Cloning, Nucleotide Sequence, and Expression of the Bacillus subtilis ans Operon, Which Codes for L-Asparaginase and L-Aspartase

DONGXU SUN AND PETER SETLOW*

Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06030

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L-Aspartase was purified from Bacillus subtilis, its N-terminal amino acid sequence was determined to construct a probe for the aspartase gene, and the gene (termed ansB) was cloned and sequenced. A second gene (termed ansA) was found upstream of the ansB gene and coded for L-asparaginase. These two genes were in an operon designated the ans operon, which is 80% cotransformed with the previously mapped aspHI mutation at 215°. Primer extension analysis of in vivo ans mRNA revealed two transcription start sites, depending on the growth medium. In wild-type cells in log-phase growth in 2× YT medium (tryptone-yeast extract rich medium), the ans transcript began at −67 relative to the translation start site, while cells in log-phase growth or sporulating (t4 to t5) in 2× SG medium (glucose nutrient broth-based moderately rich medium) had an ans transcript which began at −73. The level of the −67 transcript was greatly increased in an aspH mutant grown in 2× YT medium; the −67 transcript also predominated when this mutant was grown in 2× SG medium, although the −73 transcript was also present. In vitro transcription of the ans operon by RNA polymerase from log-phase cells grown in 2× YT medium and log-phase or sporulating cells grown in 2× SG medium yielded only the −67 transcript. Depending on the growth medium, the levels of asparaginase and aspartase were from 2- to 40-fold higher in an aspH mutant than in wild-type cells, and evidence was obtained indicating that the gene defined by the aspHI mutation codes for a trans-acting transcriptional regulatory factor. In wild-type cells grown in 2× SG medium, the levels of both aspartase and asparaginase decreased significantly by t6 of sporulation but then showed a small increase, which was mirrored by changes in the level of β-galactosidase from an ansB-lacZ fusion. The increase in the activities of ans operon enzymes between t4 and t5 of sporulation was found primarily in the forespore, and the great majority of the increased was found in the mature spore. However, throughout sporulation the only ans transcript detected was the −73 form, and no sporulation-specific RNA polymerase tested yielded a −73 transcript in vitro.

Aspartase catalyzes the conversion of L-aspartate to fumarate and ammonium ion. This enzyme has been purified from several gram-negative bacteria, and its gene has been cloned and sequenced (17, 38–40). In Bacillus subtilis, aspartase is present in vegetatively growing cells, and its synthesis is induced by aspartate (13). Not surprisingly, aspartase is required for slow growth on aspartate as the sole carbon source. A mutant of B. subtilis (aspHI) which grows rapidly on aspartate has been isolated, and the aspartase level in this mutant is much higher than that in wild-type cells (13). In addition to its presence in vegetatively growing cells of Bacillus species, aspartase has also been found in sporulating cells. Strikingly, aspartase in sporulating cells is found almost exclusively in the forespore compartment, with little if any enzyme in the mother cell (2, 3). Given the current interest in mechanisms responsible for forespore-specific gene expression during sporulation, we decided to clone and sequence the aspartase gene from B. subtilis to facilitate studies on the regulation of expression of this gene. In this communication, we describe the purification of aspartase from B. subtilis and the cloning and nucleotide sequencing of its gene. We further show that this gene and the gene encoding L-asparaginase are in the same operon and describe studies of the regulation of expression of this operon.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this work are listed in Table 1. The SS-Na medium used for growing cells with aspartase as the sole carbon source was prepared as previously described (13). In some experiments, cells were grown at 37°C in basal salts solution minimal medium (5) with glucose (0.5%) as a carbon source and 10 mM NaCl (BSG medium) and containing NH₄Cl (0.4%), aspartate (0.2%), or asparagine (0.2%) as a nitrogen source. The medium used for sporulation experiments was 2× SG (a glucose nutrient broth-based moderately rich medium) (19).

Purification and sequence analysis of aspartase. Cells of strain PS935 (aspHI) were grown at 37°C in 10 liters of 2× YT medium (a tryptone-yeast extract rich medium) (20) and harvested at an optical density at 600 nm (OD₆₀₀) of ~4 by centrifugation (10 min; 10,000 × g). The cells were washed twice with 50 mM Tris-HCl (pH 7.5), and the pellet was frozen and stored at −60°C. Frozen cells (20 g) were thawed and resuspended in 200 ml of cold 50 mM Tris-HCl (pH 7.5)–1 mM dithiothreitol–1 mM EDTA–1 mM phenylmethylnitrobenzenesulfonyl fluoride. The cells were broken by two passes through a French pressure cell at 10,000 lb/in², and the suspension was centrifuged (10 min; 14,000 × g) to remove cell debris. To each 100 ml of the supernatant fraction obtained, 10 ml of 11% (wt/vol) streptomycin sulfate was added, with stirring. After the suspension was allowed to stand for 20 min, the precipitate was removed by centrifug-
gation (25 min; 10,000 × g). Solid ammonium sulfate was added, with stirring, to the supernatant fraction to 55% saturation. After 30 min of stirring, the sample was again centrifuged (25 min; 10,000 × g), the pellet was dissolved in 100 ml of buffer I (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride), and the sample was dialyzed overnight at 4°C against two 1-liter changes of buffer I. The dialyzed sample was applied to a DEAE-cellulose column (2.5 by 10 cm) equilibrated with buffer I, and proteins were eluted with a linear KCl gradient (0 to 1 M; 250 ml of each solution) in the same buffer; 4-ml fractions were collected. Fractions with high aspartase activity were pooled and dialyzed overnight at 4°C against 2 liters of buffer I containing 0.1 M KCl and 5% glycerol, and proteins were precipitated with ammonium sulfate at 55% saturation as described above. The pellet was dissolved in 2 ml of buffer I containing 0.01 M KCl and 5% glycerol and loaded on a Sephacryl S-300 gel filtration column (1.3 by 60 cm) equilibrated with the same buffer, and 1-ml fractions were collected. Fractions with high aspartase activity were pooled and loaded on a Red-A agarose (Amicon) column (1.2 by 5 cm) equilibrated with buffer I containing 0.05 M KCl, 10 mM MgCl₂, and 5% glycerol. After a wash with 20 ml of this buffer, aspartase was eluted with a KCl gradient of 0.05 to 0.35 M. Fractions (2 ml) were assayed for aspartase activity, aliquots were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was silver stained (23).

For determination of the N-terminal amino acid sequence of aspartase, ~0.3 mg of Red-A agarose-purified enzyme was run on an 8% SDS-polyacrylamide gel. The proteins were transferred to polyvinylidine difluoride paper (Immobilon; Millipore Corp.), the paper was lightly stained with Coomassie blue, and the aspartase band was cut out and sequenced as previously described (35).

**Enzyme assays.** The two methods used in this work for measuring aspartase activity were essentially those described by Ijima et al. (13). The assay measuring the production of fumarate by its A₂₄₀ was used during aspartase purification. The assay measuring ammonia production was done as follows. Cells in 1 ml of 50 mM Tris-HCl (pH 7.5) were vortexed with 40 μl of toluene, and aspartase activity in the treated cells was determined by measuring the production of ammonia with an ammonia color reagent (Sigma). One unit of aspartase was defined as the amount of enzyme that produced 1 nmol of fumarate (or ammonia) in 1 min at 30°C. Preliminary experiments showed that similar levels of aspartase activity were found with either French press-disrupted cells or toluene-permeabilized cells (data not shown).

Asparaginase was assayed by determining ammonia production with cells treated with toluene as described above. The reaction mixture (0.5 ml) contained 0.1 M borate-NaOH buffer (pH 9.3), 10 mM EDTA, and 20 mM L-asparagine; the reaction was started by the addition of the proper amount of toluene-treated cells. After 10 to 30 min at 30°C, the reaction was stopped by the addition of an equal volume of ammonium color reagent (Sigma), and the mixture was centrifuged for 1 min in a microcentrifuge. The optical density of the supernatant fractions was measured at 420 nm (when activity was low) or at 470 nm (when activity was high) within 5 min of centrifugation. One unit of asparaginase was defined as the amount of enzyme that produced 1 nmol of ammonia in 1 min.

β-Galactosidase and glucose dehydrogenase were assayed as described previously (22). In experiments measuring β-galactosidase in sporulating cells at hour 20 of sporulation (t₂₀), cells were assayed first following direct lysozyme treatment to determine mother cell activity and then again after decoating of spores and lysozyme treatment to determine forespore activity (22). In experiments measuring aspartase and asparaginase activities in spores, spores were decoated and lysed as described above, and the lysate was passed through a tuberculin syringe needle to reduce its viscosity and dialyzed against buffer I plus 20% glycerol prior to assays. Protein was determined by the method of Lowry et al. (21).

**Cloning of the ans operon.** A homologous probe to detect the aspartase gene was prepared by the polymerase chain reaction (PCR). Two oligonucleotides, representing the degenerate coding sequences of residues 3 to 8 and 17 to 22 of aspartase, were used as primers (see Fig. 2A); *B. subtilis* 168 DNA (50 ng) was used as a template. Other reagents were obtained in a kit (GeneAmp) from the Perkin-Elmer Cetus Corp. and were used in accordance with the manufacturer’s instructions. The PCR was run for 30 cycles with the following parameters: denaturation, 1 min at 94°C; anneal-
ing, 1 min at 45°C for the first cycle and at 50°C thereafter; and elongation, 3 min at 72°C. The PCR product was run on a 3% low-melting-temperature agarose gel, the band of the correct size was cut out, and the DNA was extracted. A small portion (0.2% of the total) of this DNA was used as a template for a second PCR run as described above for 20 cycles but with [32P]dATP (0.05 mCi) at 2 μM and dGTP, dCTP, and dTTP each at 200 μM. The labeled DNA product was purified by phenol extraction and ethanol precipitation. With this PCR probe, the initial fragment cloned was a 2-kb PstI fragment cloned in plasmid pUC18 and identified by colony hybridization in Escherichia coli JM83 (20). With this 2-kb PstI fragment as a probe, we further cloned in pUC18 a new 600-bp HindII fragment that contained 300 bp of a new sequence upstream from the 2.0-kb PstI fragment. Finally, with this 600-bp HindII fragment as a probe, we cloned in pUC18 a 1-kb PstI fragment that contained the promoter region of the aspartase gene.

The 3′ end of the aspartase gene was cloned by an inverse PCR (28) after repeated failures of direct cloning. A forward primer (18-mer; 5′-CACAGGGCAATCTGTCGCG 3′; corresponding to positions 2678 to 2695 in Fig. 3) and a backward primer (20-mer; 3′-ACTAGTGTCGACAGTAAAGGC 5′; corresponding to the complementary strand of positions 2610 to 2629 in Fig. 3) were synthesized on the basis of the sequence of the 2-kb PstI fragment near the 3′ end. Chromosomal DNA was cut with HaeIII or RsaI and ligated at low concentrations (10 μg/ml) to promote the formation of circular molecules. The ligated DNAs (30 ng) were phenol extracted and used as templates in a PCR (20 cycles) run as described above but with an annealing temperature of 55°C, and the products were run on a 1.5% low-melting-temperature agarose gel. PCR products of correct sizes were cut out, phenol extracted, ethanol precipitated, and used directly for nucleotide sequencing. On the basis of the DNA sequence thus obtained, another primer, termed the downstream primer (20-mer; 3′-CCTAATCTTACACGGTGTG 5′), was synthesized; this primer corresponded to the complementary strand of positions 2995 to 2974 (not shown in Fig. 3). This primer and the forward primer were used in a standard PCR with uncut chromosomal DNA as a template. The correct DNA product was extracted following agarose gel electrophoresis and directly sequenced from both directions with the primers described above for the PCR.

**DNA sequencing.** The nucleotide sequence of the ans operon was determined by the chain termination method (30) with a Sequenase kit (U.S. Biochemicals) in accordance with the manufacturer’s instructions. DNAs were routinely subcloned in pUC18 or pUC19 prior to being sequenced. Double-stranded plasmid DNAs purified by CsCl gradient centrifugation were directly sequenced with commercially available primers or primers made on the basis of known ans sequences. For sequencing the PCR products, 7 μl of gel-purified DNA dissolved in water was mixed with 5 μl of primer (10 ng/μl) and 3 μl of 5× sequencing buffer (U.S. Biochemicals). The mixture was boiled for 10 min and immediately transferred to ice-cold water to obtain maximum annealing between the primer and the template. After being cooled, the mixture was allowed to stand at room temperature for 2 min prior to the initiation of sequencing reactions.

**The construction of ans-lacZ fusion strains.** For the construction of an ans-lacZ fusion, the 0.8-kb EcoRI fragment was isolated from plasmid pPS1304 (see Fig. 2). This fragment contains 28 codons from the N terminus of ansB, two-thirds of the ansA coding region, and the PstI-EcoRI portion of the pUC18 polylinker and was ligated into the EcoRI site of plasmid pJF751 (8) to generate a translational ansB-lacZ fusion. Transformants were isolated in Escherichia coli RR101, and a clone with the insert in the correct orientation was identified by digestion with MluI. The resulting plasmid, pPS1311, was transformed into B. subtilis strains as previously described (22), and colonies resistant to chloramphenicol (3 μg/ml) were isolated. These transformants contained plasmids which had been integrated into the chromosome through a Campbell-type recombination, as confirmed by Southern blot analysis of chromosomal DNA (data not shown). Note that in these ansB-lacZ fusion strains, the ansB gene has been interrupted; therefore, these strains are AnsB−.

A translational ansA-lacZ fusion was constructed by cloning the 0.6-kb HindII fragment of pPS1366 (see Fig. 2) into the Smal site of pJF751. Transformants were isolated in E. coli RR101, and a clone with the plasmid in the correct orientation was identified by MluI digestion. The resulting plasmid, pPS1364, was transformed into B. subtilis as described above, and its correct integration was confirmed by Southern blot analysis of chromosomal DNA from one transformant. Since the 0.6-kb HindII fragment lacks the promoter region for the ans operon (see Fig. 2), these ansA-lacZ fusion strains are both AnsA− and AnsB−. While it was clear from the Southern blot analysis that this pJF751 derivative had been integrated into the ansA gene, the behavior of this fusion, in particular, the levels of β-galactosidase produced in wild-type strains, was not reproducible, and it is possible that some alteration in the promoter region had taken place.

**In vitro transcription.** RNA polymerase containing α4 was purified from vegetative cells of strain PS832 (wild type) grown in 2× YT medium as previously described (31, 35). RNA polymerase was also purified from strains PS683 (spoIII) and PS832 grown in 2× SG medium and harvested at t5 of sporulation (35). In addition to RNA polymerase containing α4 (Es4), the enzyme preparations from the latter two strains also contained Es9 (PS683) and Es9 plus EsG (PS832) (35). Plasmid pPS1521 (1 μg), in which the 0.7-kb PstI-HindII fragment of pPS1367 was cloned between the PstI and HindII sites of pUC19, was cut with various restriction enzymes and used as a template in a runoff transcription assay carried out as previously described (33). The labeled nucleotide used was [α-32P]dATP. Runoff transcription products were analyzed by electrophoresis on a 6% polyacrylamide sequencing gel, and their sizes were estimated by comparison of their mobilities with those of RNA size markers as described previously (33).

**Primer extension.** RNA was extracted from vegetative cells or sporulating cells grown in either 2× YT or 2× SG medium and purified as described previously (22). A synthetic 25-mer oligonucleotide (3′-GTCACACTTCCCCCTTAAAACCGGCG 5′) complementary to codons 15 to 25 of the ansA gene was used as a primer. Labeling of the primer and primer extension analysis with equal amounts of RNA were performed as previously described (27). The sizes of the extended products were determined by comparison with a DNA sequencing ladder obtained from plasmid pPS1367 with the same primer (27).

**Nucleotide sequence accession number.** The B. subtilis ans sequence has been assigned GenBank accession number M63264.
TABLE 2. Purification of *B. subtilis* aspartase$^a$

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$^a$ Aspartase was purified from 20 g of frozen cells as described in Materials and Methods.

RESULTS

**Purification of aspartase.** Aspartase was purified from the *aspH* mutant of *B. subtilis*, since this strain produces significantly more enzyme activity than does the wild-type strain (Table 2) (9). After the gel filtration step, the partially purified enzyme was run on Red-A agarose, a resin that has a high affinity for *E. coli* aspartase (17). However, we were unable to elute *B. subtilis* aspartase from Red-A agarose with L-aspartate (up to 10 mM), in contrast to *E. coli* aspartase; consequently, a KCl gradient (0.05 to 0.35 M) was used. The enzyme activity eluted from this column in a rather broad peak (Fig. 1A) which coeluted with a band of about 51 kDa, as seen by SDS-PAGE (Fig. 1B). Since the distribution of this 51-kDa band paralleled the aspartase activity and since the subunit molecular weight of aspartase from both *E. coli* and *Pseudomonas fluorescens* is 50 to 52 kDa (38, 40), we tentatively assumed that the 51-kDa band was aspartase. Consequently, a large aliquot of the enzyme run on Red-A agarose was run on an SDS-polyacrylamide gel, and the 51-kDa band was subjected to protein sequence analysis. The sequence of the first 22 amino acid residues (Fig. 2A) showed significant homology with the amino-terminal sequences of aspartases from *E. coli* and *P. fluorescens* (see below), further suggesting that the 51-kDa band was indeed *B. subtilis* aspartase.

**Cloning of the ans operon.** To make a probe for cloning the *B. subtilis* aspartase gene, we synthesized two oligonucleotides based on the amino-terminal amino acid sequence of aspartase (Fig. 2A). These oligonucleotides were used in a PCR with *B. subtilis* chromosomal DNA as a template; the initial PCR product was used as a template in a second PCR to make a probe. Hybridization of this probe to Southern blots of *B. subtilis* DNA digested with one or two restriction enzymes showed that there was only one sequence in the genome that hybridized to the probe (data not shown) and allowed the initial cloning of a 2-kb *PstI* fragment (pPS1304; Fig. 2B). This 2-kb fragment contained a sequence coding for 444 amino acids of aspartase and lacking only a small region at the carboxy-terminal end (Fig. 3). The region upstream of the aspartase coding sequence showed no apparent promoter sequences but rather a second open reading frame (Fig. 3). Consequently, we cloned this upstream region by chromosomal walking and eventually cloned and sequenced an additional 1 kb (Fig. 2B). This additional 1 kb of upstream sequence contained the complete upstream open reading frame as well as the promoter for the aspartase gene (Fig. 2B and 3; also see below).

Attempts to clone the 3’ end of the aspartase gene were unsuccessful despite multiple efforts with different *E. coli* or *B. subtilis* plasmids as cloning vectors. Therefore, we synthesized DNA fragments containing the 3’ end of the gene through an inverse PCR, in which chromosomal DNA cut with HaeIII or Rsal was religated under dilute conditions and used as a template in a PCR to produce linear DNA products. Southern blot analysis of HaeIII- or Rsal-digested chromosomal DNA with the 260-bp *NdeI*-*PstI* fragment (Fig. 2B) as a probe (data not shown) predicted that an inverse PCR with HaeIII- or Rsal-cut DNA should generate 550- or 850-bp fragments, respectively. Fragments of these sizes were obtained from the inverse PCR and directly sequenced. Using the sequence thus obtained as a guide, we

![Figure 1](http://jb.asm.org/)

**FIG. 1.** Chromatography of aspartase on Red-A agarose (A) and analysis of column fractions by SDS-PAGE (B). (A) Enzyme from the Sephacryl S-300 step in the aspartase purification was applied to a Red-A agarose column, eluted with a KCl gradient (broken line), and assayed (closed circles) as described in the text. (B) Aliquots (5 μl) of fractions (numbers at top) eluted from the Red-A agarose column in (panel A) were run on 8% SDS-PAGE and silver stained. The labeled horizontal arrows indicate the migration positions of molecular mass markers.
suggested an additional primer to use in a normal PCR with uncut chromosomal DNA as a template to generate a 296-bp product, which was also sequenced (Fig. 2B). This fragment contained the missing C terminus of the aspartase gene as well as a potential transcription terminator (see below).

**Nucleotide sequence of the ans operon.** The nucleotide sequence of both open reading frames as well as significant flanking DNA was determined in both directions (Fig. 3). For PCR products, at least two independently prepared PCR fragments were sequenced in both directions to avoid possible (although rare in bulk PCR products) errors introduced by the TaqI polymerase. The gene coding for aspartase was located between nucleotides 1379 and 2806 and encoded 475 amino acid residues with a calculated molecular weight of 52,486. This size is in good agreement with the size of aspartase determined by SDS-PAGE.

The open reading frame upstream of aspartase was analyzed by comparing its amino acid sequence with bacterial polypeptide sequences in GenBank. A significant homology was found between the open reading frame and the asparagusine from *Erwinia chrysanthemi* (data not shown). The unknown polypeptide also showed significant identity (25 to 30%) with the two asparaginases from *E. coli* (see below). These results suggested that this open reading frame might encode *B. subtilis* asparaginase (see below). Since the coding sequences for both asparaginase and aspartase have only 44 nucleotides between them, we tentatively surmised that the asparaginase and aspartase genes are in the same operon. As asparaginase operons in *E. coli* and *E. chrysanthemi* have been termed *ans* operons, we have designated this *B. subtilis* operon the *ans* operon. The asparaginase and aspartase genes are termed *ansA* and *ansB*, respectively.

Analysis of the nucleotide sequence at the 5′ end of the *ansA* gene revealed a perfect −10 region for σ70 recognition at positions 267 to 272 and a less conserved −35 region for σ70 recognition at positions 244 to 249 (26) (singly underlined regions in Fig. 3). At the 3′ end of the *ansB* gene, a region of dyad symmetry with a calculated ΔG of −27.6 kcal/mol (ca. −115.5 kJ/mol) was found between positions 2821 and 2860 (Fig. 3). This sequence was followed by two T residues and

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**FIG. 2.** N-terminal amino acid sequence of aspartase and primers used for PCR to generate a probe for the N-terminal coding region of the aspartase gene (A) and organization and restriction map of the *ans* operon (B). (A) The amino acid sequence of the N terminus of *B. subtilis* aspartase determined by automated sequence analysis is given in the one-letter code. Also shown are the two degenerate oligonucleotides synthesized on the basis of the amino acid sequence and subsequently used in a PCR to synthesize a probe for the aspartase gene. (B) The thick line is the region of the *ans* operon sequenced completely and shown in Fig. 3. Restriction enzyme cleavage sites are shown above this thick line: A, AccI; E, EcoRI; H, HindIII; Ha, HaeIII; Hc, HincII; Hp, HpaI; R, RsaI; M, MluI; N, NaeI; S, SstI; H, HindIII; B, BclI; R, RsaI; and X, XbaI. The large open arrows represent the coding regions of the *ansA* and *ansB* genes, from N to C termini. Plasmids pPS1304, pPS1366, and pPS1367 represent *PstI*, HindIII-HindIII, and *PstI* fragments, respectively, cloned in pUC18 or pUC19 as described in the text. The PCR-generated fragment contains the C-terminal coding region of the aspartase gene and was generated as described in the text.
The nucleotide sequence of the ans operon and flanking regions and predicted coding sequences for asparaginase (ansA) and aspartase (ansB). The numbers to the left of each line give the number of the first nucleotide in that line. The amino acid sequences predicted from the nucleotide sequences are given in the one-letter code, with the residue above the base relative to each codon. Asterisks denote stop codons. Sequences upstream of ansA which exhibit significant or perfect homology with the consensus -35 and -10 recognition sequences of σ^70-dependent promoters (22) are singly underlined. Sequences prior to translation start sites which exhibit significant homology with the 3′ end of 16S rRNA and which are likely to be ribosome binding sites are doubly underlined. The inverted repeat sequence with opposing arrowheads below the sequence is in boldface and underlined. The numbers to the right of each line denote the first nucleotide of the inverted repeat sequence. Some nucleotides are capitalized to denote significant homology.

**FIG. 3.** Nucleotide sequence of the ans operon. The inverted repeat sequence with opposing arrowheads below the sequence is in boldface and underlined. The numbers to the right of each line denote the first nucleotide of the inverted repeat sequence. Some nucleotides are capitalized to denote significant homology.

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The inverted repeat sequence with opposing arrowheads below the sequence is in boldface and underlined. The numbers to the right of each line denote the first nucleotide of the inverted repeat sequence. Some nucleotides are capitalized to denote significant homology.

The nucleotide sequence of the ans operon and flanking regions and predicted coding sequences for asparaginase (ansA) and aspartase (ansB). The numbers to the left of each line give the number of the first nucleotide in that line. The amino acid sequences predicted from the nucleotide sequences are given in the one-letter code, with the residue above the base relative to each codon. Asterisks denote stop codons. Sequences upstream of ansA which exhibit significant or perfect homology with the consensus -35 and -10 recognition sequences of σ^70-dependent promoters (22) are singly underlined. Sequences prior to translation start sites which exhibit significant homology with the 3′ end of 16S rRNA and which are likely to be ribosome binding sites are doubly underlined. The inverted repeat sequence downstream of ansB which may be a transcription stop signal is marked with opposing arrowheads below the sequence. The two 5′ ends of ans mRNA found in vivo at -67 and -73 relative to the translation start site of ansA are in boldface type.
containing chloramphenicol (3 μg/ml) and assayed for asparaginase activity; 39 (78%) had wild-type asparaginase levels, while 11 (22%) had levels of the enzyme similar to that in PS935 (aspHI). Likewise, when PS1316 (ansB-lacZ aspHI Cm') was used as the donor of chromosomal DNA to transform PS832 (wild type), 82% of the Cm' transformants showed the AspH phenotype, as judged by their high levels of asparaginase, and 18% remained wild type. It appears that the aspHI mutation and the ans operon are closely linked, with a cotransformation frequency of about 80%. This value indicates that the ansB gene is at least 1.5 to 3 kb away from the aspHI mutation (11) and suggests that the aspHI mutation is not in the promoter for the ans operon (see below). This suggestion is consistent with the aspHI mutation defining a trans-acting factor involved in the regulation of the ans operon (see below).

Evidence that ansA and ansB are in an operon. As noted above, analysis of the nucleotide sequence of ansA and ansB was consistent with these genes being in an operon. Analysis of the levels of both enzymes in the aspHI mutant was consistent with this model, as both aspartase and asparaginase activities were elevated 7- to 10-fold in the aspHI mutant grown in rich medium (Table 3). Similarly, integration of a plasmid into the ansB gene inactivated aspartase but not asparaginase, while integration of a plasmid into ansA inactivated both asparaginase and aspartase (Table 3). Note that while the latter integration strain carried a nonfunctional ansA gene, it also carried a complete ansB gene. Since the latter was not expressed, the promoter of the ansB gene is probably upstream of the ansA gene. While plasmid integration into ansB abolished aspartase synthesis and plasmid integration into ansA abolished the synthesis of both aspartase and asparaginase, strains PS1313 (AnsB') and PS1362 (AnsA^- AnsB') grew normally in 2× YT medium and grew and sporulated normally in 2× SG medium. However, neither strain grew well, if at all, on S5-Na plates, in which L-aspartate is the sole carbon source. Similarly, strain PS1362 (AnsA^- AnsB') could not grow with asparagine as the sole carbon source, and this strain grew poorly, if at all, with asparaginase as the sole nitrogen source.

Further evidence that ansA and ansB are cotranscribed as an operon came from a determination of the 5' ends of ans mRNA by primer extension analysis. Despite repeated attempts with several primers, no mRNA with a 5' end to 300 nucleotides upstream of ansB was detected at any stage of growth in different media (data not shown). However, an mRNA with a 5' end just upstream of ansA was readily detected (Fig. 4). Indeed, two different 5' ends were found depending on the growth medium. Cells in log-phase growth in 2× YT medium contained only mRNA whose 5' end was at -67 relative to the translation start site (Fig. 3 and 4). In contrast, cells in 2× SG medium, whether in log-phase growth or at t₀, t₂, or t₅ of sporulation (Fig. 4 and data not shown),...
FIG. 5. Analysis of transcripts produced in vitro from the ans operon. Plasmid pPS1521 was cut with the appropriate restriction enzymes and transcribed in vitro with RNA polymerase, and transcripts were analyzed by gel electrophoresis as described in Materials and Methods. (A) Sequence of the ans operon in the region of the in vitro transcription start site (residues 268 to 347 in Fig. 3). The two T residues in larger boldface type represent the 5' ends of ans mRNAs found in vivo. The sequence was taken from Fig. 3. The doubly underlined ATG is the translation start codon, and the singly underlined region is the ribosome binding site. The horizontal arrow over the second boldfaced T residue indicates the only in vitro transcription start site found. The symbols used for different DNA regions were as follows: ■, plasmid DNA; ◊, plasmid polylinker; □, DNA upstream of the ansA coding sequence; ◊, ansA coding sequence. The sizes of in vitro transcripts from Smal (S)- or EcoRI (E)-cut plasmid pPS1521 are those expected if the T residue marked by the arrow is the transcription start site. (B) Autoradiogram of in vitro transcripts on an ansA template. Plasmid pPS1521 was cut with EcoRI (E) or Smal (S) and transcribed in vitro with RNA polymerase from log-phase cells of strain PS832 grown in 2× YT medium (ErA) or t3 sporulating cells of strain PS83 grown in 2× SG medium (Et3), and transcripts were analyzed by gel electrophoresis and detected by autoradiography. The sizes adjacent to the lanes are the sizes of RNA markers. nt, nucleotides.

shown), contained a slightly longer mRNA beginning at -73. However, in vitro transcription of ans operon DNA by RNA polymerase from cells grown in either 2× YT or 2× SG medium, including enzyme from sporulating cells, yielded only a transcript beginning at -67 (Fig. 5). No transcript beginning at -73 was detected, even when gels were run for times much longer than those shown in Fig. 5 and with smaller amounts of sample to preclude the possibility that the strong -67 transcript was masking the presence of the -73 transcript (data not shown). The RNA polymerase preparations tested included enzyme from vegetative cells containing predominantly EoA (Fig. 5B) as well as enzyme isolated at t3 of sporulation from both the wild-type strain and strain PS683 (spoIIIGA) (Et3; Fig. 5B; also, data not shown). Note that the t3 RNA polymerase preparations contained holoenzymes with several sporulation-specific σ factors (i.e., EoF and EoG in the wild-type strain and EoH in strain PS683) in addition to EoA. Since Et3 yielded the same pattern of ans transcripts as did EoA (Fig. 5B), it appears likely that EoA is the only holoenzyme transcribing ans DNA in vitro (see Discussion). With none of the RNA polymerase preparations tested, including the t3 enzyme, was a transcript observed beginning just upstream of ansB when an ansB template was used for in vitro transcription (data not shown).

Effect of the aspH1 mutation on ans operon expression. As noted above, the aspH1 mutation elevated levels of both asparaginase and aspartase (Table 3). This mutation also increased β-galactosidase expression from ansA-lacZ fusions (data not shown) and ansB-lacZ fusions (Table 3). Analysis of the mRNA from the aspH1 mutant grown in either 2× YT or 2× SG medium showed that in 2× YT
medium, the level of the −67 transcript was greatly increased, and that in 2× SG medium, the level of this transcript was also high, although the −73 transcript was also present (albeit possibly at a slightly reduced level) (Fig. 4B, lanes 4 to 6). The increased ans mRNA levels in the aspHI mutant indicated that this mutation increases ans transcription and thus that the locus defined by the aspHI mutation is, or encodes, a transcriptional regulator. One explanation for the effects of the aspHI mutation is that it alters the promoter sequence of the ans operon. However, we have sequenced −500 nucleotides of the region upstream of the ans operons of both strains PS832 (wild type) and PS935 (aspHI) and have found no differences (data not shown). Indeed, the finding noted above that the aspHI and wild-type strains have identical nucleotide sequences in the 500 bp upstream of the ans operon, suggests that the aspHI mutation defines a more distant regulatory region than does the ans promoter, possibly one coding for a trans-acting transcriptional factor.

To test directly whether the effect of the aspHI mutation on the ans operon expression was cis or trans acting, we first attempted to construct strains with an ansA-lacZ fusion at the amyE locus. However, for unknown reasons, neither translational nor transcriptional ansA-lacZ fusions were expressed at the amyE locus (data not shown). Consequently, we analyzed the expression of the ans operon in wild-type and aspHI mutant strains in which the ans promoter was moved −7 kb away from any cis-acting upstream or downstream sequences (Fig. 6). Note that both the aspHI and wild-type strains have identical nucleotide sequences in the 500 bp upstream of the ans operon, as discussed above. Analysis of asparaginase levels in the ansB-lacZ fusion-carrying strains PS1313 (wild type) and PS1316 (aspHI) showed that the aspHI strain has eight times more asparaginase than did the wild-type strain (Table 3). Since the ans promoter in strain PS1316 was moved −7 kb away from all ans downstream sequences (Fig. 6A), this result indicates that the aspHI mutation is unlikely to define a cis-acting downstream site. Unfortunately, we could not use the ansA-lacZ fusion carried by strains PS1362 (wild type) and PS1477 (aspHI) for a similar analysis, because the ansA fragment used for the construction of the ansA-lacZ fusion was entirely within the ansA coding sequence. Consequently, PS1362 and PS1477 only carried a single ans promoter adjacent to all upstream B. subtilis DNA (Fig. 6B). Therefore, we constructed plasmid pPS1557, in which the 0.3-kb NaeI-HincII fragment containing the ans promoter and a small amount of the ansA N-terminal coding region (Fig. 3) was cloned in Smal-cut plasmid pJF751, placing the lacZ gene in-frame with the ansA gene and under the control of the ans promoter. Plasmid pPS1557 was then used to transform both wild-type and aspHI strains to chloramphenicol resistance through integration of the plasmid at the ans locus (Fig. 6C). Since the NaeI-HincII fragment used to create pPS1557 contains the ans promoter, the resulting B. subtilis transformants, strains PS1559 (wild type) and PS1560 (aspHI), contained two ans promoters (Fig. 6C), one for the ansA-lacZ fusion and adjacent to upstream B. subtilis DNA and a second for the intact ans operon and −7 kb downstream and immediately preceded by only about 200 bp of upstream B. subtilis DNA. Analysis of the asparaginase and aspartase levels in strains PS1559 (wild type) and PS1560 (aspHI) revealed that PS1560 had 7- to 10-fold-higher enzyme levels than did PS1559 (data not shown). This result indicates that the locus defined by the aspHI mutation can act on the ans promoter from a distance of at least 7 kb, again suggesting that the aspHI mutation defines a locus encoding a trans-acting factor regulating the transcription of the ans operon. Since there are only about 200 bp of B. subtilis DNA upstream of the intact ans operon in strain PS1560, this result further suggests that the target for the trans-acting factor defined by the aspHI mutation is within these 200 bp.

Levels of asparaginase and aspartase during growth and sporulation. As noted above, both asparaginase and aspartase were present in vegetative cells of wild-type B. subtilis. However, the enzyme levels varied significantly, depending on the growth medium, being highest in 2× YT medium, lower in 2× SG medium, and lowest in a minimal glucose medium with ammonia as the nitrogen source (Table 4). As noted in part previously (4, 13), with either aspartate or asparagine as the nitrogen source in a minimal medium, both aspartase and asparaginase are induced to significant levels. In complex media, the enzyme levels in the aspHI mutant were three- to sevenfold higher than those in the wild-type strain, and the levels of both aspartase and asparaginase were high in cells grown with glucose plus ammonia (Table 4). Surprisingly, the levels of both enzymes were slightly

FIG. 6. Structure of chromosomal DNA in the ans region of various strains used to test whether the aspHI mutation alters ans expression in cis or in trans. The chromosomal DNA structures represented are not drawn to scale, but the same symbols for different chromosomal segments are used throughout; open boxes represent plasmid DNA. The closed circles labeled Pans indicate the promoter regions of the ans operon. The strains represented in panel A are PS1313 (wild type) and PS1316 (aspHI); those represented in panel B are PS1362 (wild type) and PS1477 (aspHI); and those represented in panel C are PS1559 (wild type) and PS1560 (aspHI). The details of the construction of these strains and their analysis are described in the text.
TABLE 4. Asparaginase and aspartase specific activities in cells grown in different mediaa

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Activityb in:</th>
<th>PS832 (wild type)</th>
<th>PS935 (aspHI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asparaginase</td>
<td>Aspartase</td>
<td>Asparaginase</td>
</tr>
<tr>
<td>2× YT</td>
<td>32</td>
<td>14</td>
<td>108</td>
</tr>
<tr>
<td>2× SG</td>
<td></td>
<td></td>
<td>94 (500)</td>
</tr>
<tr>
<td>Log-phase cells</td>
<td></td>
<td>(47)</td>
<td>(24)</td>
</tr>
<tr>
<td>Spores</td>
<td>6 (33)</td>
<td>8 (43)</td>
<td>23 (133)</td>
</tr>
<tr>
<td>BSG + NH₄</td>
<td>&lt;2</td>
<td>&lt;1</td>
<td>82</td>
</tr>
<tr>
<td>BSG + Aspartate</td>
<td></td>
<td>26</td>
<td>65</td>
</tr>
<tr>
<td>BSG + Asparagine</td>
<td></td>
<td>35</td>
<td>47</td>
</tr>
</tbody>
</table>

a Cells and spores were grown, harvested, and assayed by the ammonia release assay in toluene-permeabilized cells as described in Materials and Methods.

b Activity is given as units per milliliter of cells or spores at an OD₆₅₀ of 1.0. Numbers in parentheses are units per milligram of protein in extracts prepared by lysozyme lysis.

c Cells were in the mid-log phase (OD₆₅₀, 0.5 to 0.8).

decreased upon growth of the aspHI mutant with aspartate or asparagine as the nitrogen source (Table 4). The ratios of asparaginase to aspartase also varied significantly in different growth media (Table 4). However, the reasons for these differences, which were observed in several experiments, are not clear.

During growth of the wild-type strain in sporation medium, the specific activities of both asparaginase and aspartase decreased to a minimum at about t₀ of sporation, increased slightly by t₁ to t₂, and remained at this level for 2 to 4 h (Fig. 7). Enzyme specific activities also fell during growth in 2× YT medium and were much lower in stationary phase cells (data not shown). In 2× SG medium, most of the increase in enzyme specific activities at t₁ to t₂ was recovered in the mature spore, in which enzyme specific activities were almost as high as or higher than in vegetative cells (Table 4). The decrease in the levels of ans operon enzymes late in growth and their increase midway in sporulation were mirrored in large part by changes in β-galactosidase from an ansB-lacZ fusion, although the increase in β-galactosidase took place slightly later than did the increase in asparaginase and aspartase (Fig. 8A). The reason for the discrepancy in the timing of these increases is not clear, although it may involve differences in the rates of degradation of β-galactosidase, asparaginase, and aspartase during sporulation (see Discussion). Analysis of ansB-lacZ expression in spoIII mutants showed that there was a slow steady increase in β-galactosidase after t₁ in a spoIIIIC mutant, that this increase was smaller in a spoIIIG mutant, and that a spoIIIIE mutant showed no increase (Fig. 8). Indeed, the amount of β-galactosidase seen in the spoIIIIE mutant at t₅ was identical to that seen in the spoIIIIE mutant without the lac fusion (data not shown). Comparison of the maximum levels of β-galactosidase during sporulation of all four strains, after subtraction of endogenous enzyme activity, showed that the spoIIIIE mutant made less than 10% of maximum wild-type levels, while the spoIIIC and spoIIIG mutants made 60 and 45% of maximum wild-type levels, respectively (Fig. 8).

To determine the location of the β-galactosidase synthesized during sporulation of the wild-type strain carrying an ansB-lacZ fusion (strain PS1313), we analyzed the enzyme at t₀, at which time many mother cells were still not lysed. Less than 30% of the total β-galactosidase activity of strain PS1313 at t₀ was found in the mother cell fraction; the majority was located in the spores (data not shown). These results suggest that most of the ansB-directed β-galactosidase was synthesized inside forespores after the two cellular compartments were formed. The specific activity of β-galactosidase in spores of strain PS1313 at t₀ was 12.5 Miller units (data not shown), a level comparable to the level (14 to 16 Miller units) found at t₁ to t₂ (Fig. 8A).

In contrast to the behavior of asparaginase and aspartase activities in the wild-type strain, during sporulation of the aspHI mutant the levels of both asparaginase and aspartase increased about 30% at t₀ to t₁ and rose even higher by t₅ of sporation (Fig. 9). Approximately 80% of the β-galactosidase present at t₀ of sporation of the aspHI mutant carrying an ansB-lacZ fusion (strain PS1816) was in the mother cell, and the specific activity in the mother cell was more than threefold higher than that in the forespore (data not shown). That most of the ans operon expression during sporulation in the aspHI mutant was in the mother cell was also consistent with the specific activities of both asparaginase and aspartase being only two- to fourfold higher in aspHI mutant spores than in wild-type spores (Table 4).

DISCUSSION

In this paper, we report the purification of asparaginase and the cloning and sequencing of the ans operon from B. subtilis. The finding that the genes coding for asparaginase and aspartase are in the same operon in B. subtilis is possibly not surprising because of their functional correlation; i.e., the product of asparaginase is the substrate of aspartase. Cotranscription of the ansA and ansB genes as part of an operon may make it easier to coordinate asparaginase and
aspartase activities and thus maintain a balance between aspartate and asparagine levels in cells. However, in *E. coli* K-12, the *ansA, ansB*, and *aspA* genes, which code for asparaginase I, asparaginase II, and aspartase, respectively, are located in separate cistrons (14, 15, 42). While asparaginase I is a constitutive enzyme located in the cytoplasm, aspartase and the periplasmically located asparaginase II are inducible under anaerobic growth conditions. The latter two enzymes are regulated by the fnr gene product, a positive regulator of anaerobic respiration (16). It has been suggested that asparaginase II and aspartase in *E. coli* may be involved in either using asparagine as an anaerobic electron acceptor of simply providing aspartate and fumarate from exogenous asparagine (15, 16, 36). In *B. subtilis*, it appears that only one type of asparaginase is present, since no asparaginase activity could be detected in strains with an interrupted *ansA* gene and these strains could not grow with asparagine as the sole nitrogen source.

Comparison of the amino acid sequence of *B. subtilis* aspartase with those of aspartases from other bacteria (Fig. 10) shows that the *B. subtilis* aspartase is 45 and 50% identical to the aspartases from *E. coli* and *P. fluorescens*, respectively. The *B. subtilis* aspartase also exhibits high sequence identity (37%) with the *B. subtilis* fumarase (Fig. 10), as has been previously found with *E. coli* aspartase and class II fumarase (43). This finding is consistent with the suggestion made previously that genes for aspartase and fumarase were generated by a gene duplication event (43). Given the striking homology between aspartases and fumarases from both gram-negative and gram-positive organisms, it is possible that the gene duplication event which gave rise
to aspartase and fumarase genes took place prior to the divergence of these two groups of bacteria.

In *E. coli*, there are two types of asparaginase, which differ in their $K_m$ values, cellular localization, and pattern of regulation (12, 42). Comparison of the primary sequence of asparaginase from *B. subtilis* with that of either of the two *E. coli* enzymes showed less homology than that found for aspartases (Fig. 11), as more gaps were needed to align the asparaginase sequences. The homology between the *B. subtilis* enzyme and *E. coli* asparaginase I was 29%, a little higher than that between the *B. subtilis* enzyme and *E. coli* asparaginase II (24%) or between the two *E. coli* asparaginases (21.4%). It has been proposed, on the basis of substrate analog binding assays, that residues 133 to 142 in *E. coli* asparaginase II are a part of the active site (29). While good homology (six identical residues) was seen in this region between *B. subtilis* asparaginase and asparaginase I of *E. coli*, only two or three identical residues were seen in this region between these two enzymes and *E. coli* asparaginase II (doubly underlined region in Fig. 11).

These amino acid sequence comparisons suggest that the asparaginase of *B. subtilis* is more closely related to asparaginase I than to asparaginase II of *E. coli*. In addition, the $K_m$ of *B. subtilis* asparaginase in a crude extract was found to be $>1 	imes 10^{-2} M$ (34), closer to that of *E. coli* asparaginase I ($3.5 	imes 10^{-3} M$) than to that of *E. coli* asparaginase II (1.15 $\times 10^{-3} M$) (12, 14). These observations suggest that *B. subtilis* asparaginase and *E. coli* asparaginase I may have similar functions in the two bacteria. *E. coli* asparaginase II, but not asparaginase I, has been used for many years in the treatment of certain lymphomas (6). The high $K_m$ of *B. subtilis* asparaginase makes it unlikely to be a promising
candidate for this latter purpose. Nevertheless, further study of the  B. subtilis  enzyme may prove useful, possibly by generating an asparaginase with a higher activity, a lower $K_m$ and fewer side effects.

Analysis of the DNA sequences upstream of the 5' ends of  ans  mRNA present in vivo indicated that the transcript beginning at -67 relative to the translation start site has a perfect $\sigma^A$ consensus -10 recognition sequence and a -35 sequence with some homology to the -35 consensus sequence recognized by $\sigma^A$ (Fig. 12). That this transcript is indeed produced by  EcA  is consistent with its generation by RNA polymerase containing $\sigma^A$ in vitro. However, the efficiency of utilization of this promoter can clearly be regulated in vivo, as the level of the -67 transcript was increased by the  aspH1  mutation and abolished by growth in 2x SG medium, in which only the larger  ans  transcript was observed. Examination of sequences centered 10 and 35 nucleotides upstream of the transcription start site for the larger, -73 transcript revealed no similarities with consen-
sus recognition sequences for any known  B. subtilis  $\sigma$ factor (Fig. 12 and data not shown) (18, 25). While its transcription could be driven by an as-yet-uncharacterized $\sigma$ factor, we have not obtained any production of this larger transcript in vitro with any RNA polymerase preparation tested. Consequently, generation of the larger transcript might involve association of the  ans  promoter with a regulatory factor; alternatively, the larger transcript could be produced by processing from an even larger precursor, perhaps one generated under the direction of an alternative $\sigma$ factor. Further upstream of the start site of the -73 transcript are additional sequences which match fairly well with the consensus -10 and -35 recognition regions of  EcA  (singly underlined regions in Fig. 12). Utilization of this putative promoter would result in transcription initiation at -82 relative to the translation start site. However, no transcript originating from this site was seen either in vitro or in vivo.

![Sequence of the promoter region of the  ans  operon](caption)

**FIG. 12.** Sequence of the promoter region of the  ans  operon. The sequence shown was taken from Fig. 3 and represents nucleotides 201 to 281. The two T residues found at the 5' end of  ans  mRNA in vivo are shown in larger boldface type; nucleotide sequences centered 10 and 35 nucleotides upstream of the shorter, -67, transcript (10 and -35 sequences) are heavily underlined. Beneath these sequences are shown the consensus -10 and -35 recognition sequences and their spacing for RNA polymerase containing $\sigma^A$ (26). Potential -10 and -35 sequences for the longer, -73 transcript are heavily overlined. Sequences further upstream with fairly good homology to the -10 and -35 sequences recognized by $\sigma^A$ are thinly underlined. Note that no transcript utilizing the latter putative promoter has been observed.
Interestingly, the citG gene, which encodes fumarase in B. subtilis, also has two promoter regions, termed P1 and P2 (7, 24). The P1 promoter region is functional in both E. coli and B. subtilis, while the P2 promoter region is functional only in B. subtilis and is strictly dependent on promoter, the product of the spoOH gene (7). However, the expression of the ans operon in either 2× YT or 2× SG medium is not affected by a spoOH mutation (34).

Both aspartase and asparaginase activities, as well as ansA-lacZ and ansB-lacZ expression and the level of the ~67 transcript, are enhanced by the aspH1 mutation, suggesting that this mutation regulates the expression of the ans operon at the transcriptional level. Our results concerning the distance between the ansB gene and the aspH1 mutation, as well as the effect on ans expression of moving the promoter away from upstream and downstream sequences and the identity between the 500 nucleotides upstream of the ans operon in both aspH1 and wild-type strains, are most consistent with the aspH1 mutation defining a trans-acting factor regulating the expression of the ans operon. Since only one allele has been analyzed in the aspH locus, we cannot determine whether the wild-type product is normally a positive or a negative regulator of the ans operon or if it is indeed a DNA binding protein. However, as the aspH1 locus may be close to the ans operon, its cloning and analysis should be an easily approached problem.

We began our studies of the aspartase gene because of evidence that this gene showed preferential expression in forespores in sporulating cells. This evidence appears to be correct, as shown by analysis of both asparaginase and aspartase activities during sporulation, as well as the expression of an ansB-lacZ fusion. However, it also appears likely that the forespore-specific transcription of the ans operon is unrelated to sporulation per se but is due to changes in the nitrogen environment of the developing spores. These changes in the forespore nitrogen environment may be a general limitation of the nitrogen supply or an increased level of either substrate for the enzymes of the operon, asparagine and aspartate. Indeed, both general nitrogen limitation and high levels of asparagine or aspartate resulted in significant activation of ans operon expression in wild-type log-phase cells (4) (Table 4). Presumably, ans expression in the aspH1 mutant is essentially constitutive and thus relatively insensitive to both general nitrogen supply and levels of aspartate and asparagine. As cells grow in either 2× YT or 2× SG medium (with or without eventual sporulation), there is a significant decrease in ans operon expression, a decrease which does not take place in the aspH1 mutant, in which normal nitrogen regulation of this operon is blocked. This decreased ans expression in wild-type cells may be due to decreased substrate induction, as the high levels of asparagine and aspartate initially present in these rich growth media undoubtedly fall as growth proceeds. In wild-type cells, there may be a decrease in ans transcription, with an attendant dilution of ans operon enzymes by further cell growth, plus degradation of preexisting ans operon enzymes as well as β-galactosidase from the ansB-lacZ fusion. Indeed, different rates of degradation of aspartase, asparaginase, and β-galactosidase from an ansB-lacZ fusion early in sporulation may explain the differences in the kinetics of changes in these activities at this time. There is much evidence for the degradation of enzymes, including β-galactosidase from lacZ fusions, beginning late in B. subtilis growth and continuing early in sporulation, with the degradation primarily occurring in the mother cell compartment (10, 37). However, within the developing forespore the nitrogen environment may be very different from that in the encompassing mother cell, resulting in a slight turning on of the synthesis of ans operon enzymes. This turning on, as well as the stability of ans operon enzymes within the forespore and their instability in the mother cell, would then lead to significant levels of both aspartase and asparaginase in forespores and mature spores relative to mother cells. The presence of significant ans operon expression in a spoHG mutant, ans operon expression during sporulation by use of a transcript also formed in vegetative cells, and the lack of production of this transcript in vitro by any sporulation-specific RNA polymerase in the forespore, suggest that forespore-specific ans expression is not driven by forespore-specific gene products. It is striking that forespore-specific ans expression was abolished in a spoHIE mutant, since this mutation has been suggested to somehow break down the permeability barrier between mother cell and forespore (32), which would in turn equalize the nitrogen environment in the two compartments. It will be of interest to determine the specific details of how the ans operon is regulated in response to the availability of nitrogen sources and how this nitrogen availability may vary between mother cell and forespore.

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