JOURNAL OF BACTERIOLOGY, Apr. 2002, p. 2148–2154 Vol. 184, No. 8
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.8.2148–2154.2002

Bacillus subtilis 168 Contains Two Differentially Regulated Genes Encoding L-Asparaginase

Susan H. Fisher* and Lewis V. Wray, Jr.

Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts 02118

Received 27 November 2001/Accepted 18 January 2002

Expression of the two *Bacillus subtilis* genes encoding L-asparaginase is controlled by independent regulatory factors. The *ansZ* gene (formerly *yccC*) was shown by mutational analysis to encode a functional L-asparaginase, the expression of which is activated during nitrogen-limited growth by the TnrA transcription factor. Gel mobility shift and DNase I footprinting experiments indicate that TnrA regulates *ansZ* expression by binding to a DNA site located upstream of the *ansZ* promoter. The expression of the *ansA* gene, which encodes the second L-asparaginase, was found to be induced by asparagine. The *ansA* repressor, AnsR, was shown to negatively regulate its own expression.

L-Asparaginase catalyzes the conversion of L-asparagine to L-aspartate and ammonium. In *Bacillus subtilis*, two different studies indicate that the synthesis of asparaginase is regulated in response to the nutritional status of the cell. In one study, elevated levels of asparaginase were shown to be present in cells growing either in rich medium or in glucose minimal medium containing aspartate or asparagine as the sole nitrogen source (25). In addition, the *ansA* gene was shown to encode an L-asparaginase. The *ansA* gene is located in an operon with *ansB*, which encodes L-aspartase (25). Expression of the *ansAB* operon is repressed by AnsR, and the activity of AnsR has been proposed to be regulated by either asparagine or aspartate (26).

Asparaginase synthesis was also shown to be elevated in the absence of asparagine or aspartate when cells are grown with a limiting nitrogen source: e.g., glucose minimal medium containing glutamate, proline, or urea as the sole nitrogen source (1). High-level expression of many *B. subtilis* genes during nitrogen-limited growth is due to regulation by GlnR or TnrA (10). TnrA and GlnR are members of the MerR family of transcription factors. Since the sequences of their DNA-binding domains are highly similar, both proteins bind to DNA sites that have a common consensus sequence (TGT–A–––––––T–ACA) (10, 34). GlnR represses transcription when cells are grown in the presence of excess nitrogen, while TnrA functions as an activator or repressor of gene expression when nitrogen is limited (3, 23, 30, 31, 35). Although asparaginase expression is activated during nitrogen-limited growth, no TnrA or GlnR binding sites are present in the promoter region of the *ansAB* operon. This observation raised the possibility that either TnrA or GlnR indirectly regulates *ansAB* expression or that *B. subtilis* contains a second asparaginase, the synthesis of which is controlled by TnrA or GlnR. In this report, we demonstrate that *ansZ* (formerly *yccC*) encodes a functional L-asparaginase, the expression of which is activated during nitrogen-limited growth by TnrA. In addition, we demonstrate that AnsR regulates both *ansAB* and its own expression in response to asparagine availability.

* Corresponding author. Mailing address: Department of Microbiology, Boston University School of Medicine, 715 Albany St., Boston, MA 02118. Phone: (617) 638-5498. Fax: (617) 638-4286. E-mail: shfisher@bu.edu.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists *B. subtilis* strains used in this study. Derivatives of strains 168, SF168G, and SF62 containing the *lacZ* transcriptional fusions were constructed with plasmid DNA as previously described (32). Transformation with selection for spectinomycin resistance was used to transfer the *ansB*::*spc* and *ansZ*::*spc* mutations. The *codY* mutation was transferred by transformation with selection for the genetically linked spectinomycin resistance gene. Transformants containing the *codY* mutation were identified by poor growth on Difco sporulation plates. Transformation with selection for erythromycin and tetracycline resistance, respectively, was used to transfer the *ansR*::*erm* and *ansA*::*tet* mutations.

Cell growth, media, and enzyme assays. The methods used for bacterial cultivation in the MOPS (morpholinepropanesulfonic acid) minimal medium of Neidhardt et al. (20) have been described previously (2). Glucose was added to a final concentration of 0.5%. All nitrogen sources were added at 0.2% to minimal growth medium, except where noted.

Extracts for enzyme assays were prepared from cells grown to mid-log growth phase (70 to 90 Klett units). β-Galactosidase was assayed in crude extracts as previously described (2). β-Galactosidase activity was always corrected for endogenous β-galactosidase activity present in *B. subtilis* cells containing the promoterless *lacZ* gene from pSFL7 integrated at the *amyE* site. Cells harvested for L-asparaginase assays were washed twice with buffer I (50 mM KPO_4 [pH 8.4], 10 mM EDTA [pH 8.0]) containing 150 mM NaCl and stored at −80 until assayed. Cell extracts were prepared by resuspending the thawed cells in 1 ml of buffer I, incubating the cells with 0.2 mg of lysozyme per ml for 20 min at 4°C, and sonication. After lysis, the cell debris was removed by centrifugation, and the supernatant was used for enzyme assays. L-Asparaginase activity was measured as the L-asparagine-dependent production of ammonium. The cell extracts were added to the reaction mixture (25 mM KPO_4 [pH 8.4], 10 mM EDTA [pH 8.0], 20 mM L-asparaginase) and incubated for 20 min at 30°C, and then the ammonium concentrations were determined by using a modified Berthelot reaction (29). One unit of L-asparaginase activity produced 1 μmol of NH_4 per min.

Plasmids and *lacZ* fusions. Three different subclones of the *ansA* promoter were constructed. Plasmids pANS4, pANS6, and pANS15 contain, respectively, *Bsp*HI-*Hin*cII, *Bsp*HI-*Xmn*I, and *Bsp*HI-*Msl*I DNA fragments from pPS1367 (25) cloned between the *Nco*I and *Stu*I sites of pLEW424 (32). To construct *ansA-lacZ* fusions, *Eco*RI-*Hin*dIII *ansA* promoter fragments from pANS6, pANS4, and pANS15 were cloned into pSFL7 to give pANS10, pANS12, and pANS19, respectively. Plasmid pANS5 contains the *ansR* promoter and was constructed by inserting a *Hin*cII-*Bsp*HI DNA fragment from pPS1367 between the *Ecl*136II and *Nco*I sites of pLEW424. The *ansR-lacZ* fusion plasmid pANS25 was constructed by cloning the *Eco*RI-*Hin*dIII *ansR* promoter fragment from pANS5 into pSFL7.

A 417-bp DNA fragment containing the *ansZ* promoter region was obtained by PCR amplification of *B. subtilis* chromosomal DNA with *Pwo* DNA polymer-

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

Strain	Genotype[a]	Source, reference, or derivative[b]
168	*trpC2*	This laboratory
SF62	*tnrA62*::Tn*917 trpC2*	31
PS37	*unkU*::*spc* Δ*codY trpC2*	P. Serror
SF168G	Δ*glnR57 trpC2*	30
PS1833	*ansR*::*erm trpC2*	26
SF168NR	*ansR*::*erm trpC2*	168 × PS1833 DNA
SF168NZ	*ansZ*::*spc trpC2*	168 × pANS48 DNA
SF168NB	*ansB*::*spc trpC2*	168 × pANS30 DNA
SF168NA	*ansA*::*tet*	168 × pANS29 DNA

[a] Genotype symbols are those of Biaudet et al. (4), with *ansZ* (formerly *yccC*) added.

[b] Strains were derived by transforming the first strain listed with DNA from the second strain or plasmid listed.

ase by using primers ANSZ1 (5′-TCCATAACTCATAACATTCCCACC) and ANSZ2 (5′-AGCTATCGTGCCTCCTGTCG). Plasmid pANS41 contains a 200-bp *Mfe*I-*Bsa*WI fragment from the PCR-generated DNA cloned into pMTL23P (7). The *ansZ* promoter DNA in pANS41 was sequenced to confirm the fidelity of the PCR amplification. Plasmid pANS43 contains an *Xba*I-*Sph*I DNA fragment of the *ansZ* promoter from pANS41 cloned into pJDC9 (8). An *ansZ-lacZ* fusion was constructed by cloning an *Eco*RI-*Hin*dIII DNA fragment from pANS43 into pSFL7 (30).

Inactivation of the chromosomal *ansA*, *ansB*, and *ansZ* genes. A DNA fragment containing the *ansA* gene was obtained by PCR amplification of *B. subtilis* chromosomal DNA with primers ANSA2 (5′-TACGGAATTCATACCTTCTC GCAACCCATC) and ANSA3 (5′-CGTAAAGCTTAAGATCCGAAGACTGA CCTAGC). Plasmid pANS28 was constructed by cloning an *Sna*BI-*Hin*dIII DNA fragment from the PCR product into pJDC9. Located within the *ansA* coding sequence of pANS28 is a unique *Pst*I site. Plasmid pANS29 contains a *Pst*I-*Pst*I tetracycline resistance gene cassette from pBEST309 (16) inserted into the *Pst*I site of pANS28. Plasmid pANS30 contains a disruption of the *ansB* gene and was constructed by inserting a spectinomycin resistance gene cassette into the *Nsi*I site of pPS1304 (25). The chromosomal *ansA* and *ansB* genes were disrupted by transforming *B. subtilis* cells with DNA from plasmids pANS29 and pANS30, respectively.

A DNA fragment containing the *ansZ* gene was obtained by PCR amplification of *B. subtilis* DNA with primers ANSZ3 (5′-CGTAGAATTCATATAAAG CAGGTGTTGTCGGCG) and ANSZ4 (5′-TTTTAAGATTCAGAGGTCATT ATTGGTCC). Plasmid pANS47 contains an *Eco*RI-*Hin*dIII fragment from the PCR-generated DNA cloned into pJDC9. A unique *Eco*RV site lies within the *ansZ* coding sequence. Plasmid pANS48 was constructed by inserting a spectinomycin-resistant *Eco*RV-*Hin*cII DNA fragment from pDG1726 (13) into the *Eco*RV site of pANS47. The chromosomal *ansZ* gene was disrupted by transforming *B. subtilis* cells with pANS48 DNA.

In vitro analysis of DNA binding. The binding of purified TnrA to the *ansZ* promoter region utilized a 208-bp *Xba*I-*Acc*65I DNA fragment from pANS41. The downstream end of this DNA fragment was labeled by a fill in reaction with Klenow DNA polymerase and [α-^{32}P]dGTP. Gel mobility shift and DNase I footprinting experiments were performed as previously described (34). The binding of TnrA to the *ansA* promoter region was tested with a 232-bp *Bam*HI-*Pst*I DNA fragment from pANS4.

Primer extension experiments. 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 (11). Primer extensions were performed as previously described (11) with two oligonucleotide primers, ANSZ6 (5′-AAAA ACAAACAATAGTGCGG) and ANSZ7 (5′-GTGCGGTAAAAAGTACGAG C), which are complementary to the 5′ end of the *ansZ* coding region.

RESULTS

ansZ encodes a functional L-asparaginase. The *ansZ* gene (previously called *yccC*) was identified by the *B. subtilis* genome sequencing project and was proposed to encode an L-asparaginase (18). The deduced amino acid sequence of *ansZ* shows 59% identity to the L-asparaginase from *Erwinia chrysanthemi* (19) and 53% identity to L-asparaginase II from *Escherichia coli* (5). The N-terminal amino acid sequence of YccC has been predicted to function as a lipoprotein signal peptide (27). An inverted repeat sequence with structural similarity to factor-independent transcriptional terminators is located immediately downstream of the *ansZ* coding region, while the divergently transcribed *lmrAB* operon is located upstream of *ansZ*. Together, these observations suggest that *ansZ* is expressed as a monocistronic transcript. A DNA sequence with similarity to the TnrA binding site consensus sequence is centered 76 nucleotides upstream of the *ansZ* translational start codon (Fig. 1).

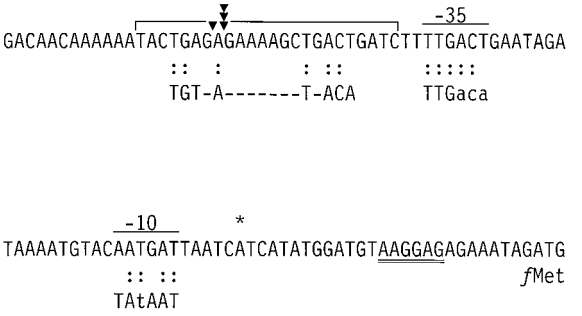

FIG. 1. Nucleotide sequence of the *ansZ* promoter region. The consensus sequences for the −10 and −35 regions of σ^A-dependent promoters (14) and the TnrA binding site (33) are shown below the nucleotide sequence. The uppercase letters in the −10 and −35 consensus sequences are used to indicate bases that are highly conserved in *B. subtilis* σ^A-dependent promoters (14). The transcriptional start site is indicated by an asterisk. The putative ribosomal binding site is double underlined, and the translational start codon for *ansZ* has the label *f*Met located below it. The region protected from DNase I digestion by TnrA is indicated by the horizontal bracket above the DNA sequence. The DNase I-hypersensitive sites are shown as solid triangles.

To determine whether *ansZ* encodes a functional L-asparaginase the synthesis of which is nitrogen regulated, the *ansZ* gene was mutationally inactivated and asparaginase activity was determined for wild-type and Δ*ansZ* cells grown in glucose minimal medium containing either excess (glutamate plus ammonium) or limiting (glutamate) nitrogen. In wild-type cells, asparaginase levels are 10-fold higher in extracts of glutamate-grown cells than in cells grown with glutamate plus ammonium as the nitrogen source (Table 2). These data agree with previously published results indicating that asparaginase expression

TABLE 2. Asparaginase expression in wild-type or *ansA* and *ansZ* mutant strains

Relevant genotype[a]	Asparaginase sp act (U/mg of protein) in cells grown on[b]:	
	Excess nitrogen (glutamate + NH_4Cl)	Limited nitrogen (glutamate)
Wild type	5.1	51.8
Δ*ansZ*	3.4	5.5
Δ*ansA* Δ*ansZ*	≤1.6	≤1.6

[a] All strains are derivatives of strain 168.

[b] Data are the average of three or more determinations, which did not vary by more than 15%. Cultures were grown in MOPS glucose minimal medium containing the indicated nitrogen sources.

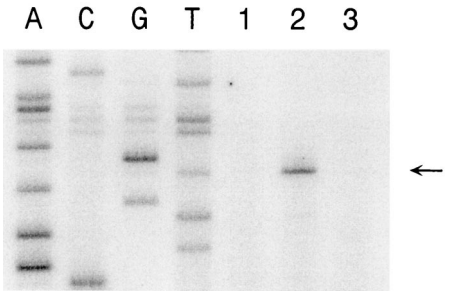

FIG. 2. Primer extension analysis of *ansZ*. *B. subtilis* RNA used in the primer extension was isolated from wild-type cells grown in glucose minimal medium containing either glutamate plus ammonium (lane 1) or glutamate (lane 2) as the nitrogen source. *S. cerevisiae* RNA was used in the primer extension shown in lane 3. The ANSZ7 oligonucleotide primer was used for dideoxynucleotide sequencing of pANS41 (lanes A, C, G, and T) and for primer extensions. The primer extension product is denoted by the arrow. Identical results were obtained with the ANS6 oligonucleotide primer (data not shown).

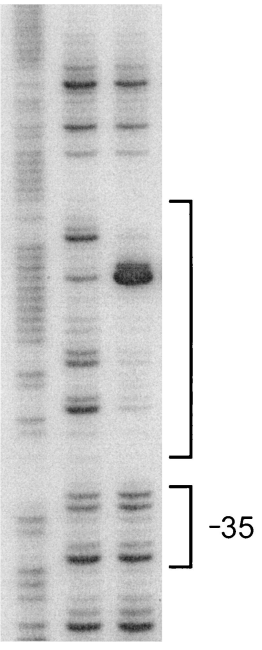

FIG. 3. DNase I footprinting of the *ansZ* promoter with TnrA. Lane R, G+A molecular size standards. Lanes 1 and 2 were treated with DNase I in the absence (lane 1) or presence (lane 2) of 2.5 μM TnrA. The TnrA-protected region and the −35 promoter region are bracketed.

is nitrogen regulated in *B. subtilis* (1). The levels of asparaginase present in extracts of glutamate-grown Δ*ansZ* cells are 10-fold lower than that seen in extracts of wild-type cells (Table 2). In contrast, similar levels of L-asparaginase are present in extracts of wild-type and Δ*ansZ* cells grown in medium containing glutamate plus ammonium as the nitrogen source (Table 2). These results indicate that *ansZ* encodes an L-asparaginase that is preferentially expressed in nitrogen-limited cells. As noted above, the deduced amino acid sequence of AsnZ contains a putative protein secretion sequence. Nonetheless, *ansZ*-encoded asparaginase activity was detected in soluble cell extracts. No experiments were performed to determine whether AnsZ is also secreted.

Because the *B. subtilis ansA* gene also encodes an L-asparaginase (25), the level of asparaginase activity was also examined in a Δ*ansA* Δ*ansZ* double mutant. Since no detectable asparaginase activity was present in extracts of the Δ*ansA* Δ*ansZ* double mutant (Table 2), the low levels of asparaginase present in extracts of Δ*ansZ* cells must be due to *ansA* expression. Thus, *ansA* and *ansZ* encode the only asparaginases detectable in *B. subtilis* under these conditions.

Identification of the *ansZ* transcriptional start site. Primer extension analysis was used to identify the apparent transcriptional start site for the *ansZ* promoter. These experiments used RNA isolated from cells grown in glucose minimal medium containing either limiting (glutamate) or excess (glutamate plus ammonium) nitrogen sources and an oligonucleotide complementary to the *ansZ* coding region. The primer extension product obtained with RNA from glutamate-grown cells (Fig. 2) corresponds to an mRNA with a 5′ end located 27 bp upstream of the *ansZ* start codon (Fig. 1). In contrast, no primer extension product was obtained with RNA isolated from cells grown with excess nitrogen (glutamate plus ammonium) (Fig. 2). The transcriptional regulation observed in the primer extension experiments is in agreement with the results obtained with asparaginase assays (Table 2) and with transcriptional *ansZ-lacZ* fusion studies described below. Examination of the DNA sequence upstream of the transcriptional start site reveals that while the −35 promoter region is highly similar to the σ^A consensus sequence (14), the −10 region contains two mismatches with highly conserved nucleotides (Fig. 1). This observation suggests that the *ansZ* promoter is a nonoptimal σ^A-dependent promoter with a low level of intrinsic transcriptional activity.

Identification of the *ansZ* TnrA binding site. Gel mobility shift analysis was used to determine whether TnrA directly interacts with the *ansZ* promoter region. In these experiments, purified TnrA bound to *ansZ* promoter region DNA with an equilibrium dissociation binding constant (K_d) of 340 nM (data not shown). The affinity of TnrA for its *ansZ* binding site is significantly less than its affinity for the *amtB* (previously called *nrgA*) promoter, which has a K_d of 8 nM (34). The TnrA binding site in the *ansZ* promoter region has two mismatches with the TnrA consensus sequence (Fig. 1), which may account for the low in vitro affinity of TnrA for this site.

A DNase I protection experiment demonstrated that purified TnrA protects DNA upstream of the *ansZ* promoter region from DNase I digestion (Fig. 3). This protected region includes the TnrA consensus sequence and extends from position −37 to −70 on the nontemplate strand (Fig. 1). In addition, the binding of TnrA to the *ansZ* promoter DNA gives rise to DNase I-hypersensitive sites at positions −52 and −53 on the nontemplate strand. DNase I-hypersensitive sites were previously observed at the same relative position in the TnrA footprint of the *amtB* promoter (34). The TnrA-binding site in the *ansZ* promoter is centered 49 bp upstream of the transcriptional start site, which is similar to what has been observed for other TnrA-activated genes (33).

Nitrogen regulation of *ansZ* expression. The expression of *ansZ* was examined in more detail with the (*ansZ-lacZ*)*45* transcriptional fusion. This *lacZ* fusion contains an *ansZ* pro-

TABLE 3. β-Galactosidase expression of *lacZ* fusions in wild-type and mutant strains

lacZ fusion[a]	Relevant genotype	β-Galactosidase sp act (U/mg of protein) in cells grown on[b]:		
		Excess nitrogen	Limiting nitrogen	
		Glutamate + NH_4Cl	Aspartate	Glutamate
(*ansZ-lacZ*)*45*	Wild type	0.1	124	382
	tnrA	0.1	0.1	0.1
	ansR	0.2	ND[c]	111
	ansR ansB	0.2	ND	269
	ansB	0.1	ND	303
(*amtB-lacZ*)*416*	Wild type	0.06	119	167
(*amtB-lacZ*)*435*	Wild type	0.05	11.7	29.4

[a] All strains are derivatives of strain 168 containing *lacZ* fusions integrated as a single copy at the *amyE* locus. The (*amtB-lacZ*)*416* and (*amtB-lacZ*)*435 lacZ* fusions have been described previously as *nrgA-lacZ* fusions (33).

[b] Data are the average of three or more determinations, which did not vary by more than 20%. Cultures were grown in MOPS glucose minimal medium containing the indicated nitrogen sources.

[c] ND, not determined.

TABLE 4. Culture doubling time for wild-type and mutant strains

Relevant genotype	Doubling time (min) of cells grown on[a]:		
	Glutamate + NH_4Cl	Aspartate	Glutamate
Wild type	65	90	160
ansR	62	ND[b]	85
ansR ansB	65	ND	275
ansB	60	ND	315

[a] Cells were grown in MOPS minimal medium containing glucose as the carbon source and the indicated nitrogen sources. Doubling times are the averages of two to four independent determinations, which did not vary by more than 25%.

[b] ND, not determined.

moter fragment that extends from −121 to +83 with respect to the transcriptional start site. Since β-galactosidase levels were 3,800-fold higher in cells grown with limiting nitrogen (glutamate) than in extracts of cells grown with excess nitrogen (glutamate plus ammonium) (Table 3), expression of the *ansZ-lacZ* fusion is nitrogen regulated. The observation that expression of the (*ansZ-lacZ*)*45* fusion is not elevated in a *tnrA* mutant grown with glutamate as the nitrogen source (Table 3) indicates that TnrA is required for the activation of *ansZ* expression during nitrogen-limited growth. GlnR does not regulate *ansZ* expression, because similar levels of *ansZ* expresion were seen in wild-type and *glnR* cells grown with either excess or limiting nitrogen (data not shown). The CodY repressor protein regulates the expression of many genes involved in nitrogen metabolism (10, 21). The highest levels of CodY-dependent repression occur in cells grown with amino acids. Two experimental observations indicate that *ansZ* expression is not subject to nutritional regulation by CodY. First, the addition of 0.2% Casamino Acids to cells growing in glucose minimal medium containing glutamate plus ammonium as the nitrogen source did not alter *ansZ-lacZ* expression (data not shown). Second, no difference in *ansZ-lacZ* expression was observed in wild-type and *codY* cells grown in glucose minimal medium containing glutamate plus ammonium plus 0.2% Casamino Acids as the nitrogen source (data not shown).

Although aspartate is a limiting nitrogen source, expression of the (*ansZ-lacZ*)*45* fusion was threefold higher in glutamate-grown cultures than in aspartate-grown cultures (Table 3). Two observations indicate that glutamate-grown cultures are more nitrogen limited than aspartate-grown cultures. First, glutamate-grown cells grow more slowly than aspartate-grown cells (Table 4). Second, the level of glutamine synthetase, a key enzyme in nitrogen assimilation, is higher in glutamate-grown *B. subtilis* cultures than in aspartate-grown cultures (1). Thus, increased expression of the (*ansZ-lacZ*)*45* fusion in glutamate-grown cells compared to aspartate-grown cells most likely results from glutamate being a poorer source of nitrogen than aspartate. To test this hypothesis, the expression of two other TnrA-dependent nitrogen-regulated *lacZ* fusions was examined. The wild-type *amtB* promoter in the (*amtB-lacZ*)*416* fusion contains an optimal TnrA binding site (K_d of 8 nM) (34). The TnrA binding site in the (*amtB-lacZ*)*435* fusion contains 2-bp changes that reduce the in vitro TnrA affinity (K_d of 650 nM) and in vivo expression (33, 34). The (*amtB-lacZ*)*416* and *435* fusions have β-galactosidase levels that are 1.4- and 2.5-fold higher, respectively, in glutamate-grown cells than in aspartate-grown cells (Table 3). Thus, the increased level of *ansZ* expression in glutamate-grown cells compared to aspartate-grown cells most likely results from glutamate being a poorer source of nitrogen than aspartate rather than from some intrinsic property of the *ansZ* promoter.

Regulation of *ansA* expression by AnsR. The AnsR-dependent regulation of *ansA* expression was examined with the (*ansA-lacZ*)*12* transcriptional fusion (Fig. 4). The *ansA* promoter fragment in this *lacZ* fusion extends from −118 to +87 with respect to the transcriptional start site. As expected, the (*ansA-lacZ*)*12* fusion is negatively regulated by AnsR, because high-level β-galactosidase expression from the (*ansA-lacZ*)*12* fusion occurs in the *ansR* mutant during growth on all nitrogen sources (Table 5). The expression of *ansA* has been proposed to be induced by either asparagine or aspartate (25). Asparagine was found to induce *ansA* expression, because β-galactosidase levels in wild-type cells are 23- and 40-fold higher, re-

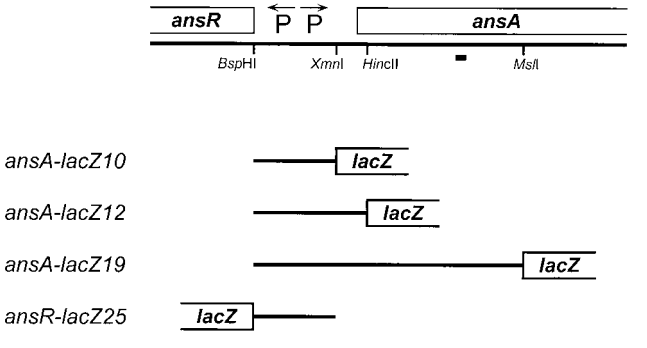

FIG. 4. Physical structure of the *ansR-ansA* promoter region. The *ansR* and *ansA* promoters are indicated by the letter P with an arrow above it. The black box below the line indicates the putative *cre* site. The DNA fragments used to construct *ansA*- and *ansR-lacZ* fusions are diagrammed at the bottom.

TABLE 5. β-Galactosidase expression from *ansA-lacZ* and *ansR-lacZ* fusions

lacZ fusion[a]	Relevant genotype	β-Galactosidase sp act (U/mg of protein) in cells grown on[b]:					
		Excess nitrogen				Limiting nitrogen	
		Glutamate + NH_4Cl	Glutamate + NH_4Cl + Asparagine[c]	Glutamate + NH_4Cl + Aspartate[c]	Asparagine	Aspartate	Glutamate
(*ansA-lacZ*)*12*	Wildtype	13	301	16	523	16	10
	ansR	574	639	ND[d]	634	589	ND
(*ansA-lacZ*)*10*	Wildtype	1.0	76	1.6	ND	ND	0.9
(*ansR-lacZ*)*25*	Wildtype	4	28	5	49	3	ND
	ansR	55	55	ND	50	49	ND

[a] All strains are derivatives of strain 168 containing the indicated *lacZ* fusions integrated as a single copy at the *amyE* locus (see Fig. 4).
[b] Data are the average of three or more determinations, which did not vary by more than 15%. Cultures were grown in MOPS glucose minimal medium containing the indicated nitrogen sources.
[c] Minimal medium containing glutamate plus ammonium as nitrogen sources was supplemented with either 0.02% aspartate or 0.02% asparagine.
[d] ND, not determined.

spectively, in cells grown with either asparagine or glutamate plus ammonium plus asparagine as the nitrogen source than in cells grown with glutamate plus ammonium as the nitrogen source (Table 5). Since similar levels of *ansA* expression occurred in cells grown in medium containing glutamate plus ammonium, glutamate plus ammonium plus aspartate, or aspartate alone as the nitrogen source (Table 5), aspartate does not appear to induce *ansA* expression. Thus, our data indicate that AnsR regulates *ansA* expression in response to asparagine availability.

It has been suggested that AnsR represses expression of *ansA* by binding to an inverted repeat sequence located between nucleotides +9 and +33 (25). To determine if this sequence is required for the AnsR-dependent regulation of *ansA*, the (*ansA-lacZ*)*10* fusion was constructed (Fig. 4). This *lacZ* fusion contains an *ansA* promoter DNA fragment that extends from −118 to +23 and lacks the downstream half of the inverted repeat sequence that is present in the (*ansA-lacZ*)*12* fusion. Since asparagine induces the expression of β-galactosidase from both the (*ansA-lacZ*)*10* and (*ansA-lacZ*)*12* fusions (Table 5), this sequence does not appear to be required for AnsR repression.

Role of carbon and nitrogen transcriptional regulatory factors in *ansA* expression. Carbon catabolite repression of many *B. subtilis* genes is mediated at *cis*-acting sites called carbon repression elements (*cre*) (24). The *cre* sites for several *B. subtilis* genes are located considerable distances downstream of their transcriptional start sites (6, 12, 17, 32, 37). It has been previously noted that a putative *cre* site (TGATAACGATTA CA) is located 185 bp downstream of the *ansA* transcriptional start site (15). To see whether this is a functional *cre* site, β-galactosidase expression from the (*ansA-lacZ*)*19* fusion, which contains this *cre* site (Fig. 4), was examined in cells grown in minimal medium containing glutamine as the nitrogen source and either excess (glucose) or limited (citrate) carbon. Since carbon limitation did not elevate *ansA* expression in either uninduced or asparagine-induced cultures (data not shown), *ansA* expression does not appear to be subject to regulation by carbon catabolite repression.

The observation that similar levels of β-galactosidase expression from the (*ansA-lacZ*)*12* fusion were seen in wild-type cells grown with the excess (glutamate plus ammonium) and limiting (glutamate) nitrogen indicates that TnrA and GlnR most likely do not regulate *ansA* expression (data not shown). Moreover, no significant binding of TnrA to the *ansA* promoter region was observed in gel mobility shift experiments (data not shown). There is also no evidence that CodY regulates *ansA* expression. The addition of 0.2% Casamino Acids to medium containing glutamate plus ammonium as the nitrogen source did not repress *ansA* expression. Similar levels of *ansA* expression were seen in wild-type and *codY* cells grown with glutamate plus ammonium plus 0.2% Casamino Acids as the nitrogen source (data not shown).

AnsR regulates its own expression. The AnsR protein belongs to a family of transcriptional repressors which includes some phage repressors that are known to be autogenously regulated (28). The (*ansR-lacZ*)*25* fusion (Fig. 4) was used to examine the regulation of *ansR* expression. β-Galactosidase expression from the (*ansR-lacZ*)*25* fusion is induced by asparagine in wild-type cells and is derepressed in an *ansR* mutant (Table 5). These results indicate that AnsR regulates its own expression in response to asparagine availability.

Role of AnsR in *ansZ* expression. The ability of AnsR to regulate *ansZ* expression in response to asparagine availability was examined. Two experimental observations argue that the expression of *ansZ* is not induced by asparagine. First, β-galactosidase expression from the (*ansZ-lacZ*)*45* fusion is not altered by addition of 0.02% asparagine to glucose minimal medium containing glutamate plus ammonium as the nitrogen source (data not shown). Second, (*ansZ-lacZ*)*45* cells grown with either glutamate plus ammonium or asparagine as the nitrogen sources contain the same low levels of β-galactosidase (Table 3) (data not shown). However, when expression of the (*ansZ-lacZ*)*45* fusion was examined in an *ansR* mutant strain, a surprising result was obtained. While the *ansR* mutation had no effect on *ansZ* expression under conditions of nitrogen excess, the level of *ansZ* expression in glutamate-grown cells is 3.5-fold lower in the *ansR* mutant than in the wild-type strain (Table 3).

There are two possible explanations for the altered *ansZ* expression seen in the *ansR* mutant during nitrogen-limited growth. First, AnsR might function as a transcriptional activa-

tor of *ansZ* expression during nitrogen-limited growth. Alternatively, the *ansR* mutation could indirectly alter *ansZ* expression under these growth conditions. The latter hypothesis is supported by the observation that the *ansR* mutant, which has higher levels of asparaginase and aspartase than wild-type cells, grows more rapidly than the wild-type cells in glutamate-grown cultures (Table 4). In contrast, wild-type and *ansR* cultures grew with similar doubling times when glutamate plus ammonium was the nitrogen source (Table 4). Since the amino group of glutamate can be readily transferred to aspartate by transamination, the elevated levels of aspartase in the *ansR* mutant may be responsible for altered *ansZ* expression in glutamate-grown *ansR* cells. To test this hypothesis, *ansZ* expression was examined in *ansR ansB* and *ansB* mutant strains. During growth in medium containing glutamate as the sole nitrogen source, the expression of the (*ansZ-lacZ*)*45* fusion is 2.5- to 3-fold higher, respectively in the *ansR ansB* and *ansB* mutants than in the *ansR* strain (Table 3). Moreover, the *ansR ansB* and *ansB* cultures grow more slowly than the wild-type cultures with glutamate as the sole nitrogen source (Table 4). All of this indicates that lower levels of *ansZ* expression in glutamate-grown *ansR* cells result from overexpression of aspartase and that aspartase plays a significant role in the utilization of glutamate as a nitrogen source in *B. subtilis*.

DISCUSSION

Two genes, *ansA* and *ansZ*, encode functional L-asparaginase enzymes in *B. subtilis*. The expression of these two genes is regulated in response to different nutritional signals. When cells are grown in the presence of inducing amounts of asparagine, AnsR repression of the *ansAB* operon is relieved and AnsA is expressed at high levels. Since the *ansAB* operon encodes both L-asparaginase and L-aspartase, high-level *ansAB* expression causes asparagine to be catabolized to fumarate and two molecules of ammonium. In contrast, expression of the monocistronic *ansZ* gene is activated during nitrogen-limited growth by the nitrogen regulatory factor TnrA. Under these growth conditions, asparagine would be degraded to ammonium and aspartate by L-asparaginase. Other microorganisms are known to contain multiple L-asparaginases. Both *Escherichia coli* and *Klebsiella aerogenes* contain two L-asparaginases, a high-affinity periplasmic enzyme and a low-affinity cytoplasmic enzyme (22). In *E. coli*, synthesis of the cytoplasmic asparaginase I is constitutive, while expression of the periplasmic asparaginase II is activated during anaerobiosis, where fumarate generated from asparagine degradation functions as an electron acceptor. In contrast, the *K. aerogenes* periplasmic asparaginase is subject to nitrogen regulation. Two L-asparaginases are also present in *Saacharomyces cerevisiae* (9). The cytoplasmic asparaginase I is synthesized constitutively, while expression of extracellular asparaginase II is elevated during nitrogen-limited growth (9). Interestingly, the amino acid sequence of the *B. subtilis* AnsA enzyme closely resembles the sequence of *E. coli* asparaginase I, while the sequence of AnsZ is more similar to that of *E. coli* asparaginase II. There is no evidence that expression of the *B. subtilis* asparaginase genes is elevated under anaerobic conditions (36).

During growth on minimal medium containing an amino acid as the sole nitrogen source, ammonium must be generated by amino acid catabolism, because glutamine synthesis requires ammonium. If degradation of an amino acid used as the sole nitrogen source produces ammonium inefficiently, the growth rate of the culture slows due to nitrogen limitation. For instance, the doubling time of *B. subtilis* cells grown in glucose minimal medium containing glutamate as the sole nitrogen source is considerably longer than that of cultures growing with both glutamate and ammonium as nitrogen sources (Table 4). The observation that the doubling time of a glutamate-grown *ansB* strain, which lacks aspartase, is significantly longer than that of a wild-type strain (Table 4) argues that aspartase plays a major role in ammonium generation in glutamate-grown cells. This pathway most likely involves the conversion of glutamate to aspartate by glutamate-oxaloacetate transaminase, followed by the degradation of aspartate to fumarate and ammonium by aspartase. Since *ansB* cells still are able to grow with glutamate as the sole nitrogen source, albeit at a slower rate than wild-type cells, other pathways for generating ammonium from glutamate must also be present in *B. subtilis*.

The role of aspartase in the generation of ammonium from glutamate provides an explanation for the reduced *ansZ* expression seen in the *ansR* mutant (Table 3). Since the *ansR* mutant contains higher levels of aspartase than wild-type cells, ammonium would be generated from glutamate at a faster rate in *ansR* cells than in wild-type cells. The increased flux of nitrogen from glutamate to ammonium in glutamate-grown *ansR* cells would partially relieve nitrogen limitation, resulting in faster growth rates (Table 4) and lower levels of *ansZ* expression (Tables 3). Since introduction of the *ansB* mutation into the *ansR* strain blocks production of ammonium from glutamate via aspartate, growth of the *ansB ansR* double culture becomes more nitrogen-limited than that of the *ansR* culture. As a result, *ansZ* is expressed at higher levels in glutamate-grown *ansB ansR* cells than in *ansR* cells.

While aspartate and glutamate are limiting nitrogen sources, TnrA-dependent promoters are not activated in aspartate-grown cells to the same level as glutamate-grown cells (Table 3). As noted above, this difference reflects the fact that aspartate-grown cells are not as limited for nitrogen as glutamate-grown cells. It is noteworthy that the relative levels of expression in aspartate-grown cells compared to that in glutamate-grown cells are not the same for all TnrA-activated promoters. The wild-type *amtB* promoter is expressed in aspartate-grown cells at 70% of the level seen in glutamate-grown cells. In contrast, the *ansZ* and *amtB435* promoters are expressed at relatively lower levels (32 and 40%, respectively) in aspartate-grown cells than in glutamate-grown cells (Table 3). These differences in expression reflect the affinity of these promoters for TnrA. The wild-type *amtB* promoter has a much higher affinity for TnrA than the *ansZ* and *amtB435* promoters. Thus, in aspartate-grown cells, promoters with high-affinity TnrA-binding sites are activated to a greater extent than promoters with low affinity for TnrA. These differences in the affinity of various promoters for TnrA would result in a graded response to nitrogen availability and raise the possibility that during a shift to nitrogen-limited conditions, some TnrA-activated promoters would be expressed before others.

ACKNOWLEDGMENTS

We are grateful to Peter Setlow for strain PS1833 and plasmids pPS1304 and pPS1367. We thank Jaclyn Brandenburg and Erin Bitner for providing technical assistance.

This research was supported by Public Health Service research grant GM51127 from the National Institutes of Health.

REFERENCES

1. **Atkinson, M. R., and S. H. Fisher.** 1991. Identification of genes and gene products whose expression is activated during nitrogen-limited growth in *Bacillus subtilis*. J. Bacteriol. **173:**23–27.
2. **Atkinson, M. R., L. V. Wray, Jr., and S. H. Fisher.** 1990. Regulation of histidine and proline degradation enzymes by amino acid availability in *Bacillus subtilis*. J. Bacteriol. **172:**4758–4765.
3. **Belitsky, B. R., L. V. Wray, Jr., S. H. Fisher, D. E. Bohannon, and A. L. Sonenshein.** 2000. Role of TnrA in nitrogen source-dependent repression of *Bacillus subtilis* glutamate synthase gene expression. J. Bacteriol. **182:**5939–5947.
4. **Biaudet, V., F. Samson, C. Anagnostopoulos, S. D. Erlich, and P. Bessières.** 1996. Computerized map of *Bacillus subtilis.* Microbiology **142:**2669–2729.
5. **Bonthron, D. T.** 1990. L-Asparaginase II of *Escherichia coli* K-12: cloning, mapping and sequencing of the *ansB* gene. Gene **91:**101–105.
6. **Bryan, E. M., B. W. Beall, and C. P. Moran, Jr.** 1996. A σ^E-dependent operon subject to catabolite repression during sporulation in *Bacillus subtilis*. J. Bacteriol. **178:**4778–4786.
7. **Chambers, S. P., S. E. Prior, D. A. Barstow, and N. P. Minton.** 1988. The pMTL *nic*$^-$ cloning vectors. I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. Gene **68:**139–149.
8. **Chen, J. D., and D. A. Morrison.** 1988. Construction and properties of a new insertion vector, pJDC9, that is protected by transcriptional terminators and useful for cloning of DNA from *Streptococcus pneumoniae*. Gene **64:**155–164.
9. **Dunlop, P. C., G. M. Meyer, D. Ban, and R. J. Roon.** 1978. Characterization of two forms of asparaginase in *Saccharomyces cerevisiae*. J. Biol. Chem. **253:**1297–1304.
10. **Fisher, S. H.** 1999. Regulation of nitrogen metabolism in *Bacillus subtilis*: vive la différence! Mol. Microbiol. **32:**223–232.
11. **Fisher, S. H., and L. V. Wray, Jr.** 1989. Regulation of glutamine synthetase in *Streptomyces coelicolor.* J. Bacteriol. **171:**2378–2383.
12. **Grundy, F. J., A. J. Turinsky, and T. M. Henkin.** 1994. Catabolite regulation of *Bacillus subtilis* acetate and acetoin utilization genes by CcpA. J. Bacteriol. **176:**4527–4533.
13. **Guérout-Fleury, A.-M., K. Shazand, N. Frandsen, and P. Stragier.** 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene **167:**335–336.
14. **Helman, J. D.** 1995. Compilation and analysis of *Bacillus subtilis* σ^A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. Nucleic Acids Res. **23:**2351–2360.
15. **Hueck, C. J., W. Hillen, and M. H. Saier.** 1994. Analysis of *cis*-active sequence mediating catabolite repression in Gram-positive bacteria. Res. Microbiol. **145:**503–518.
16. **Itaya, M.** 1992. Construction of a novel tetracycline resistance gene cassette useful as a marker on the *Bacillus subtilis* chromosome. Biosci. Biotech. Biochem. **56:**685–686.
17. **Kraus, A., C. Hueck, D. Gärtner, and W. Hillen.** 1994. Catabolite repression of the *Bacillus subtilis xyl* operon involves a *cis* element functional in the context of an unrelated sequence, and glucose exerts additional *xylR*-dependent repression. J. Bacteriol. **176:**1738–1745.
18. **Kumano, M., A. Tamakoshi, and K. Yamane.** 1997. A 32 kb nucleotide sequence from the region of the lincomycin-resistance gene (22°-25°) of the *Bacillus subtilis* chromosome and identification of the site of the *lin-2* mutation. Microbiology **143:**2775–2782.
19. **Minton, N. P., H. M. S. Bullman, M. D. Scawen, T. Atkinson, and H. J. Gilbert.** 1986. Nucleotide sequence of the *Erwinia chrysanthemi* NCPPB 1066 L-asparaginase gene. Gene **46:**25–35.
20. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. **119:**736–747.
21. **Ratnyke-Lecamwassam, M., P. Serror, K.-W. Wong, and A. L. Sonenshein.** 2001. *Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels. Genes Dev. **15:**1093–1103.
22. **Reitzer, L. J.** 1996. Sources of nitrogen and their utilization. p. 380–390. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. American Society for Microbiology, Washington, D.C.
23. **Schreier, H. J., S. W. Brown, K. D. Hirschi, J. F. Nomellini, and A. L. Sonenshein.** 1989. Regulation of *Bacillus subtilis* glutamine synthetase gene expression by the product of the *glnR* gene. J. Mol. Biol. **210:**51–63.
24. **Stülke, J., and W. Hillen.** 1999. Carbon catabolite repression in bacteria. Curr. Opin. Microbiol. **2:**195–201.
25. **Sun, D., and P. Setlow.** 1991. Cloning, nucleotide sequence, and expression of the *Bacillus subtilis ans* operon, which codes for L-asparaginase and L-aspartase. J. Bacteriol. **173:**3831–3845.
26. **Sun, D., and P. Setlow.** 1993. Cloning and nucleotide sequence of the *Bacillus subtilis ansR* gene, which encodes a repressor of the *ans* operon coding for L-asparaginase and L-aspartase. J. Bacteriol. **175:**2501–2506.
27. **Tjalsma, H., A. Bolhuis, J. D. H. Jongbloed, S. Bron, and J. M. van Dijl.** 2000. Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. Microbiol. Mol. Biol. Rev. **64:**515–547.
28. **Waldor, M. K., E. J. Rubin, G. D. N. Pearson, H. Kimsey, and J. J. Mekalanos.** 1997. Regulation, replication, and integration functions of the *Vibrio cholerae* CTXφ are encoded by region RS2. Mol. Microbiol. **24:**917–926.
29. **Wong, B. L., and C. R. Shobe.** 1974. Single-step purificationof urease by affinity chromatography. Can. J. Microbiol. **20:**623–630.
30. **Wray, L. V., Jr., A. E. Ferson, and S. H. Fisher.** 1997. Expression of the *Bacillus subtilis ureABC* operon is controlled by multiple regulatory factors including CodY, GlnR, TnrA, and Spo0H. J. Bacteriol. **179:**5494–5501.
31. **Wray, L. V., Jr., A. E. Ferson, K. Rohrer, and S. H. Fisher.** 1996. TnrA, a transcriptional factor required for global nitrogen regulation in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **93:**8841–8845.
32. **Wray, L. V., Jr., F. K. Pettengill, and S. H. Fisher.** 1994. Catabolite repression of the *Bacillus subtilis hut* operon requires a *cis*-acting site located downstream of the transcription initiation site. J. Bacteriol. **176:**1894–1902.
33. **Wray, L. V., Jr., J. M. Zalieckas, A. E. Ferson, and S. H. Fisher.** 1998. Mutational analysis of the TnrA-binding sites in the *Bacillus subtilis nrgAB* and *gabP* promoter regions. J. Bacteriol. **180:**2943–2949.
34. **Wray, L. V., Jr., J. M. Zalieckas, and S. H. Fisher.** 2000. Purification and *in vitro* activities of the *Bacillus subtilis* TnrA transcription factor. J. Mol. Biol. **300:**29–40.
35. **Wray, L. V., Jr., J. M. Zalieckas, and S. H. Fisher.** 2001. *Bacillus subtilis* glutamine synthetase controls gene expression through a protein-protein interaction with transcription factor TnrA. Cell **107:**427–435.
36. **Ye, R. W., W. Tao, L. Bedzyk, T. Young, M. Chen, and L. Li.** 2000. Global gene expression profiles of *Bacillus subtilis* grown under anaerobic conditions. J. Bacteriol. **182:**4458–4465.
37. **Zeng, X., A. Galiner, and H. H. Saxild.** 2000. Catabolite repression of *dra-nupC-pdp* operon expression in *Bacillus subtilis*. Microbiology **146:**2901–2908.