operon, Which Encode Genes Required for Nitrate Assimilation in *Bacillus subtilis*†

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The divergently transcribed *nasA* gene and *nasB* operon are required for nitrate and nitrite assimilation in *Bacillus subtilis*. The β-galactosidase activity of transcriptional *lacZ* fusions from the *nasA* and *nasB* promoters was high when cells were grown in minimal glucose medium containing poor nitrogen sources such as nitrate, proline, or glutamate. The expression was very low when ammonium or glutamine was used as the sole nitrogen source. The repression of the genes during growth on good sources of nitrogen required wild-type glutamine synthetase (GlnA), but not GlnR, the repressor of the *glnRA* operon. Primer extension analysis showed that the −10 region of each promoter resembles those of σ^A-recognized promoters. Between the divergently oriented *nasA* and *nasB* promoters is a region of dyad symmetry. Mutational analysis led to the conclusion that this sequence is required in *cis* for the activation of both *nasA* and *nasB*. The derepression of these genes in a *glnA* mutant also required this sequence. These results suggest that an unidentified transcriptional activator and glutamine synthetase function in the regulation of *nasA* and the *nasB* operon.

Bacillus subtilis can use nitrate as the sole source of nitrogen (10). Genes that function in nitrate assimilation in *B. subtilis* (*nasA* to *nasF*), including those encoding nitrate reductase and nitrite reductase, were isolated, and their primary structures were determined (26). Most bacteria possess elaborate mechanisms for controlling genes required for nitrogen utilization in response to changes in nitrogen source availability. In enteric bacteria such as *Escherichia coli*, the expression of these genes is controlled by the global nitrogen regulatory (Ntr) system. NRII (NtrB) phosphorylates NRI (NtrC) in response to the intracellular ratio of glutamine to α-ketoglutarate. NRI (NtrC) phosphate binds to enhancer-like elements upstream of the Ntr-regulated promoters which are recognized by σ^{54} RNA polymerase (21). In *Klebsiella aerogenes* (4, 20, 27) and *Klebsiella pneumoniae* (5), the genes encoding the enzymes involved in nitrate assimilation are induced by nitrate or nitrite and repressed by ammonium. The repression by ammonium is mediated by Ntr (20), and the induction by nitrate in *K. pneumoniae* was recently shown to be controlled by an antitermination mechanism (19). In *Synechococcus* sp., ammonium represses the genes encoding nitrate/nitrite reductases, a process which involves the assimilation of ammonium nitrogen through glutamine synthetase (16, 17). A mutation in *ntcA*, resulting in loss of nitrate/nitrite reductases, glutamine synthetase, and methylammonium transport, was isolated from *Synechococcus* sp. (39). Inspection of the NtcA amino acid sequence showed that it belongs to the Crp family of bacterial transcriptional activators (38).

It is unlikely that a global Ntr system exists in the spore-forming soil bacterium *B. subtilis* (10, 13). One domain of LevR, a regulatory protein controlling the levanase operon in *B. subtilis*, is similar to the central domain of NtrC (8). RocR (6), a positive regulator of arginine catabolism, was also shown to belong to the NtrC/NifA family. A homolog of σ^{54}, or RpoN, which is required for the transcription of many nitrogen-regulated genes in enteric bacteria, also exists in *B. subtilis* (9). However, *B. subtilis* σ^{54} is probably not responsible for the transcription of general nitrogen-controlled genes but is responsible only for the levanase operon and the genes that function in the catabolism of arginine, ornithine, isoleucine, or valine. Two types of nitrogen-dependent control have been observed in *B. subtilis*. One was observed in studies of the *hut* operon, which functions in the utilization of histidine as a nitrogen source (2, 3), and in studies of *citB* (aconitase) (14) and the *dciA* operon (34, 35). In these cases, expression is under amino acid-dependent repression in addition to carbon catabolite repression. In the case of the *dciA* operon, a single mechanism may be responsible for amino acid repression and carbon catabolite repression, since mutations near the transcriptional start site relieve repression by amino acids as well as by glucose (34). A mutation, *cod*, that relieves amino acid and glucose repression of *dciA* expression was isolated, suggesting that *cod* may encode a regulator of *dciA* transcription, which is responsive to nutritional conditions (34). In the case of the *hut* operon and *citB*, amino acid repression was shown to be independent of carbon catabolite repression (2, 14) and glutamine synthetase (2). The second type of control requires the product of *glnA*, glutamine synthetase (11). In this form of regulation, expression is sensitive to nitrogen availability. Nitrogen-regulated genes in *B. subtilis*, normally repressed in medium containing ammonium or glutamine, are constitutively expressed in *glnA* mutants. Genes under this type of control include the *glnRA* operon (glutamine synthetase) (29), the genes encoding asparaginase (1) and urease (1), and the *nrg* (nitrogen regulated) genes (1). Among the *glnA* alleles that cause the derepression of nitrogen-regulated genes in the presence of glutamine are those that result in near-total absence of the enzyme (33). Strains with *glnA* mutations that confer altered catalytic and/or regulatory properties (7, 31, 33) also express glutamine synthetase at a high level under repressing conditions. These results suggest that catalytic activity of glutamine

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† This paper is dedicated to the memory of Fang Yang.

‡ Deceased.

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source or reference[a]
Strains derived from JH642		
JH642	*trpC2 pheA1*	J. Hoch
OKB105	*pheA1 sfp*	23
LAB1166	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pZS5	
LAB1167	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pZS6	
LAB1581	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pFY6	
LAB1583	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pFY8	
LAB1589	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pFY9	
LAB1600	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pPH1	
LAB1655	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pMMN163	
LAB1656	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pMMN164	
LAB1685	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pMMN171	
LAB1686	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pMMN172	
LAB1706	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pMMN180	
LAB1707	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pMMN181	
LAB1714	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pMMN183	
LAB1715	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pMMN184	
LAB1728	*trpC2 pheA1* Δ*nasB*::pMMN192	
LAB1798	*trpC2 pheA1* Δ*nasA*::pMMN202	
LAB1803	*trpC2 pheA1* Δ*nasB*::pMMN192 SPβ*c2del2*::Tn*917*::pZS5	
LAB1804	*trpC2 pheA1* Δ*nasA*::pMMN202 SPβ*c2del2*::Tn*917*::pZS5	
LAB1805	*trpC2 pheA1* Δ*nasB*::pMMN192 SPβ*c2del2*::Tn*917*::pZS6	
LAB1806	*trpC2 pheA1* Δ*nasA*::pMMN202 SPβ*c2del2*::Tn*917*::pZS6	
Strains isogenic to SF10		
HJS31	Δ*glnR57*	29
SF10	Wild type	S. Fisher
SF73	*glnA73*	12
LAB1207	*glnA73* SPβ*c2del2*::Tn*917*::pZS6	
LAB1208	Δ*glnR57* SPβ*c2del2*::Tn*917*::pZS6	
LAB1214	*glnA73* SPβ*c2del2*::Tn*917*::pZS5	
LAB1215	Δ*glnR57* SPβ*c2del2*::Tn*917*::pZS5	
LAB1223	SPβ*c2del2*::Tn*917*::pZS5	
LAB1224	SPβ*c2del2*::Tn*917*::pZS6	
LAB1877	SPβ*c2del2*::Tn*917*::pMMN163	
LAB1878	SPβ*c2del2*::Tn*917*::pMMN164	
LAB1879	SPβ*c2del2*::Tn*917*::pMMN183	
LAB1880	SPβ*c2del2*::Tn*917*::pMMN184	
LAB1881	*glnA73* SPβ*c2del2*::Tn*917*::pMMN163	
LAB1882	*glnA73* SPβ*c2del2*::Tn*917*::pMMN164	
LAB1883	*glnA73* SPβ*c2del2*::Tn*917*::pMMN183	
LAB1884	*glnA73* SPβ*c2del2*::Tn*917*::pMMN184	

[a] Unless otherwise noted, all listed strains were derived from this work.

synthetase is involved in the nitrogen regulation of gene expression.

In this report, data are presented which demonstrate that the transcription of the *nasB* operon encoding nitrate/nitrite reductase subunits and *nasA*, which is transcribed in the opposite direction with respect to the *nasB* operon, is regulated in response to nitrogen source availability in a manner resembling this second, *glnA*-dependent mode of nitrogen control. The identification of a *cis*-acting element required for full expression and regulation of *nasA*/*B* expression is also reported.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *B. subtilis* strains used in this study are listed in Table 1, which contains strains of different genetic backgrounds.

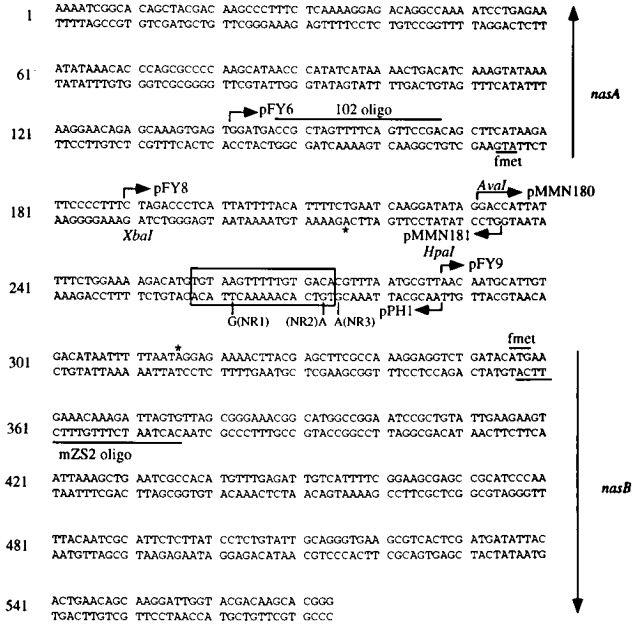


FIG. 1. Nucleotide sequence of the promoter region of *nasA*/*B*. Translation start sites are marked as fmet. The directions of *nasA* and *nasB* translation are shown by arrows in the right margin. Transcription start sites of divergent transcription units, determined by primer extension analysis with 102 and mZS2 oligonucleotides as shown in Fig. 2, are marked as asterisks. The dyad symmetry sequence is boxed. The letters below the dyad symmetry indicate the sites of base substitutions (NR1 to NR3) introduced in three mutagenesis experiments. Arrows with plasmid names show endpoints of deletions constructed.

Some strains are derived from JH642, and some are isogenic to SF10. LAB1223, LAB1224, LAB1877, LAB1878, LAB1879, and LAB1880 were constructed from SF10. LAB1207, LAB1214, LAB1881, LAB1882, LAB1883, and LAB1884 were constructed from SF73. LAB1208 and LAB1215 were constructed from HJS31. Construction of a *nasA* deletion mutant (LAB1798) is described elsewhere (26). A *nasB* deletion mutant (LAB1728) was constructed by replacing an internal *nasB* fragment (0.6 kb) with a phleomycin resistance (Phleo^r) gene. Minimal glucose medium (MMG) is composed of 0.5% glucose, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.1% sodium citrate, 0.012% $MgSO_4$, 10 mM $MnCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM $FeSO_4$, and 50 μg of auxotrophic amino acid requirement per ml if necessary. For the nitrogen source, 0.2% NH_4Cl, KNO_3, glutamate, proline, or glutamine was added. Glutamine was freshly prepared and filter sterilized. For preparation of agar plates, 1.2% Noble Agar (Difco Laboratories, Detroit, Mich.) was used. DS (Difco sporulation) medium was described previously (23). DSG medium was DS medium supplemented with 1% glucose, and DSGG medium was DS medium supplemented with 1% glucose and 0.2% glutamine. Plasmids pTKlac (18), pZS3, pZS4, and pZS5 are described in reference 26. DNA fragments carrying various promoter regions were cloned in front of the *lacZ* gene of plasmid pTKlac. The recombinant plasmid was introduced into the SPβ prophage site on the *B. subtilis* chromosome as previously described (41).

Measurement of β-galactosidase specific activity. *B. subtilis* cells carrying *lacZ* fusions were grown in DS, DSG, and DSGG media, and 1 ml of cells was withdrawn every 30 min for measurement of β-galactosidase activity. Cells cultured in MMG supplemented with different nitrogen sources were collected at the mid to late log phase of the growth curve. Preliminary results showed that activities of *nasA*- and *nasB-lacZ* fusions were constant during the growth phase in MMG. β-Galactosidase activity was measured as previously described (22).

Primer extension. RNA was isolated from *B. subtilis* OKB105 (23, 24), a surfactin-producing strain derived from JH642. In surfactin-producing strains, *nasA* and *nasB* expression is strongly induced postexponentially in DSG medium (25). OKB105 cells were grown in DSG medium, and RNA was isolated from cells at T_1 (1 h after the end of the exponential phase) and T_5. RNA was also isolated from cells cultured in MMG supplemented with nitrate or ammonium. *nasA* and *nasB* transcription start sites were determined by primer extension as previously described (24). Two different 20-mer oligonucleotides were used as primers: a 20-mer corresponding to bases 148 to 167 (oligo 102 in Fig. 1) for the determination of the *nasA* transcription start site, and an oligonucleotide complementary to bases 357 to 376 (oligo mZS2 in Fig. 1) for the transcription initiation site of the *nasB* operon. Nucleotide sequence determination was performed by the dideoxy-chain termination method (28) with the above primers.

Deletion of the promoter region. Plasmids pFY6, pFY8, pFY9, and pMMN180

TABLE 2. β-Galactosidase activities of *nasA/B-lacZ* fusions in wild-type cells grown with various nitrogen sources

Nitrogen source	β-Galactosidase activity (Miller units) of[a]:	
	LAB1166 (*nasB-lacZ*)	LAB1167 (*nasA-lacZ*)
Nitrate	10.3 ± 0.07	18.2 ± 0.9
Ammonium	<0.01	0.40 ± 0.19
Nitrate + ammonium	0.07 ± 0.01	0.64 ± 0.03
Proline	17.7 ± 2.6	50.0 ± 3.1
Glutamate	14.2 ± 0.7	50.7 ± 1.6
Glutamine	0.07 ± 0.01	1.13 ± 0.08

[a] Average of two or three independent cultures. The error is the standard deviation.

TABLE 3. β-Galactosidase activities of *nasA/B-lacZ* fusions in *glnA* and *glnR* mutants

Strain	β-Galactosidase activity (Miller units) of[a]:			
	nasB-lacZ		*nasA-lacZ*	
	Glutamate	Glutamine	Glutamate	Glutamine
LAB1223 (wt)[b]	3.17 ± 0.1	<0.01		
LAB1224 (wt)			31.8 ± 0.3	0.37 ± 0.02
LAB1214 (*glnA*)	NA[c]	42.9 ± 5.3		
LAB1207 (*glnA*)			NA	72.3 ± 2.5
LAB1215 (*glnR*)	5.68 ± 0.38	<0.01		
LAB1208 (*glnR*)			18.7 ± 1.3	0.57 ± 0.11

[a] Average of two or three independent cultures. The error is the standard deviation.
[b] wt, wild type.
[c] NA, not applicable.

are deletion derivatives of pZS5 (26). Plasmids pPH1 and pMMN181 are pZS6 (26) derivatives. pFY6 was constructed by unidirectional deletion of pZS5 with the Erase-a-Base system (Promega Corp., Madison, Wis.). The deletion endpoints were determined by nucleotide sequencing. To create pFY8, pZS5 was digested with *Xba*I, a fill-in reaction was performed with Sequenase (United States Biochemical Corp., Cleveland, Ohio), and then the plasmid was further digested with *Bam*HI. The resulting 380-bp DNA fragment was inserted into pTKlac digested with *Sma*I and *Bam*HI. pFY9 was constructed by inserting a *Hpa*I-*Bam*HI fragment (290 bp) of the *nasB* promoter into pTKlac digested with *Sma*I and *Bam*HI. pZS3 containing the 570-bp promoter region was digested with *Ava*I. After filling in with Sequenase, the plasmid was further digested with *Bam*HI. A 340-bp fragment was inserted into pTKlac digested with *Sma*I and *Bam*HI to construct pMMN180. A 230-bp fragment from the same digest was used to construct pMMN181. pZS6 was partially digested with *Hpa*I and then cleaved with *Sma*I. pPH1 was then constructed by self-ligation of the *Sma*I-*Hpa*I cleavage plasmid. The resultant plasmids were introduced into the SPβ locus as described previously (41).

Oligonucleotide-directed mutagenesis. Oligonucleotide-directed mutagenesis was done as previously described (24) with M13mp9 containing the 570-bp promoter fragment shown in Fig. 1. Three oligonucleotides (18-mer) complementary to the sequence around nucleotides 250 to 280 were used for the mutagenesis of the dyad symmetry sequence: NR1 (5′-CACAAAAACGTACA CATG-3′), NR2 (5′-TAAACGTATCACAAAAAC-3′), and NR3 (5′-CATTA AACATGTCACAAA-3′) (the mutations introduced are underlined). The mutagenized DNA fragments were isolated from the replicative forms of the mutagenized M13 clones and inserted into pTKlac in both orientations. Plasmids pMMN163 and pMMN164 are pZS6 and pZS5 derivatives, respectively, carrying the NR1 mutation in the dyad symmetry. Plasmids pMMN183 and pMMN184 have the NR2 mutation; pMMN171 and pMMN172 have the NR3 mutation.

RESULTS

Expression of *nasA* and *nasB* is nitrogen regulated. The *nasBCDEF* operon in *B. subtilis* was found to encode the enzymes required for nitrate and nitrite assimilation and the enzyme for synthesis of the siroheme cofactor of nitrite reductase (26). *nasA*, whose transcription is in the opposite direction to that of *nasBCDEF*, is also required for nitrate assimilation (26). The expression of *nasA* and *nasB* was monitored by assaying β-galactosidase activity of cells harboring *nasA-lacZ* and *nasB-lacZ* in the prophage of SPβ (SPβ::pZS6 and SPβ::pZS5, respectively, constructed as described in reference 26). Expression of neither *lacZ* fusion was detected until early stationary phase, and expression of both increased sharply after T_2 (2 h after the end of the exponential growth phase) in DSG medium, but no expression was detected either in DS medium or in DSGG medium (data not shown). This result suggests that induction of the fusions carried by *nasA/B* was due to nitrogen limitation. Next, the expression of *nasA/B* was examined in cells cultured in MMG supplemented with various nitrogen sources. Both *lacZ* fusions were expressed at constant levels during growth and stationary phase in MMG with nitrate, glutamate, or proline as a nitrogen source, but the expression was significantly reduced in MMG supplemented with ammonium or glutamine (Table 2). In the presence of both nitrate and ammonium, the β-galactosidase activity was also reduced, suggesting that regulation is exerted through negative control depending on the availability of readily utilized nitrogen sources rather than by induction involving a nitrate-dependent mechanism.

Many nitrogen-regulated genes in *B. subtilis* are known to be controlled by a mechanism that requires wild-type glutamine synthetase, the product of *glnA* (10, 13). Expression of these genes is derepressed in *glnA* mutants. Although *glnA* expression is repressed by the *glnR* product, *glnR* mutations do not cause derepression of other nitrogen-regulated genes. To test whether *nasA* and *nasB* expression is derepressed by the *glnA* mutation or the *glnR* mutation, SPβ::pZS5 and SPβ::pZS6 were used to lysogenize cells of strains SF73 (*glnA73*) and HJS31 (Δ*glnR57*) as well as an isogenic wild-type strain, SF10. In SF73, both fusions were highly expressed in MMG containing glutamine (Table 3) and also were completely derepressed during both exponential and stationary growth phases in DSGG medium (data not shown). However, in the *glnR* mutant as well as the wild-type strain, the *lacZ* fusions were still repressed in the presence of glutamine. These results indicate that the expression of *nasA-lacZ* and *nasB-lacZ* is under global nitrogen control through glutamine synthetase.

Mapping of transcription start sites. To determine transcription start sites of *nasA* and *nasB*, RNA was isolated from OKB105 cells cultured in MMG containing nitrate or ammonium and in DSG medium. RNA purified from cells grown in MMG with nitrate and those harvested at T_5 from DSG medium gave primer extension products corresponding to *nasA* and *nasB* transcripts, but no signal was detected with RNA samples from cells cultured in ammonium medium or a T_1 culture grown in DSG medium (Fig. 2). This result is in agreement with the result obtained by measuring β-galactosidase activity from transcriptional *nasA-lacZ* and *nasB-lacZ* fusions. Since the apparent start sites in cells from MMG-nitrate and DSG medium are identical, the activation of the genes by poor nitrogen sources and postexponential induction in DSG medium involves the use of the same promoters. There is a 99-bp space between the divergent transcription start sites. The −10 regions of the putative promoters show homology to those of σ^A-recognized promoters. (In the case of *nasA*, the sequence is TATCCT, with four of six matches; for *nasB*, it is CATAAT, with five of six matches.) The potential −35 region (TTTTCC), with three of six matching the σ^A consensus sequence, is separated by 17 bases from the putative −10 region in *nasA*. However, no appropriately positioned −35 region could be identified in *nasB*.

Deletion analysis of regulatory regions. In an attempt to

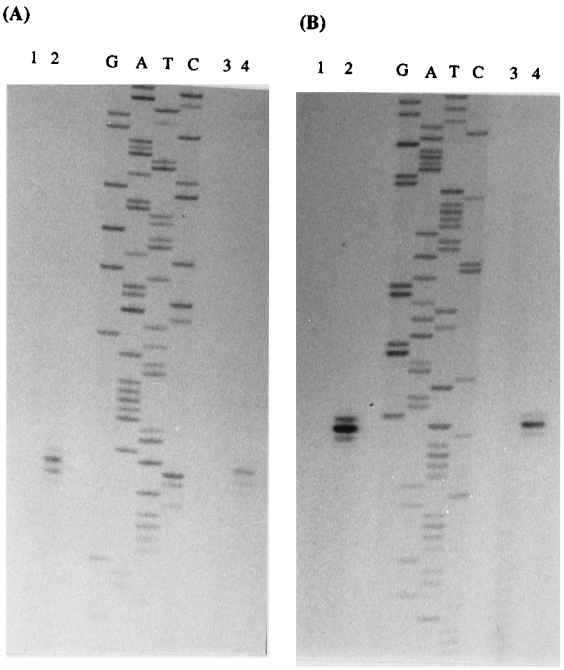


FIG. 2. Primer extension analysis of *nasA* and *nasB* transcription. (A) Determination of the *nasB* transcription start site with the mZS2 oligonucleotide shown in Fig. 1 as primer. (B) Determination of the *nasA* transcription start site with the 102 oligonucleotide as primer. Dideoxy-sequencing ladders (G, A, T, and C) obtained with the same primers used for primer extension analysis are shown. RNA was isolated from cells cultured in DSG medium and harvested 1 h after the end of the exponential growth phase (T_1) (lane 1) and at T_5 (lane 2) and in MMG medium with ammonium (lane 3) or with nitrate (lane 4). The nucleotide positions of the start sites are shown in Fig. 1.

determine the minimal regions required for transcription from the divergently oriented promoters, deletion analysis was carried out. Figure 3 shows a schematic diagram of the promoters and the deletion mutations constructed, along with the corresponding β-galactosidase activities from cells grown in MMG with glutamine (repressing) or glutamate (inducing). Upstream deletions to position −84 of the *nasB* promoter (pFY6, pFY8, and pMMN180) did not affect *nasB-lacZ* expression in cells grown in glutamate medium, but the expression in cells grown with glutamine as a nitrogen source was 20- to 40-fold higher in LAB1706 than in the strains carrying fusions with more upstream DNA (LAB1166, LAB1581, and LAB1583). This may suggest either that a negative regulatory site is present in the region between positions −84 and −128 or that the absence of transcription in the opposite direction (*nasA-lacZ*) enhances *nasB* transcription initiation in glutamine medium. Deletion up to position −28 of the *nasB* promoter (pFY9) resulted in loss of activity. The higher activity detected in this construct (LAB1589) as well as in LAB1707 (*nasA-lacZ*) under the repressing conditions (glutamine-containing medium) is puzzling, since the potential −35 region of the promoter was deleted. The possibility of alternative transcriptional start sites in these fusions remains to be examined. Similarly, the deletions up to position −71 of the *nasA* promoter (pPH1) did not cause significant change, except for the observed higher expression in cells grown in glutamine medium compared with LAB1167 cells, again suggesting that a negative regulatory region was deleted in pPH1. Loss of *nasA-lacZ* expression in glutamate medium was observed by deletion of DNA to a point 18 bp upstream of the transcription start site, which probably eliminates promoter activity. These results show that the sequences downstream from position −71 of the *nasA* promoter and position −84 of the *nasB* promoter are sufficient for the transcriptional activation of these genes.

Mutational analysis of a dyad symmetry element. In the region between 41 and 59 bp upstream of each transcription start site, a sequence exhibiting dyad symmetry (GTGTNAN$_7$TNACAC) was found, which might be a *cis*-acting element involved in the observed regulation. To study the involvement of the dyad symmetry in the expression of the divergently oriented transcription units, three mutations which reduce symmetry in the sequence were introduced (Fig. 4). The NR1 mutation resulted in a 4- to 6-fold reduction in *nasA/B* expres-

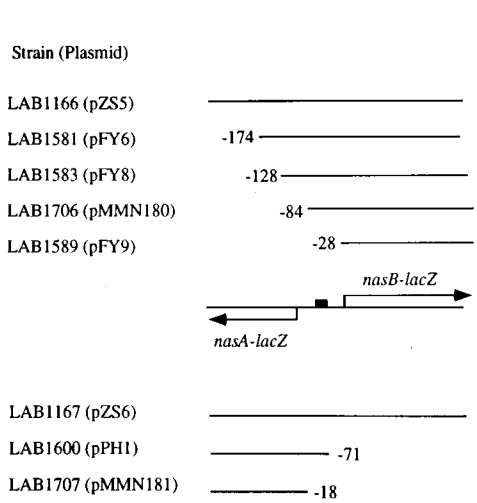
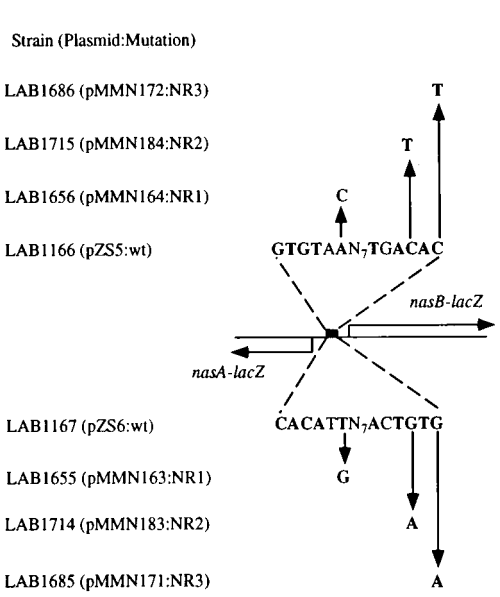


FIG. 3. β-Galactosidase activity directed by various deletion derivatives of *nasA* and *nasB* promoters. The divergent *lacZ* transcription directed by *nasA* and *nasB* promoters is shown by arrows. The solid box represents the region of dyad symmetry shown in Fig. 1. The lines above and below represent the fragments of deleted DNA that were inserted into pTKlac. The positions of the deletion endpoints are indicated next to the lines. The resultant plasmids are shown in parentheses. These plasmids were integrated as a single copy at the SPβ prophage site in the chromosome, and the resultant strains are indicated as LAB numbers. The promoter activities were measured in cells cultured in MMG supplemented with glutamate or glutamine. The averages of two or three independent cultures are shown along with the standard deviation. Regulation ratios (Reg. Ratio) were calculated by dividing the enzyme activities in cultures grown in the presence of glutamate by those in cultures grown in the presence of glutamine.



Strain (Plasmid:Mutation)	*nasB-lacZ* (Miller Unit) Glutamate	Glutamine	Reg. Ratio
LAB1686 (pMMN172:NR3) T	9.38 ± 0.14	0.41 ± 0.01	22.9
LAB1715 (pMMN184:NR2) T	1.72 ± 0.02	2.52 ± 0.02	0.7
LAB1656 (pMMN164:NR1) C	2.10 ± 0.30	0.61 ± 0.01	3.4
LAB1166 (pZS5:wt)	14.5 ± 0.4	0.08 ± 0.007	181
	nasA-lacZ (Miller Unit) Glutamate	Glutamine	Reg. Ratio
LAB1167 (pZS6:wt)	27.2 ± 1.6	1.40 ± 0.01	19.4
LAB1655 (pMMN163:NR1) G	5.07 ± 0.26	1.03 ± 0.07	4.9
LAB1714 (pMMN183:NR2) A	0.57 ± 0.09	0.86 ± 0.01	0.7
LAB1685 (pMMN171:NR3) A	25.3 ± 5.0	2.03 ± 0.12	12.5

FIG. 4. β-Galactosidase activity directed by mutant derivatives of dyad symmetry sequence. The sequence of dyad symmetry (solid box) located within the intergenic region of the divergent transcripts, *nasA* and *nasB*, was subjected to site-directed mutagenesis. The base substitutions introduced by the mutagenesis are shown by arrows and letters. The fragments with the mutations were inserted into pTKlac in opposite orientations to determine the effect of the mutations on *nasA* and *nasB* transcription, and the resultant plasmids are shown in parentheses. β-Galactosidase activities were measured from the cells cultured in MMG supplemented with glutamate or glutamine. The averages of two or three independent cultures are shown along with the standard deviation. Regulation ratios (Reg. Ratio) were calculated by dividing the enzyme activities in cultures grown in the presence of glutamate by those in cultures grown in the presence of glutamine.

sion, and the NR2 mutation led to a 5- to 6-fold reduction in the case of *nasB-lacZ* and a 47-fold reduction of *nasA* promoter activity. The NR3 mutation, which changes one of the outermost bases in the dyad symmetry, did not have a severe effect on *nasA-lacZ* or *nasB-lacZ* expression. Nitrogen regulation of *nasA/B* expression was partially released in strains carrying NR1 and NR2 mutations. This result showed that the sequence $TGTNAN_7TNACA$ is required in *cis* for the transcriptional activity and regulation of both *nasA* and *nasB* promoters. To examine the effect of the dyad symmetry mutations on *nasA* and *nasB* transcription in the *glnA* mutant, the mutant promoters fused to *lacZ* were introduced into cells of a *glnA* strain as well as those of the isogenic wild type by SPβ specialized transduction. Table 4 shows that the activities of the mutant promoters in the *glnA* cells were as low as those detected in the wild-type strain. This indicates that the derepression caused by the *glnA* mutation requires the dyad symmetry sequence.

Effects of *nasA* and *nasB* mutations on *nasA/B* transcription. The possibility that transcription in either orientation is subject to autoregulation was examined. Two deletion mutants, Δ*nasA* and Δ*nasB*, were constructed (as described in reference 26) and lysogenized with SPβ::pZS5 and SPβ::pZS6. The *nasA*- and *nasB*-directed β-galactosidase activity was measured in the resulting lysogens (Table 5). In both deletion mutants, transcription from either promoter was 2.5- to 4-fold higher

TABLE 4. Expression of *nasA/B-lacZ* fusions with mutant promoters in a *glnA* mutant

Strain	Activity (Miller units) of[a]: *nasB-lacZ* Glutamate	*nasB-lacZ* Glutamine	*nasA-lacZ* Glutamate	*nasA-lacZ* Glutamine
LAB1223 (wt/pZS5)[b]	11.6 ± 0.5	0.05 ± 0.007		
LAB1224 (wt/pZS6)			45.2 ± 5.3	1.55 ± 0.20
LAB1878 (wt/pMMN164)	2.63 ± 0.01	0.32 ± 0.15		
LAB1877 (wt/pMMN163)			7.22 ± 1.0	0.84 ± 0.02
LAB1880 (wt/pMMN184)	2.87 ± 0.4	2.02 ± 0.20		
LAB1879 (wt/pMMN183)			1.48 ± 0.15	1.10 ± 0.04
LAB1207 (*glnA*/pZS5)	NA[c]	25.9 ± 4.5		
LAB1214 (*glnA*/pZS6)			NA	145.3 ± 11.5
LAB1882 (*glnA*/pMMN164)	NA	0.48 ± 0.16		
LAB1881 (*glnA*/pMMN163)			NA	0.43 ± 0.02
LAB1884 (*glnA*/pMMN184)	NA	1.37 ± 0.10		
LAB1883 (*glnA*/pMMN183)			NA	0.68 ± 0.04

[a] Average of two or three independent cultures. The error is the standard deviation.
[b] wt, wild type.
[c] NA, not applicable.

TABLE 5. Effect of *nasA* and *nasB* deletions on the expression of *nasA/B-lacZ* fusion

Strain	Activity (Miller units) of[a]:			
	nasB-lacZ		*nasA-lacZ*	
	Glutamate	Glutamine	Glutamate	Glutamine
LAB1166 (wt)[b]	10.9 ± 1.3	<0.01		
LAB1167 (wt)			31.1 ± 7.0	1.11 ± 0.25
LAB1803 (Δ*nasB*)	30.4 ± 8.0	0.14 ± 0.1		
LAB1805 (Δ*nasB*)			87.6 ± 13.0	3.40 ± 0.70
LAB1804 (Δ*nasA*)	27.3 ± 0.81	<0.01		
LAB1806 (Δ*nasA*)			123.3 ± 5.6	1.53 ± 0.04

[a] Average of two or three independent cultures. The error is the standard deviation.
[b] wt, Wild type.

than in wild-type cells in MMG containing glutamate. This suggests that *nasA* and *nasB* transcription is partially autorepressed, being subject to negative control by the product(s) of the divergently oriented transcription units. There is an alternative possibility that a small amount of nitrate contaminating MMG-glutamate serves as an inducer and is consumed quickly in the presence of intact *nasA/B* genes, resulting in a decreased level of induction in *nasA/B*$^+$ strains. However, this possibility is unlikely, because the addition of nitrate to MMG-glutamate did not increase *nasA/B* transcription in wild-type cells (25). Another explanation for this result is that the contaminating nitrate or nitrite in the growth medium was converted to ammonium, which resulted in the slight repression of *nasA/B* expression in wild-type cells. In the mutant strains which cannot metabolize nitrate or nitrite to ammonium, higher expression might be observed.

DISCUSSION

The data reported here show that the expression of the *B. subtilis nasA* gene and *nasB* operon, encoding proteins involved in nitrate assimilation, is regulated by global nitrogen control. Low-level expression of *nasA-lacZ* and *nasB-lacZ* fusions was observed in cells grown in medium containing a preferred nitrogen source such as ammonium or glutamine, while *nas*-directed β-galactosidase activities were high when the fusion-bearing cells were grown in the presence of a poor nitrogen source such as nitrate, glutamate, or proline. This derepression by the absence of good nitrogen sources rather than induction by nitrate is similar to that of nitrate/nitrite reductases in *Synechococcus* sp. (16, 17). In *Klebsiella* spp., however, a poor nitrogen source was not sufficient to induce nitrate/nitrite reductases (4, 5), indicating that these genes are induced by the presence of nitrate or nitrite rather than by the absence of ammonium. The ammonium- and glutamine-dependent repression of *nasA* and *nasB* required wild-type glutamine synthetase but not GlnR, the *glnA* operon repressor. This regulatory pattern is also observed in the expression of other nitrogen-controlled genes in *B. subtilis*, such as *nrg-21* and *nrgAB* (1, 40). Urease and asparaginase activity is also regulated in a *glnA*-dependent manner (1). The *B. subtilis ans* operon, which codes for asparaginase and asparatase, and *ansR*, which is adjacent to *ans* but is divergently transcribed, have recently been isolated and characterized (36, 37). The phenotype of an *ansR* null mutation and the presence of a helix-turn-helix motif in the *ansR* product suggest that *ansR* encodes a repressor of the *ans* operon. In an *ansR* mutant, asparaginase expression was insensitive to repression by ammonium (37). Although asparaginase synthesis is known to be under control of a mechanism involving the *glnA* product (1), transcription of the *ans* operon has not been reported to be controlled by glutamine synthetase. Decapeptides in the carboxy-terminal regions of AnsR and GlnR proteins share six identical residues and three similar ones, leading to speculation that these conserved decapeptides can be modified in response to nitrogen availability, thus changing the affinity of the proteins for their respective DNA targets.

Both GlnR and GlnA are required for repression of *glnRA* transcription under conditions of nitrogen excess. Mutations in either gene result in constitutive expression (7, 29, 30, 33). GlnR, the repressor of the *glnRA* operon, was shown to bind to the operator *glnRA* O_1, a 21-bp symmetrical sequence between positions −40 and −60, as well as to *glnRA* O_2, a quasi-symmetrical element located between −17 and −37. *glnRA* O_2 has partial homology to *glnRA* O_1 (15, 32). The *glnRA* O_1 sequence is similar to the $TGTNAN_7TNACA$ element that may be an activator-binding site associated with *nasA/B*, as proposed herein. However, GlnR appears not to be involved in *nasA/B* regulation.

An operon (*nrgAB*) coding for a protein similar to the *E. coli glnB*-encoded PII protein (40) may also be involved in nitrogen control in *B. subtilis*. Some similarity is observed between *nasA/B* and *nrgAB*. The *nrgAB* promoter has significant similarity with the −10 region, but not the −35 region, of the consensus sequence for σ^A-dependent promoters. Immediately upstream of the −35 region is a sequence exhibiting dyad symmetry ($CATGTNAGN_5CTNACATG$), which shares homology ($TGTNAN_7TNACA$) with the *cis* regulatory sequences of *nasA/B* and *glnRA*, although it is not yet known whether the putative element is involved in the regulation of *nrgAB* expression. Δ*nrgAB* and Δ*nrgB* mutants were reported to grow more slowly on minimal medium with nitrate than wild-type cells did (40). It is possible that *nrgAB* and *nasA/B* have a common transcriptional activator or that the expression of *glnRA*, *nrgAB*, and *nasA/B* is regulated by different *trans*-acting factors. In either case, glutamine synthetase is required for the nitrogen-dependent regulation of these genes. Two models have been proposed to explain how glutamine synthetase regulates *glnRA* (29). In one model, glutamine synthetase responds to nitrogen excess by binding to GlnR, thereby forming a repressor complex with high affinity for its DNA target. A second model is that glutamine synthetase modifies GlnR in response to some metabolic consequence of nitrogen excess. At present, there is no evidence to distinguish between the two models, nor are there clues which might suggest a mechanism for how glutamine synthetase could modulate the activities of diverse *trans*-acting nitrogen-regulatory factors.

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