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

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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 *aspH1* 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 (t_1 to t_4) 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 *aspH1* 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 t_0 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 t_2 and t_5 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* (*aspH1*) 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.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this work are listed in Table 1. The S5-Na medium used for growing cells with aspartate 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 NH_4Cl (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 (*aspH1*) 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 (OD_{600}) 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 phenylmethylsulfonyl fluoride. The cells were broken by two passes through a French pressure cell at 10,000 lb/in^2, 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 centrifu-

TABLE 1. *B. subtilis* strains used

Strain	Genotype or phenotype	Source
PS604	*spoIIIC94 trpC2*	BGSC[a]
PS607	*spoIIIE36 trpC2*	BGSC
PS683 (MO428)	*spoIIIGΔ1 trpC2*	P. Stragier
PS832	Wild type, Trp^+ revertant of 168	Laboratory stock
PS935 (IA296)	*aspH1 trpC2*	BGSC
PS1313	*ansB-lacZ* $AnsB^-$ Cm^r	pPS1311[b]→PS832
PS1314	*ansB-lacZ spoIIIE36 trpC2* $AnsB^-$ Cm^r	pPS1311[b]→PS607
PS1316	*ansB-lacZ aspH1 trpC2* $AnsB^-$ Cm^r	pPS1311[b]→PS935
PS1319	*ansB-lacZ spoIIIG1110* $AnsB^-$ Cm^r MLS^{rc}	pPS1110[d]→PS1313
PS1321	*ansB-lacZ spoIIIC94 trpC2* $AnsB^-$ Cm^r	pPS1311[b]→PS604
PS1362	*ansA-lacZ* $AnsA^-$ $AnsB^-$ Cm^r	pPS1364[b]→PS832
PS1477	*ansA-lacZ aspH1 trpC2* $AnsA^-$ $AnsB^-$ Cm^r	pPS1364[b]→PS935
PS1559	*ansA-lacZ* $AnsA^+$ $AnsB^+$ Cm^r	pPS1557[b]→PS832
PS1560	*ansA-lacZ aspH1 trpC2* $AnsA^+$ $AnsB^+$ Cm^r	pPS1557[b]→PS935

[a] BGSC, *Bacillus* Genetic Stock Center.
[b] Construction of these plasmids is described in the text.
[c] MLS^r, resistance to macrolides-lincosamides-streptogramin B.
[d] pPS1110 contains a part of the *spoIIIG* gene in which a 43-bp *Pst*I-*Hin*dIII fragment is replaced by a functional *ermC* gene (33). Its integration into the chromosome inactivates the *spoIIIG* gene.

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 $MgCl_2$, 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 Iijima et al. (13). The assay measuring the production of fumarate by its A_{240} 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 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 ammonia 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 (t_{20}), 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 [α-^{32}P]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 *Pst*I fragment cloned in plasmid pUC18 and identified by colony hybridization in *Escherichia coli* JM83 (20). With this 2-kb *Pst*I fragment as a probe, we further cloned in pUC18 a 600-bp *Hin*cII fragment that contained 300 bp of a new sequence upstream from the 2.0-kb *Pst*I fragment. Finally, with this 600-bp *Hin*cII fragment as a probe, we cloned in pUC18 a 1-kb *Pst*I 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′ ACAGGGCAATCTGTCCGG 3′; corresponding to positions 2678 to 2695 in Fig. 3) and a backward primer (20-mer; 3′ ACTAGTGTCGACAGTTAGGC 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 *Pst*I fragment near the 3′ end. Chromosomal DNA was cut with *Hae*III or *Rsa*I and religated 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′ CCTACATTCTAACGGTTG 5′), was synthesized; this primer corresponded to the complementary strand of positions 2955 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.

Construction of *ans-lacZ* fusion strains. For the construction of an *ansB-lacZ* fusion, the 0.8-kb *Eco*RI 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 *Pst*I-*Eco*RI portion of the pUC18 polylinker and was ligated into the *Eco*RI 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 *Mlu*I. 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 *Hin*cII fragment of pPS1366 (see Fig. 2) into the *Sma*I site of pJF751. Transformants were isolated in *E. coli* RR101, and a clone with the plasmid in the correct orientation was identified by *Mlu*I 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 *Hin*cII 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 σ^A 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 (*spoIIIG*) and PS832 grown in 2× SG medium and harvested at t_3 of sporulation (35). In addition to RNA polymerase containing σ^A ($E\sigma^A$), the enzyme preparations from the latter two strains also contained $E\sigma^F$ (PS683) and $E\sigma^F$ plus $E\sigma^G$ (PS832) (35). Plasmid pPS1521 (1 μg), in which the 0.7-kb *Pst*I-*Hin*cII fragment of pPS1367 was cloned between the *Pst*I and *Hin*cII 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-^{32}P]ATP. 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′ GTCAACTTCCCCTTTTA CCCGACCG 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]

Purification step	Vol (ml)	Protein (mg)	U (10^3)	Recovery (%)	Sp act (U/mg of protein, 10^2)	Purification (fold)
Cell supernatant	115	4,600	2,346	100	5.1	1
Streptomycin sulfate precipitate	120	4,800	3,072	131	6.5	1.25
$(NH_4)_2SO_4$ precipitate	30	1,950	2,321	99	12	2.33
DEAE-cellulose chromatography	20	424	1,526	65	36	7.1
Gel filtration	6	36	511	22	141	27.8
Red-A agarose chromatography	18	6.8	368	16	540	106

[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.

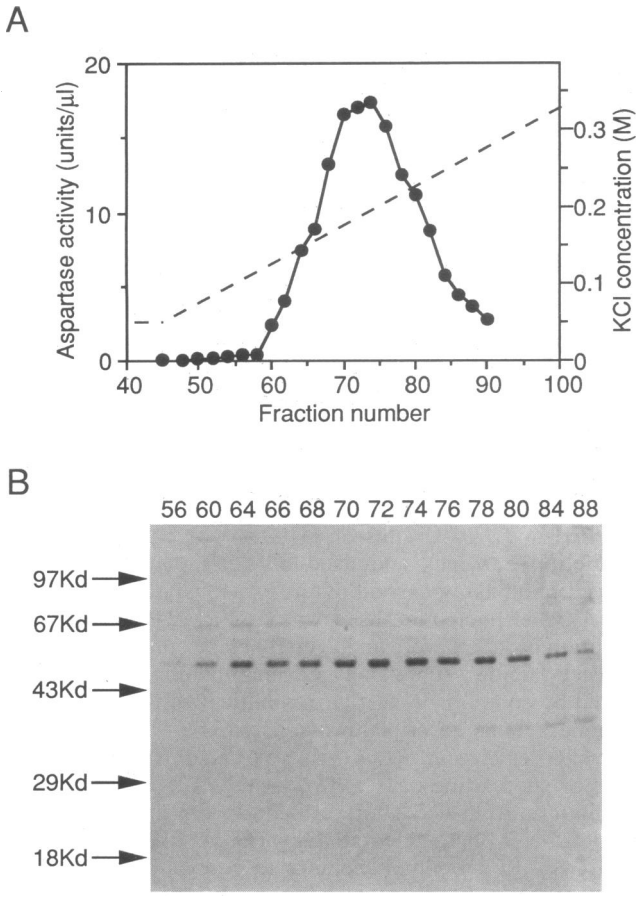

FIG. 1. Chromatography of aspartase on Red-A agarose (A) and analysis of column fractions by SDS-PAGE (B). (A) Enzyme from the Sephacryl S-300 step in the aspartase purification was applied to a Red-A agarose column, eluted with a KCl gradient (broken line), and assayed (closed circles) as described 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.

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 *Pst*I 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 *Hae*III or *Rsa*I was religated under dilute conditions and used as a template in a PCR to produce linear DNA products. Southern blot analysis of *Hae*III- or *Rsa*I-digested chromosomal DNA with the 260-bp *Nae*I-*Pst*I fragment (Fig. 2B) as a probe (data not shown) predicted that an inverse PCR with *Hae*III- or *Rsa*I-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

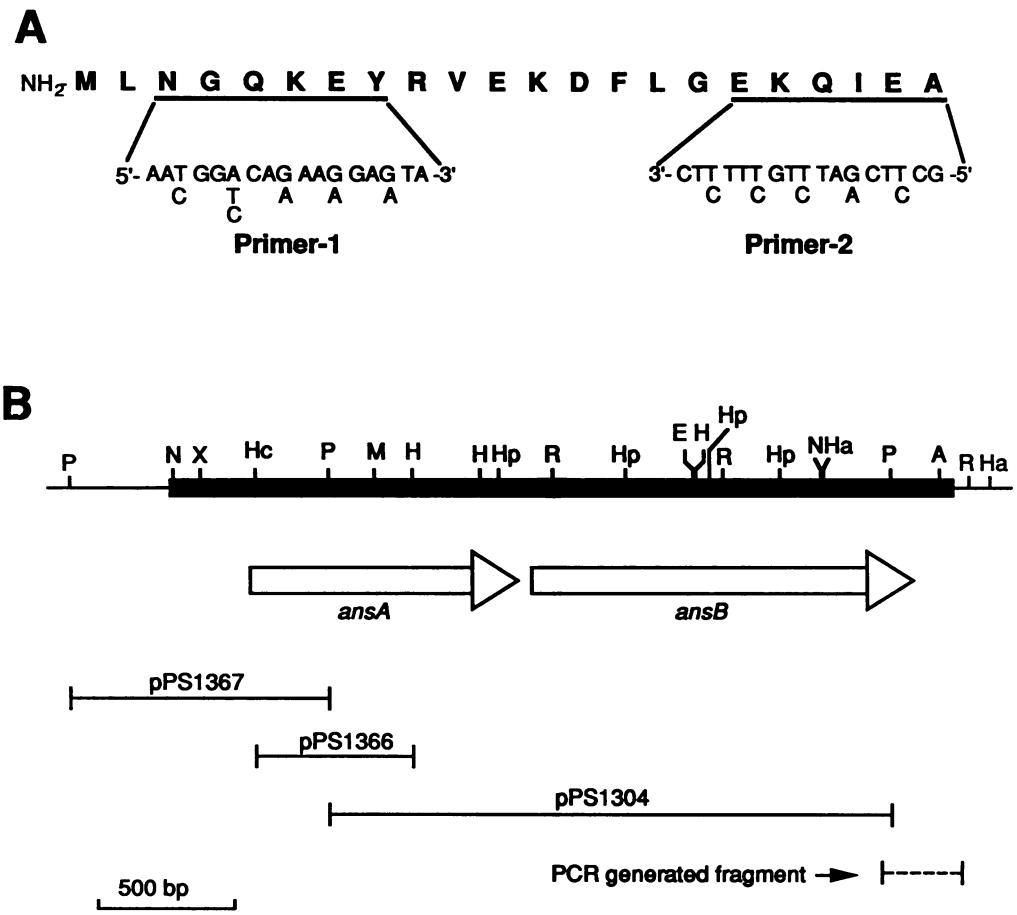

FIG. 2. N-terminal amino acid sequence of aspartase and primers used for PCR to generate a probe for the N-terminal coding region of the aspartase gene (A) and organization and restriction map of the *ans* operon (B). (A) The amino acid sequence of the N terminus of *B. subtilis* aspartase determined 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, *Acc*I; E, *Eco*RI; H, *Hin*dIII; Ha, *Hae*III; Hc, *Hin*cII; Hp, *Hin*pI; M, *Mlu*I; N, *Nae*I; P, *Pst*I; R, *Rsa*I; and X, *Xba*I. 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 *Pst*I, *Hin*cII-*Hin*dIII, and *Pst*I 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.

synthesized 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 *Taq*I 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 asparaginase 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 σ^A recognition at positions 267 to 272 and a less conserved −35 region for σ^A 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

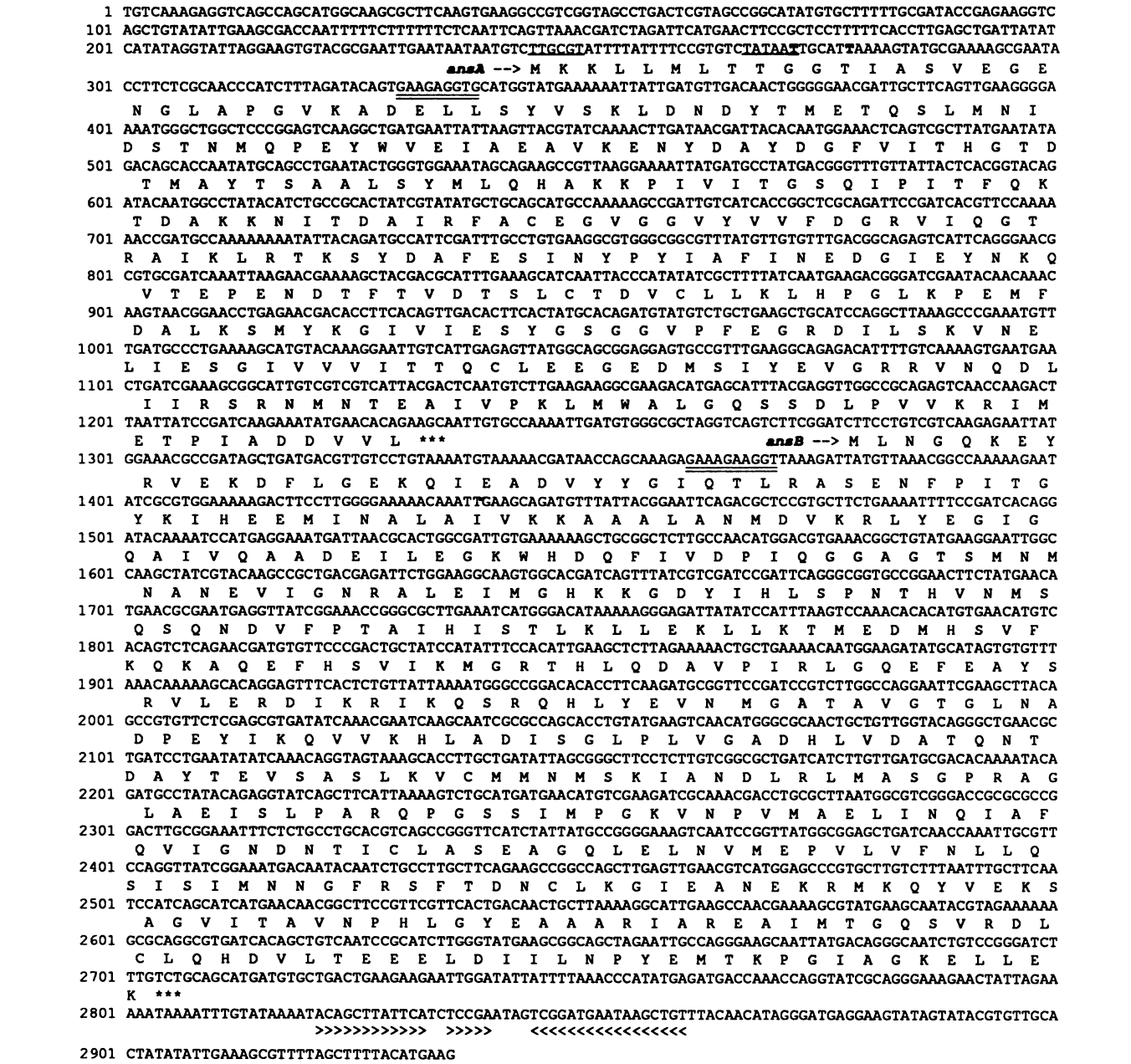

FIG. 3. Nucleotide sequence of the *ans* operon and flanking regions and predicted coding sequences for asparaginase (*ansA*) and aspartase (*ansB*). The numbers to the left of each line give the number of the first nucleotide in that line. The amino acid sequences predicted from the nucleotide sequences are given in the one-letter code, with the residue above the first base of 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^A-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.

resembled a rho-independent transcription termination signal (41). Both coding regions of the *ans* operon were preceded by sequences with significant homology to the 3′ end of 16s rRNA and presumably represented ribosome binding sites (doubly underlined residues in Fig. 3).

Chromosomal location of the *ans* operon. Analysis of the *Sfi*I fragment of *B. subtilis* chromosomal DNA which contains the *ans* operon has shown that the *ans* operon is on the same fragment as the previously identified *aspH1* mutation (1). To determine whether the *ans* operon and the *aspH1* mutation are closely linked, we used chromosomal DNA from strain PS1313 (*ansB-lacZ* Cm^r) to transform PS935 (*aspH1*) to chloramphenicol resistance. A low concentration of DNA (0.03 μg/ml) was used in the transformation reaction to minimize the uptake of multiple DNA fragments. Fifty Cm^r transformants were grown overnight in 2× YT medium

TABLE 3. Asparaginase, aspartase, and β-galactosidase activities in various strains[a]

Strain	Activity[b] of:		
	Asparaginase	Aspartase	β-Galactosidase
PS832 (wild type)	14	11	<2
PS935 (*aspH1*)	90	112	<2
PS1313 (*ansB-lacZ*)	6	<1	621
PS1316 (*ansB-lacZ aspH1*)	49	<1	1,548
PS1362 (*ansA-lacZ*)	<1	<1	36

[a] Cells were grown in 2× YT medium, and samples were harvested in the late exponential phase (OD_{600}, 2).

[b] Aspartase and asparaginase were assayed by the ammonia release assay in toluene-permeabilized cells, and values are given as nanomoles of NH_3 released per minute per milliliter of cells at an OD_{600} of 1.0. Values for β-galactosidase are given as Miller units.

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 (*aspH1*). Likewise, when PS1316 (*ansB-lacZ aspH1* Cm^r) was used as the donor of chromosomal DNA to transform PS832 (wild type), 82% of the Cm^r transformants showed the AspH phenotype, as judged by their high levels of asparaginase, and 18% remained wild type. It appears that the *aspH1* 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 *aspH1* mutation (11) and suggests that the *aspH1* mutation is not in the promoter for the *ans* operon (see below). This suggestion is consistent with the *aspH1* 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 *aspH1* mutant was consistent with this model, as both aspartase and asparaginase activities were elevated 7- to 10-fold in the *aspH1* 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 asparagine 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 10 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

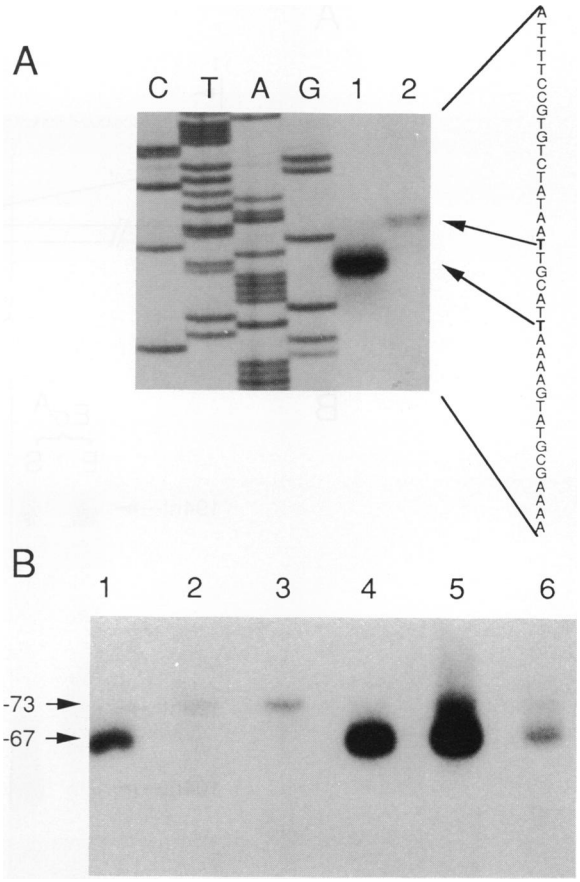

FIG. 4. Determination of the 5′ end of *ans* mRNA in vivo by primer extension analysis. (A) Analysis of 5′ ends of *ans* mRNAs by comparison of primer extension products with a DNA sequencing ladder. RNA was extracted and purified from cells of strain PS832 (wild type) either in log-phase growth in 2× YT medium (lane 1) or at t_2 of sporulation in 2× SG medium (lane 2). This RNA was then used in a primer extension analysis as described in Materials and Methods, and the extended primer was analyzed by gel electrophoresis and autoradiography. Lanes C, T, A, and G represent DNA sequencing reactions run on plasmid pPS1367 with the appropriate dideoxynucleotide triphosphate and the same labeled primer that was used in the primer extension reactions. The sequence to the right of lane 2 is the sequence in the region of the 5′ ends of *ans* mRNAs (residues 255 to 293 in Fig. 3), and the arrows indicate the residues at the 5′ ends of the extended primers in either lane 1 or lane 2. (B) Autoradiogram of primer extension products generated with different RNA samples. RNA was extracted and purified, equal amounts were subjected to primer extension analysis as described above, and the products were electrophoresed and autoradiographed. The arrows labeled −67 and −73 indicate the positions of the 5′ ends of *ans* mRNAs relative to the translation start site as determined in panel A. The RNAs used were as follows (lanes): 1 to 3, strain PS832 (wild type); 4 to 6, strain PS935 (*aspH1*). The cells used were as follows (lanes): 1 and 4, cells grown in 2× YT medium and harvested in the log phase; 2 and 5, cells grown in 2× SG medium and harvested in the log phase; 3 and 6, cells grown in 2× SG medium and harvested at t_2 of sporulation.

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 t_0, t_2, or t_4 of sporulation (Fig. 4 and data not

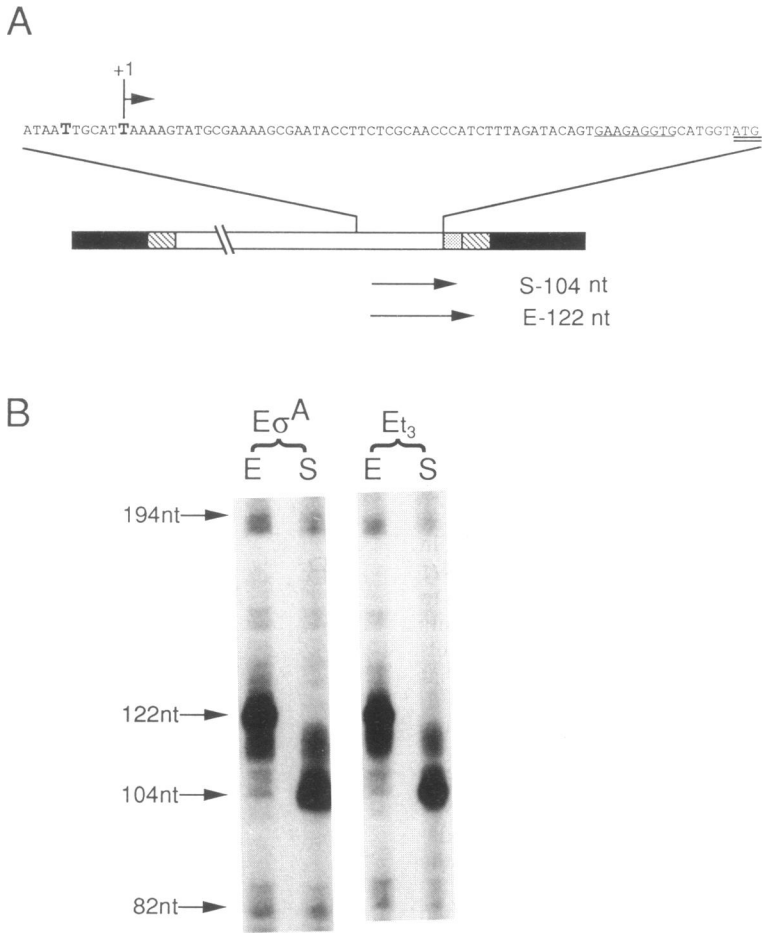

FIG. 5. Analysis of transcripts produced in vitro from the *ans* operon. Plasmid pPS1521 was cut with the appropriate restriction enzymes and transcribed in vitro with RNA polymerase, and transcripts were analyzed by gel electrophoresis as described in Materials and Methods. (A) Sequence of the *ans* 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 *Sma*I (S)- or *Eco*RI (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 *Eco*RI (E) or *Sma*I (S) and transcribed in vitro with RNA polymerase from log-phase cells of strain PS832 grown in 2× YT medium ($E\sigma^A$) or t_3 sporulating cells of strain PS683 grown in 2× SG medium (Et_3), 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 $E\sigma^A$ (Fig. 5B) as well as enzyme isolated at t_3 of sporulation from both the wild-type strain and strain PS683 (*spoIIIGΔ1*) (Et_3; Fig. 5B; also, data not shown). Note that the t_3 RNA polymerase preparations contained holoenzymes with several sporulation-specific σ factors (i.e., $E\sigma^F$ and $E\sigma^G$ in the wild-type strain and $E\sigma^F$ in strain PS683) in addition to $E\sigma^A$. Since Et_3 yielded the same pattern of *ans* transcripts as did $E\sigma^A$ (Fig. 5B), it appears likely that $E\sigma^A$ is the only holoenzyme transcribing *ans* DNA in vitro (see Discussion). With none of the RNA polymerase preparations tested, including the t_3 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

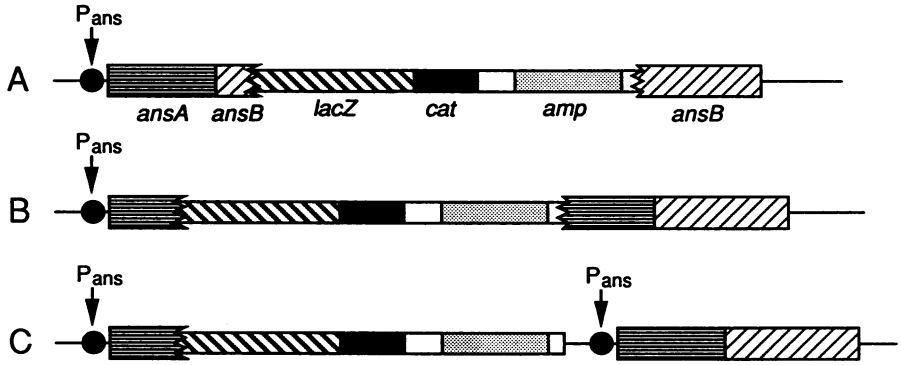

FIG. 6. Structure of chromosomal DNA in the *ans* region of various strains used to test whether the *aspH1* mutation alters *ans* expression in *cis* or in *trans*. The chromosomal DNA structures represented are not drawn to scale, but the same symbols for different chromosomal segments are used throughout; open boxes represent plasmid DNA. The closed circles labeled P_{ans} indicate the promoter regions of the *ans* operon. The strains represented in panel A are PS1313 (wild type) and PS1316 (*aspH1*); those represented in panel B are PS1362 (wild type) and PS1477 (*aspH1*); and those represented in panel C are PS1559 (wild type) and PS1560 (*aspH1*). The details of the construction of these strains and their analysis are described in the text.

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 *aspH1* mutant indicated that this mutation increases *ans* transcription and thus that the locus defined by the *aspH1* mutation is, or encodes, a transcriptional regulator. One explanation for the effects of the *aspH1* 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 (*aspH1*) and have found no differences (data not shown). Indeed, the finding noted above that the *aspH1* mutation is only 80% cotransformed with the *ans* operon suggests that the *aspH1* 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 *aspH1* mutation on *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 *aspH1* 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 *aspH1* 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 (*aspH1*) showed that the *aspH1* 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 *aspH1* 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 (*aspH1*) 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 *Nae*I-*Hin*cII fragment containing the *ans* promoter and a small amount of the *ansA* N-terminal coding region (Fig. 3) was cloned in *Sma*I-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 *aspH1* strains to chloramphenicol resistance through integration of the plasmid at the *ans* locus (Fig. 6C). Since the *Nae*I-*Hin*cII fragment used to create pPS1557 contains the *ans* promoter, the resulting *B. subtilis* transformants, strains PS1559 (wild type) and PS1560 (*aspH1*), contained two *ans* promoters (Fig. 6C), one for the *ansA-lacZ* fusion and adjacent to all upstream *B. subtilis* DNA and a second for the intact *ans* operon and ~7 kb downstream and immediately preceded by only ~200 bp of upstream *B. subtilis* DNA. Analysis of the asparaginase and aspartase levels in strains PS1559 (wild type) and PS1560 (*aspH1*) 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 *aspH1* mutation can act on the *ans* promoter from a distance of at least 7 kb, again suggesting that the *aspH1* mutation defines a locus encoding a *trans*-acting factor regulating the transcription of the *ans* operon. Since there are only ~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 *aspH1* 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 *aspH1* 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

TABLE 4. Asparaginase and aspartase specific activities in cells grown in different media[a]

Growth medium	Activity[b] in:			
	PS832 (wild type)		PS935 (*aspH1*)	
	Asparaginase	Aspartase	Asparaginase	Aspartase
2× YT	32	14	108	94 (500)
2× SG				
Log-phase cells[c]	10 (47)	5 (24)	58 (284)	32 (150)
Spores	6 (33)	8 (43)	23 (133)	14 (87)
BSG + NH_4	<2	<1	82	31
BSG + Aspartate	26	1.5	65	17
BSG + Asparagine	35	7	47	15

[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 OD_{600} 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 (OD_{600}, 0.5 to 0.8).

decreased upon growth of the *aspH1* 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 sporulation medium, the specific activities of both asparaginase and aspartase decreased to a minimum at about t_0 of sporulation, increased slightly by t_1 to t_2, 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 t_1 to t_2 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 in 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 t_3 in a *spoIIIC* mutant, that this increase was smaller in a *spoIIIG* mutant, and that a *spoIIIE* mutant showed no increase (Fig. 8). Indeed, the amount of β-galactosidase seen in the *spoIIIE* mutant at t_5 was identical to that seen in the *spoIIIE* 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 *spoIIIE* 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).

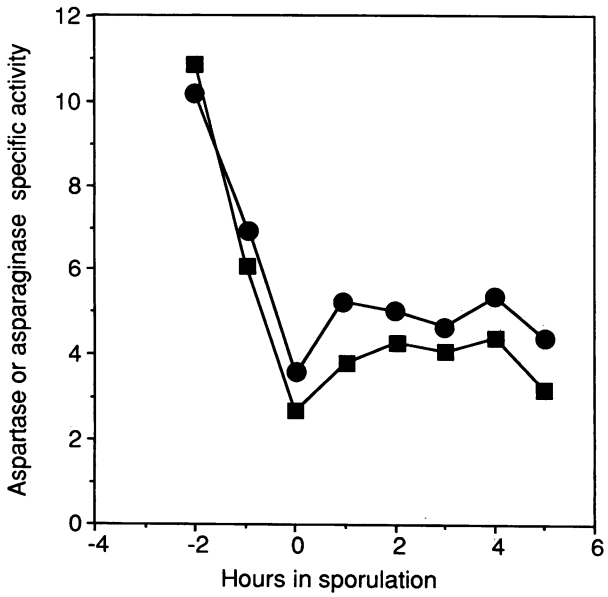

FIG. 7. Levels of aspartase (●) and asparaginase (■) during growth and sporulation of wild-type *B. subtilis*. Strain PS832 (wild type) was grown and sporulated in 2× SG medium as described in the text. Samples were removed at various times, the OD_{600} of the culture was determined (to identify the time [hour 0] of the initiation of sporulation), and the samples were isolated, made permeable with toluene, and assayed for aspartase and asparaginase.

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 t_{20}, at which time many mother cells were still not lysed. Less than 30% of the total β-galactosidase activity of strain PS1313 at t_{20} was found in the mother cell fraction; the majority was located in the spore (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 t_{20} was 12.5 Miller units (data not shown), a level comparable to the level (14 to 16 Miller units) found at t_4 to t_5 (Fig. 8A).

In contrast to the behavior of asparaginase and aspartase activities in the wild-type strain, during sporulation of the *aspH1* mutant the levels of both asparaginase and aspartase increased about 30% at t_0 to t_1 and rose even higher by t_5 of sporulation (Fig. 9). Approximately 80% of the β-galactosidase present at t_{20} of sporulation of the *aspH1* mutant carrying an *ansB-lacZ* fusion (strain PS1316) 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 *aspH1* 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 *aspH1* mutant spores than in wild-type spores (Table 4).

DISCUSSION

In this paper, we report the purification of aspartase 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

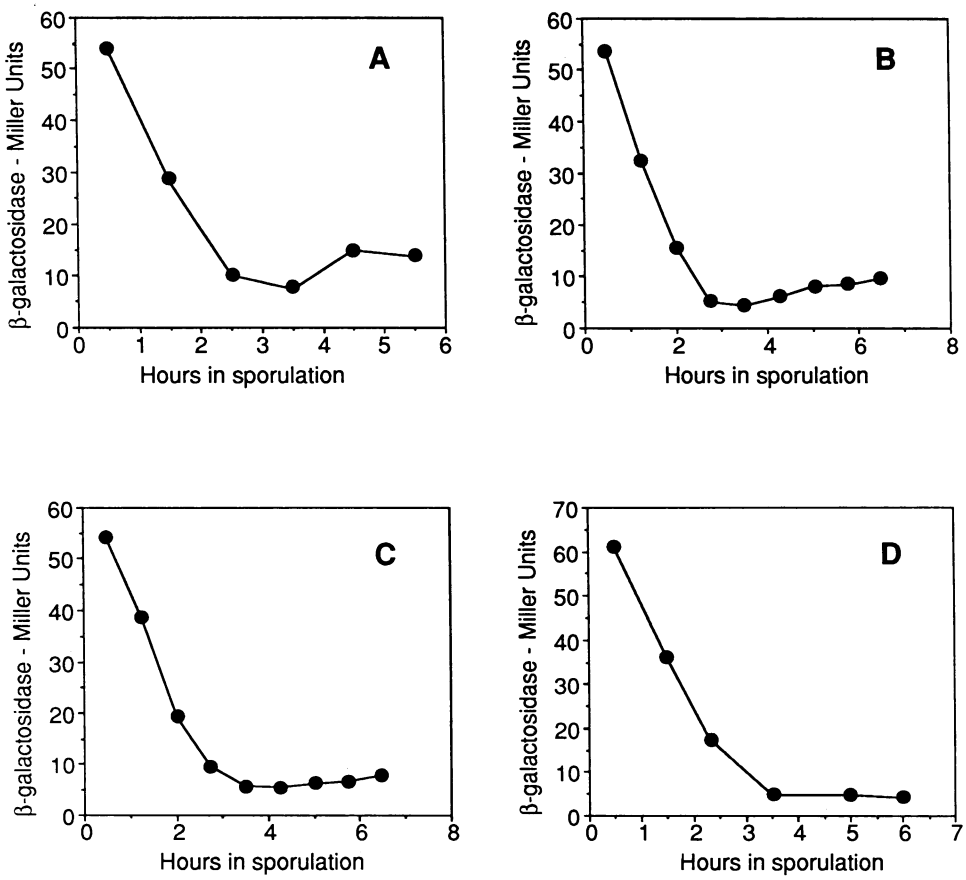

FIG. 8. Levels of β-galactosidase from an *ansB-lacZ* fusion in wild-type and Spo$^-$ stains. Strains PS832 (Spo$^+$) (A), PS1321 (*spoIIIC*) (B), PS1319 (*spoIIIG*) (C), and PS1314 (*spoIIIE*) (D) were grown and sporulated in 2× SG medium. At various times, samples were harvested and assayed for β-galactosidase. The time of initiation of sporulation is defined as the time that log-phase growth of the culture ended.

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,

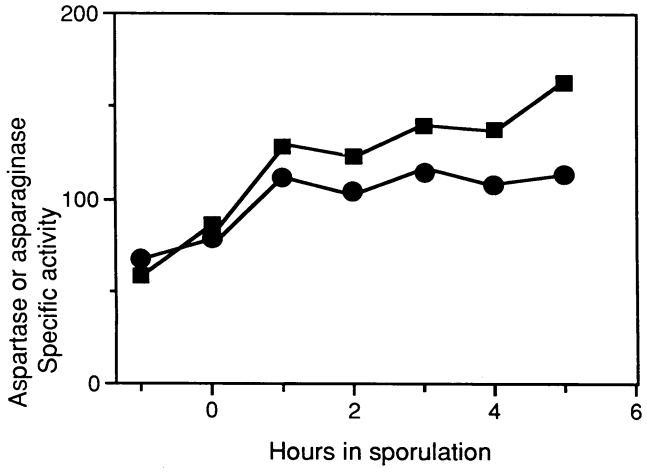

FIG. 9. Levels of asparaginase (■) and aspartase (●) during growth and sporulation of the *aspH1* mutant strain. Strain PS935 was grown and sporulated and samples were harvested and assayed as described in the legend to Fig. 7.

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 anerobic 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

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Bs-A   MLNGQKEYRVEKDFLGEKQIEADVYYGIQTLRASENFPITGYKIHE--EM    48
Ec-A     MSNNI.I.E.L..TREVP..A...VH....IV..Y.SNN..SDIP.F    47
Pf-A    MSSAASF.T...L..VLEVP.QA.........VN..RLS.VP.SHYPKL   49
Bs-F        M...I.R.TM..VKVP..KFW.A..Q.SK...K.GSE.M-PM-RV   43

Bs-A   INALAIVKKAAALANMDVKRLYEGIGQAIVQAADEILE-GKWHDQFIVDP   97
Ec-A   VRGMVM......M..KELQTIPKSVAN..IA.CD.V.NN..CM...P..V   97
Pf-A   VVG..M..Q...D..RELGQ.S.RKHA..SE.CAR-.IR.DF.EE.V..M   98
Bs-F   VK.F..L.RST....KRLGN.DVEKAE..AAVC.DV.K-..YD.N.PLVV   92

Bs-A   IQGGAGTSMNMNANEVIGNRALEIMGHKKGDYIHLSPNTHVNMSQSQNDV  147
Ec-A   Y.......V...T...LA.IG..L...Q..E.QY.N..D...KC..T..A  147
Pf-A   ........T........A.I...A...Q..E.QY.H..ND...A..T..A  148
Bs-F   W.T.S..QS...M...VA...TALLKE.NS.-QTIH..DD..R...S..T  141

Bs-A   FPTAIHIS-TLKLLEKLLKTMEDMHSVFKQKAQEFHSVIKMGRTHLQDAV  196
Ec-A   Y..GFR.A-VYSS.I..VDAINQLREG.ER..V..QDIL.....Q.....  196
Pf-A   Y....RLG-L.LGHDA..ASLDSLIQA.AA.GA..SH.L.....Q.....  197
Bs-F   ....M.VAAV.AVY.Q.VPALDQLRNTLDE..KAYNDIV.I........T  191

Bs-A   PIRLGQEFEAYSRVLERDIKRIKQSR-QHLYEVNMGATAVGTGLNADP-E  244
Ec-A   .MT.....R.F.IL.KEEV.N.QRTA-EL.L...L....I.....T-.K.  244
Pf-A   .MT.....R.FATT.GE.LA.L.TLAPEL.T...L.G..I...I....R-  246
Bs-F   .LT....ISGWVHM.D.SKEM.LEAT-DKMRALAI.G......I..H.-.  239

Bs-A   YIKQVVKHLADISGLPLVGADHLVDATQNTDAYTEVSASLKVCMMNMSKI  294
Ec-A   .SPLA..K..EVT.F.C.P.ED.IE..SDCG..VM.HGA..RLAVK....  294
Pf-A   .QALA.QR..T...Q...P.AD.IE..SDMG.FVLF.GM..RTAVKL...  296
Bs-F   FGEL.SEEITKLT.QTFSSSPNKFH.LTSH.EI.YAHGA..ALAADLM..  289

Bs-A   ANDLRLMASGPRAGLAEISLPARQPGSSIMPGKVNPVMAELINQIAFQVI  344
Ec-A   .C....LS.......N..N..EL.A......A.....VP.VV..VC.K..  344
Pf-A   .C....LS....T.IN..N.................IP.AV..V.....  346
Bs-F   ...V.WL.....C.IG..VI.ENEP...........TQS.ALTM..A.IM  339

Bs-A   GNDNTICLASEAGQLELNVMEPVLVFNLLQSISIMNNGFRSFTDNCLKGI  394
Ec-A   ...T.VTM.A.....Q.......IGQAMFE.VH.LT.ACYNLLEK.IN..  394
Pf-A   ...LALTM.A.G...Q......LIA.KIFD..RLLQRAMDMLREH.IV..  396
Bs-F   ...A..GF.ASQ.NF....FK..IIY.F...VQLLSD.MN..H.K.AV..  389

Bs-A   EANEKRMKQYVEKSAGVITAVNPHLGYEAAARIAREAIMTGQSVRDLCLQ  444
Ec-A   T..KEVCEG..YN.I.IV.YL..FI.HHNGDIVGKICAE..K...EVV.E  444
Pf-A   T...A.CREL..H.I.LV..L..YI...N.T....I.LES.RG.LE.VRE  446
Bs-F   .P.KETIQENLSN.LMLV..L...I...N..K..KL.HKE.LTLKEAA.K  439

Bs-A   HDVLTEEELDIILNPYEMTKPGIAGKELLEK                    475
Ec-A   RGL...A...D.FSVQNLMH.AYKA.RYTDESEQ                  478
Pf-A   EGL.DDAM..D..R.EN.IA.RLVPLKA                        474
Bs-F   LEL....QFNEMVK.ED.V..KA                             462
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FIG. 10. Comparison of amino acid sequences of aspartases and fumarase from several bacteria. The *B. subtilis* aspartase (Bs-A) sequence was taken from Fig. 3. The *E. coli* and *P. fluorescens* aspartase sequences (Ec-A and Pf-A, respectively) were taken from references 38 and 40, respectively. The *B. subtilis* fumarase (Bs-F) sequence was taken from references 24 and 43. Sequences were aligned to provide maximum homology with the Bs-A sequence. Residues in other sequences identical to those in the Bs-A sequence are given as dots; gaps that were introduced to maximize alignment are given as dashes. All amino acid residues are given in the one-letter code; the number to the right of each line of sequence gives the number of the last residue in the sequence.

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 $>1 \times 10^{-2}$ M (34), closer to that of *E. coli* asparaginase I (3.5×10^{-3} M) than to that of *E. coli* asparaginase II (1.15×10^{-5} 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

```
Bs-A                              MKKLLMLTTGGTIASVEGE----NGLAP  24
Ec-I                                  MQRSEQGYIP.S.H----LQRQL  19
Ec-II  MEFFKKTALAALVMGFSGAALALPNITI.A......GGGDSATKS.YTVG  50

Bs-A   GVKADELLSYVSKLDNDYTMETQSLMNIDSTNMQPEYWVEIAEAVKENYD  74
Ec-I   ALMPE----FHRPEMP.F.IHEYTPL-M..SD.TP.D.QH...DI.AHY.  64
Ec-II  K.GVEN.VNAVPQLK.IANVKGEQVV..G.QD.ND.V.LTL.KKINTDC. 100

Bs-A   AYDGFVITHGTDTMAYTSAALSYMLQHAKKPIVITGSQIPITFQKTDAKK 124
Ec-I   D......L.........AS...F..ENLG..VIV......LAELRS.GQI 114
Ec-II  KT............EE.AYF.DLTVK-CD..V.MV.ANR.S.SMSA.GPF 149

Bs-A   NITDAIRFACE-GVG---GVYVVFDGRVIQGTRAIKLRTKSYDAFESINY 170
Ec-I   .LLN.LYV.ANYPIN---E.TLF.NN.LYR.N.TT.AHADGF...A.P.L 161
Ec-II  .LYN.VVT.AD-KASANR..L..MNDT.LD.RDVT.TN.TDVAT.K.V.. 198

Bs-A   PYIAFINEDGIEYNKQVTEPENDTFTVDTSLCTDVCLLKLHPGLKPEMF- 219
Ec-I   .PLLEAGIHIRRL.TPPAPHGEGELI.HPITPQPIGVVTTY..ISADVVR 211
Ec-II  GPLGY.HNGK.D.QRTPARKSTSDTPF.V.KLNELPKVGIVYNY-ANASD 247

Bs-A   DALKSMYKG--IVIESYGSGGVPFEGRDILSKVNELIESGIVVVITTQCL 267
Ec-I   NF.RQPV.A--LILR...V.NA.-QNKAF.QELQ.ASDR.....NL...M 258
Ec-II  LPA.ALVDAGYDG.V.A.V.N.NLY-KSVFDTLATAAKT.TA..RSSR-V 295

Bs-A   EEGEDMSIYEVGRRVNQDLIIRSRNMNTEAIVPKLMWALGQSSDLPVVKR 317
Ec-I   SGKVN.GG.AT.NALAHAGV.GGAD.TV..TLT..HYL.S.EL.TETIRK 308
Ec-II  PT.ATTQDA..DD--AKYGFVA.GTL.PQKARVLLQL..T.TK.PQQIQQ 343

Bs-A   IMETPIADDVVL                                       329
Ec-I   A.SQN.RGELTPDD                                      322
Ec-II  .FNQY                                               348
```

FIG. 11. Comparison of amino acid sequences of asparaginases from *B. subtilis* and *E. coli*. All amino acid sequences are given in the one-letter code and were aligned to provide maximum sequence homology. Residues identical to those in *B. subtilis* asparaginase are given as dots; residues missing in one or two enzymes are given as dashes. Bs-A, *B. subtilis* asparaginase; Ec-I, *E. coli* asparaginase I; Ec-II, *E. coli* asparaginase II. The doubly underlined residues in the Ec-II sequence have been suggested to represent the substrate binding site (29). The number to the right of each line of sequence gives the number of the last residue in the sequence. Sequence data were taken from Fig. 3 and references 14 and 15.

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 σ^A consensus −10 recognition sequence and a −35 sequence with some homology to the −35 consensus sequence recognized by σ^A (Fig. 12). That this transcript is indeed produced by $E\sigma^A$ is consistent with its generation by RNA polymerase containing σ^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 2× 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 consensus recognition sequences for any known *B. subtilis* σ factor (Fig. 12 and data not shown) (18, 25, 27). While its transcription could be driven by an as-yet-uncharacterized σ factor, we have not obtained the 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 σ 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 $E\sigma^A$ (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.

```
                                  -35                                 -10
CATATAGGTATTAGGAAGTGTACGCGAATTGAATAATAATGTCTTGCGTATTTTATTTTCCGTGTCTATAATTGCATTAAA
                    -35                   -35          -10                  -10
                                          TTGACA       17-18nt              TATAAT
```

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 σ^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 σ^A are thinly underlined. Note that no transcript utilizing the latter putative promoter has been observed. nt, nucleotides.

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 σ^H, 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 *aspH* locus must 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 *spoIIIG* 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, including the holoenzymes $E\sigma^F$ and $E\sigma^G$, known to be active only 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 *spoIIIE* 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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