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Characterization of a *Bacillus subtilis* Sporulation Operon That Includes Genes for an RNA Polymerase σ Factor and for a Putative DD-Carboxypeptidase

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At early stages of sporulation, the *spoIIA* locus is transcribed as a tricistronic (1.7-kb) operon, coding for σ^F and for two proteins that modulate the activity of σ^F. The locus is transcribed as a longer (2.9-kb) transcript at the late stages of sporulation. We show here that the longer transcript contains an additional open reading frame whose product has extensive sequence homology with DD-carboxypeptidases; the corresponding gene is designated *dacF*. Cotranscription of a morphogene, such as *dacF*, with the gene for a σ factor suggests a way to couple transcription regulation with morphogenesis. The predicted N-terminal sequence of the DacF protein and the inhibition of sporulation by a translational *dacF-lacZ* fusion both suggest that the protein has a signal peptide for transport into or across a membrane. Expression of a *dacF-lacZ* transcriptional fusion was in the forespore. The 5′ end of the 2.9-kb transcript was determined by primer extension analysis. The region 5′ to the end showed no homology to promoters recognized by known σ factors but was homologous to the corresponding region of the forespore-specific 0.3-kb gene of *Bacillus subtilis*.

The formation of spores by *Bacillus subtilis* has become a paradigm for the analysis of cell differentiation in procaryotes. The process requires the temporally regulated expression of a large number of genes (18). A series of sporulation-specific σ factors and transcription regulators have been shown to be required. The pattern of gene expression is tied to the morphological events during spore formation. This is indicated by the evidence that different genes are transcribed in the different cell types involved in spore formation (6, 14, 32). It is also indicated by evidence that activation of RNA polymerase factor σ^E from pro-σ^E is in some way coupled to spore septum formation (1, 15, 36) and that a later morphological event may bring about activation of σ^K from pro-σ^K (5, 19). However, the actual mechanism of activation of pro-σ^E or pro-σ^K is not fully understood. Further, it may be that activation of these σ factors represents just one mechanism by which transcription regulation is coordinated with the morphological changes during sporulation and that other mechanisms will also be found. One of the most interesting problems in understanding spore formation is to elucidate those mechanisms.

We have been analyzing the regulation of transcription of the *spoIIA* locus, which is induced about 1 h after the start of spore formation (25, 29, 30, 40). The locus is expressed at this time as a tricistronic operon (11, 27, 29, 30). The third gene of the operon has been shown to code for an RNA polymerase factor, σ^F (8, 37). The first two genes of the operon code for proteins that modulate the activity of σ^F (31). A second burst of *spoIIA* transcription, detected with *lacZ* fusions, commences approximately 3 1/2 h after the start of sporulation (7). This corresponds to the time of appearance of a second *spoIIA* transcript that is substantially larger than the one detected earlier in sporulation (29, 30). We report here an analysis of the longer transcript. We show that it contains an additional long open reading frame (ORF) coding for a putative DD-carboxypeptidase, an enzyme associated with peptidoglycan synthesis. Cotranscription of a morphogene and a gene for a σ factor is one way that transcription regulation might be coordinated with morphological changes during spore formation.

(A preliminary report of this work was presented at the 5th International Conference on Genetics and Biotechnology of Bacilli, Asilomar, Calif., 1991 [41].)

MATERIALS AND METHODS

Strains. The *Escherichia coli* strain used was DH5α, F⁻ *endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1* λ⁻ *recA1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 φ80d*lacZ*ΔM15. The parental *B. subtilis* strain was MB24 *trpC2 metC3 rif-2* (40). *B. subtilis* strains containing a single copy of a plasmid integrated into the chromosome were constructed by transformation with the plasmid as donor and were maintained on medium containing 3 to 5 μg of chloramphenicol per ml. Strains with multiple copies of an integrated plasmid were maintained on medium containing 20 μg of chloramphenicol per ml. In all strains containing an integrated plasmid, the structure of the integrated plasmid was confirmed by Southern hybridizations of appropriately restricted DNA.

Plasmids. All plasmids were maintained in *E. coli* DH5α unless otherwise stated. Plasmids pHM2 (16) and pPP157 (40) were described previously. The extent of the *spoIIA* region present in different plasmids is indicated in Fig. 1. Plasmids pJF751, pJH101 (10), and pJM783 (9) were gifts from J. A. Hoch. Plasmid pPP155 was constructed by cloning a 1.8-kbp *Pvu*II fragment from pHM2 into the *Sma*I site of pJM783. Plasmid pPP159 contains a 0.7-kbp *Hin*dIII-*Eco*RI fragment from a derivative of pHM2 cloned into pUC18 cut with *Hin*dIII and *Eco*RI. Plasmid pPP209 was constructed by cloning a 0.84-kbp *Hin*dIII-*Sca*I fragment from pHM2 into pJH101 that had been cut with *Hin*dIII and *Eco*RV. Plasmid pPP212 contains a 2.4-kbp *Bgl*II fragment

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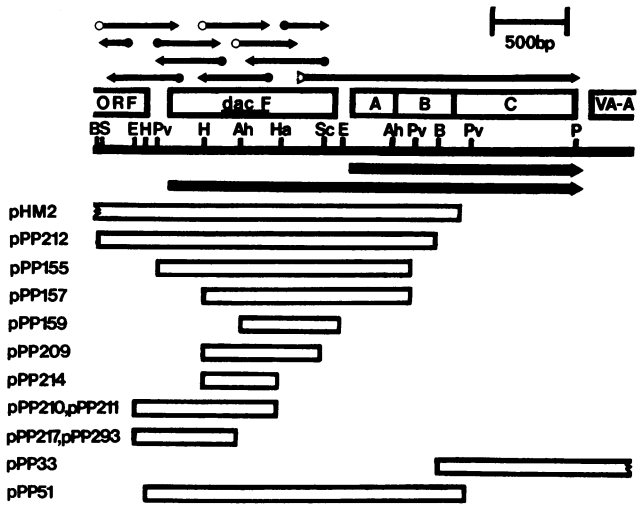

FIG. 1. Restriction map of the *spoIIA* region of the *B. subtilis* chromosome, showing the regions present in various plasmids used in this study. ORFs are shown as rectangles immediately above the restriction map; A, B, and C indicate the three ORFs of the *spoIIA* locus; VA-A indicates the first ORF of the *spoVA* locus. The sequencing strategy used is indicated at the top of the figure. The long arrow with an open trapezoid at its base above the restriction map shows the region that was sequenced previously (11); short arrows show regions sequenced (open circles indicate that M13 universal primer was used; closed circles indicate that oligonucleotides hybridizing to the corresponding regions of the *B. subtilis* DNA were used); thick arrows below the restriction map indicate the likely extents of the 1.9- and 2.9-kb transcripts. Restriction sites are indicated as follows: Ah, *Aha*III; B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Ha, *Hae*III; P, *Pst*I; Pv, *Pvu*II; S, *Sma*I; Sc, *Sca*I.

from pHM2 cloned into pUC19 that had been cut with *Bam*HI. It contains all of the *spoIIAA* gene, part of the *spoIIAB* gene, and the region extending about 2.0 kbp upstream from the *spoIIA* operon. Plasmid pPP214 was constructed by replacing the *Hin*dIII-*Eco*RV fragment in pJH101 with a 526-bp *Hin*dIII-*Hae*III fragment from pPP212. A 1,013-bp *Eco*RI-*Hae*III fragment of pPP212 was ligated to *Eco*RI-*Sma*I-digested pJM783 or *Eco*RI-*Sma*I-digested pUC19 to construct plasmids pPP211 and pPP210, respectively. A 745-bp *Eco*RI-*Aha*III fragment from pPP210 was ligated into *Eco*RI-*Sma*I-digested pJF751 to construct pPP217. The same 745-bp *Eco*RI-*Aha*III fragment (from pPP211) was ligated into *Eco*RI-*Sma*I-digested pJM783 to construct pPP293. All constructions were checked by analyzing digestion patterns obtained with the appropriate endonuclease.

DNA preparation and sequencing. DNA was prepared as described previously (40). DNA sequencing was by the dideoxy chain termination method of Sanger et al. (28), using a Sequenase kit according to the instructions of the manufacturer (United States Biochemical Corporation). Primers used were universal primers (forward and reverse) where appropriate. Primers corresponding to newly sequenced regions were also used and were synthesized with an Applied Biosystems 380B DNA synthesizer.

RNA analysis. RNA preparation and primer extension analysis were performed as described previously (40). The Northern (RNA) blot analysis of RNA fractionated by electrophoresis in agarose-formaldehyde was performed as described by Maniatis et al. (20). *B. subtilis* rRNA and mouse rRNA were used as size standards.

β-Galactosidase activity. Samples were assayed by a colorimetric method using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate as described by Nicholson and Setlow (22). Specific β-galactosidase activity is expressed as nanomoles of ONPG hydrolyzed per minute per milligram (dry weight) of bacteria.

Sporulation. Bacteria were induced to sporulate in modified Schaeffer's sporulation medium (MSSM), supplemented with chloramphenicol where indicated, as described previously (26). To determine the extent of sporulation, cultures were heated to 85°C for 20 min and survivors were enumerated by dilution and plating on sporulation agar, supplemented with chloramphenicol where appropriate. This heat-resistant count was compared with the viable count determined for the culture by sampling immediately before the heat treatment.

Germination. Spore fractions were isolated by the lysozyme-salt-detergent method of Nicholson and Setlow (22) and stored at 4°C in distilled water. Spore suspensions were heat activated at 65°C for 30 min and, after a 10-min equilibration period at 37°C, were diluted 10-fold in Penassay broth with 10 mM L-alanine to give a concentration of approximately 2×10^7 spores per ml. The optical density at 580 nm (OD_{580}) was measured. Samples were incubated at 37°C, and the OD_{580} was measured at 10-min intervals. The ability of spores to germinate was also tested in 10 mM Tris-HCl, pH 8.4, containing 10 mM L-alanine, 10 mM L-asparagine, 1 mM D-glucose, 1 mM D-fructose, and 10 mM KCl (22).

Other methods. All other methods were described previously (40).

Nucleotide sequence accession number. DNA sequences reported in this paper have been submitted to GenBank and given accession no. M85047.

RESULTS

Analysis of the RNA transcripts of the *spoIIA* operon. Savva and Mandelstam (29, 30) have shown by Northern blot analysis that two transcripts hybridized to a probe from the *spoIIA* region. The smaller transcript was present between 1 and 5 h after the start of sporulation (t_1 to t_5), and its reported size, 1.4 to 1.7 kb, corresponded reasonably to the predicted size of the tricistronic *spoIIA* operon. The larger transcript, estimated to be 2.6 kb, was first detected about 3 h after the start of sporulation. Neither transcript hybridized to a probe for the 5′ end of the locus *spoVA*, which is located immediately downstream from *spoIIA*, and Savva and Mandelstam have suggested that the 2.6-kb transcript extends upstream from the tricistronic *spoIIA* locus (29, 30). To expand on this analysis, we extracted RNA at 1-h intervals from *B. subtilis* MB24 induced to sporulate in MSSM. The RNA was fractionated by electrophoresis in an agarose-formaldehyde gel, blotted onto nitrocellulose paper, and hybridized to probes for different sections of the *spoIIA* region. Probes containing DNA internal to the tricistronic operon hybridized to two transcripts calculated to be 1.7 and 2.9 kb (Fig. 2a; a *Bgl*II-*Pst*I probe spanning *spoIIAC* and part of *spoIIAB* was used). A low basal level of the 1.7-kb transcript was present during vegetative growth; induction of that transcript began at about t_1, and the amount of transcript was maximal at about t_3. The 2.9-kb transcript was not detected until about t_4, and the maximal level was detected at t_5. The sizes and times of appearance agree reasonably with the results of Savva and Mandelstam (29, 30); it should be noted that Savva and Mandelstam employed a different sporulation

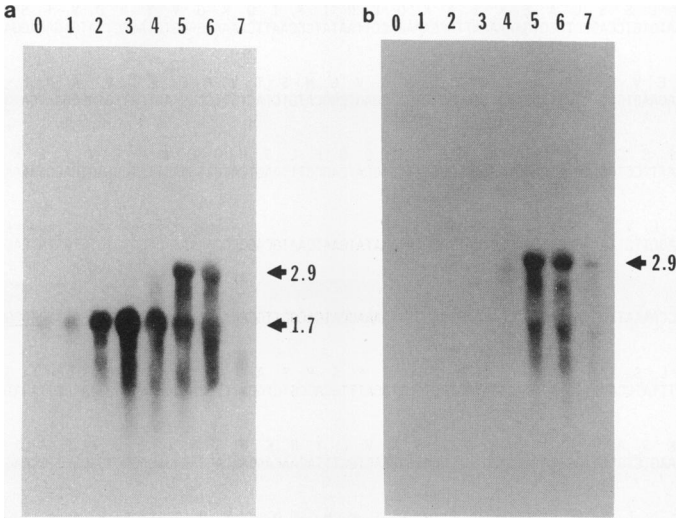

FIG. 2. Northern blot analysis of the *spoIIA* locus. RNA was extracted from MB24 at the start of sporulation (lanes 0) and at hourly intervals thereafter (lanes 1 through 7). (a) After denaturation, 20-μg samples of each RNA preparation were loaded into wells of an agarose-formaldehyde gel and subjected to electrophoresis overnight. After electrophoresis, the RNA was transferred to nitrocellulose paper. The nitrocellulose paper was hybridized with radiolabeled *Bgl*II-*Pst*I fragment spanning *spoIIAC* and part of *spoIIAB* (Fig. 1). The sizes of messages in kilobases are indicated. (b) Probe was removed from the membrane by using boiled water, and removal was confirmed by autoradiography. The nitrocellulose membrane was then rehybridized with the *Hin*dIII-*Sca*I fragment internal to *dac*F (Fig. 1). The 2.9-kb transcript is indicated.

system and that there is some variation in the time of sporulation events between their system and ours. Neither transcript hybridized to a probe for the 5′ end of the *spoVA* locus (data not shown), and only the 2.9-kb transcript (Fig. 2b) hybridized to a *Hin*dIII-*Sca*I probe internal to *dacF* (Fig. 1; see below). Appearance of the 2.9- but not the 1.7-kb transcript was blocked when the region upstream from the tricistronic *spoIIA* operon was disrupted by Campbell integration of pPP209 (Fig. 1; data not shown). Hybridization of the 2.9-kb transcript to the *Hin*dIII-*Sca*I probe and the disruption experiment established that the 2.9-kb transcript extends upstream from the 1.7-kb transcript; the hybridization of both transcripts to the *Bgl*II-*Pst*I probe suggests, but does not prove, that the two transcripts have similar 3′ ends.

DNA sequence of the region upstream from the *spoIIA* locus. The analysis of RNA transcripts had indicated that the region upstream from the *spoIIA* locus was cotranscribed with *spoIIA* at late stages of spore formation. We therefore determined the nucleotide sequence of 1.4 kbp upstream from the published sequence of the *spoIIA* region (11). The strategy used is indicated in Fig. 1. The complete sequence is shown in Fig. 3. Nucleotide 1378 corresponds to nucleotide 1 of Fort and Piggot (11). The sequence includes revisions in the region of nucleotides 5 to 15 of Fort and Piggot (11). Immediately upstream from the *spoIIA* locus is a long ORF. The ORF has the coding capacity (starting from the Met codon immediately downstream from the putative ribosome binding site) for a protein of 389 residues with a calculated molecular weight of 43,354. The free energy (ΔG) of binding for the ribosome binding site (GAAAtGGAGG) was −18.2 kcal/mol, calculated according to the rules of Tinoco et al. (38).

A similarity search of the protein base was carried out with the Bionet System by the Fasta-Mail program (24). The gene product of the unknown ORF had extensive similarity to penicillin-binding protein 5 (PBP 5) of *E. coli* (32.2% identity in a 397-amino-acid overlap) and PBP 6 of *E. coli* (34.2% identity in a 395-amino-acid overlap). Both are DD-carboxypeptidases. It also had similarity to PBP 5 of *B. subtilis*, which is a vegetative PBP and a DD-carboxypeptidase (39). The similarities are shown in Fig. 4. It is tentatively concluded that this ORF also codes for a DD-carboxypeptidase, and the gene is designated *dacF*. The first 23 amino acid residues show some conservation of sequence with the leader peptides of PBPs 5 and 6 of *E. coli* and have the general features of a leader peptide; it is inferred that the DacF protein also has a leader peptide. The serine residue marked with an asterisk in Fig. 4 has been shown to bind covalently to penicillin for several PBPs and is in a highly conserved region of PBPs (13). In addition, three other conserved regions which are present in all penicillin-interactive proteins (Fig. 4, crosses) (13) are also found in the DacF protein.

The region upstream from *dacF* has a potential stem-loop structure with a very strong similarity to Rho-independent terminators (Fig. 3). The ΔG of the stem-loop structure was calculated to be −32.8 kcal/mol. This apparent transcriptional terminator was preceded by the 3′ end of an ORF. From the DNA sequence, 114 amino acid residues of the C-terminal region have been predicted. This product has extensive homology with human purine nucleoside phosphorylase (43.6% identity in a 94-amino-acid overlap), suggesting that the *B. subtilis* gene (designated *pnp*) may code for purine nucleoside phosphorylase.

Determination of the 5′ end of the 2.9-kb transcript. From the sequence information and the Northern blot analysis, it seemed likely that the 5′ end of the 2.9-kb transcript would be located between the putative *pnp* terminator and the suggested ribosome binding site for *dacF* (residues 420 to 500 [Fig. 3]). Primer extension analysis was used to test this. Plasmid pPP212 was used for dideoxynucleotide sequence standards; pPP212 included all of *dacF* and *spoIIAA* and part of *spoIIAB* (Fig. 1). Two oligonucleotide primers that were designed to be complementary to the 2.9-kb transcript were

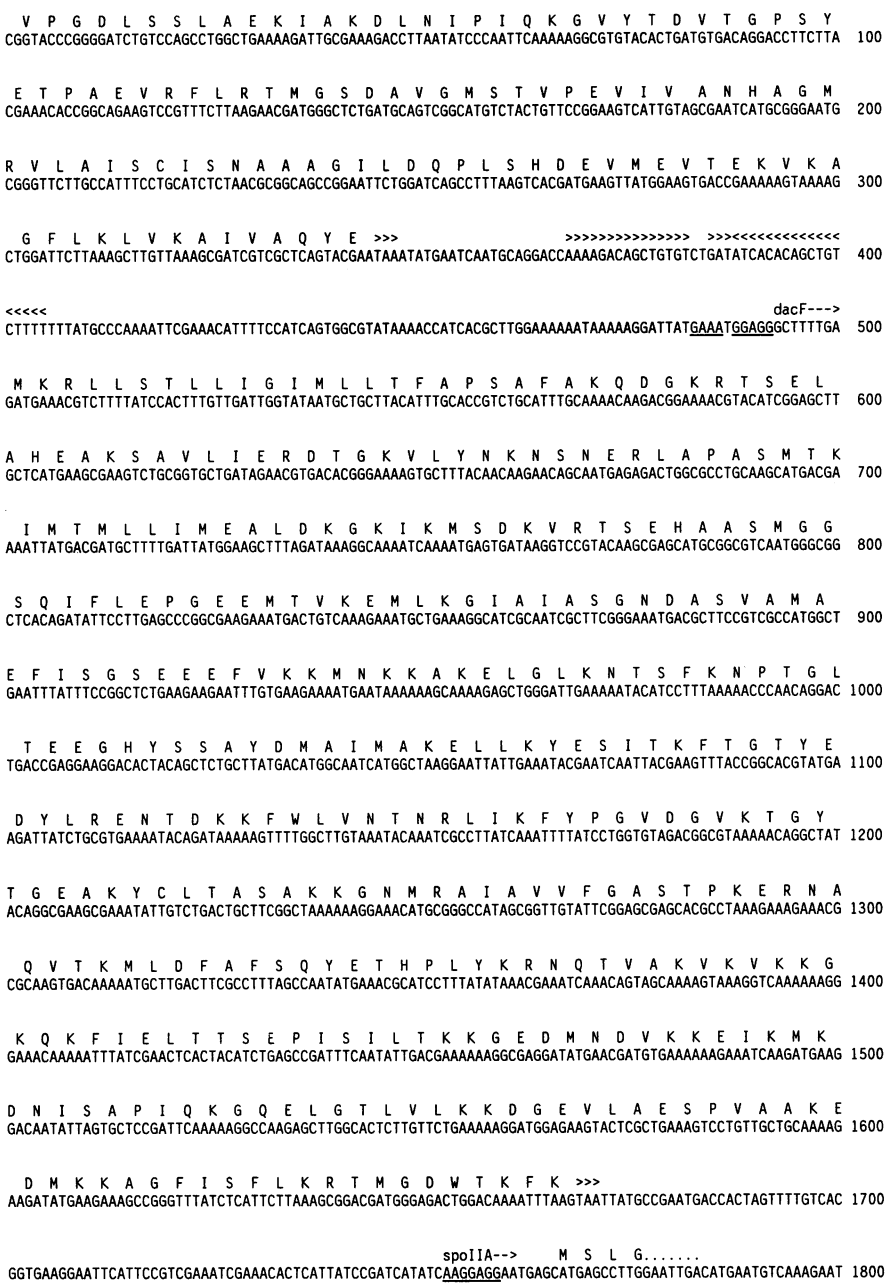

FIG. 3. Nucleotide sequence of the *spoIIA* upstream region. The amino acid sequences of long ORFs are shown below the nucleotide sequence, using the single-letter code. The putative ribosome binding site for the *dacF* is underlined. The large group of chevrons indicates the location of a potential stem-loop structure involved in transcription termination; groups of three chevrons indicate termination codons.

synthesized. RNA was extracted at hourly intervals from the end of exponential growth (see Materials and Methods). The reverse transcripts obtained with a 17-mer primer which corresponded to bases 553 to 569 in the sequence (Fig. 3) were characterized by electrophoresis with respect to the products of dideoxy sequencing reactions obtained with the same primer and pPP212. A transcriptional signal was obtained from samples taken from 4 to 7 h after the start of sporulation (t_4 to t_7) (Fig. 5). The same 5′ end of the transcript was identified with a second primer (Fig. 6), which corresponded to bases 610 to 625 in the sequence. Our best estimate from these and other experiments is that it corresponded to a G at position 477 (Fig. 3) in the nontranscribed DNA strand.

Analysis of transcriptional and translational fusions of *lacZ* to *dacF*. The 1,013-bp *Eco*RI-*Hae*III fragment containing the promoter region and part of the *dacF* structural gene (Fig. 1) was cloned into the integrative transcriptional *lacZ* fusion vector pJM783 in the correct orientation to yield pPP211. Chloramphenicol-resistant transformants of *B. subtilis* MB24 by pPP211 were selected, and an isolate was used for further study. It was confirmed by Southern hybridization that a single copy of the plasmid had integrated into the chromosome by a Campbell-like mechanism (data not

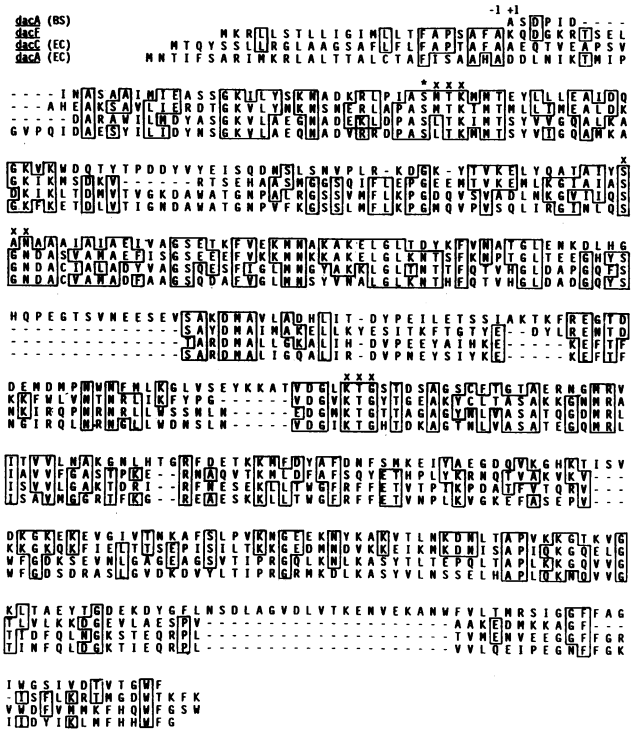

FIG. 4. Comparison of the complete amino acid sequence of the putative DD-carboxypeptidase coded for by *dacF* with the sequences of known DD-carboxypeptidases. The corresponding genes are indicated: *B. subtilis* (BS) *dacA* (39); *E. coli* (EC) *dacA* (3); and *E. coli dacC* (3). Identical amino acids are boxed; gaps introduced into the sequences are shown as dashes. The active-site serine residue is marked with an asterisk. The putative N terminus of the mature DacF protein is indicated (+1) by homology with the N terminus of the known DD-carboxypeptidases. The highly conserved regions common to all penicillin-interactive proteins are marked with crosses (13).

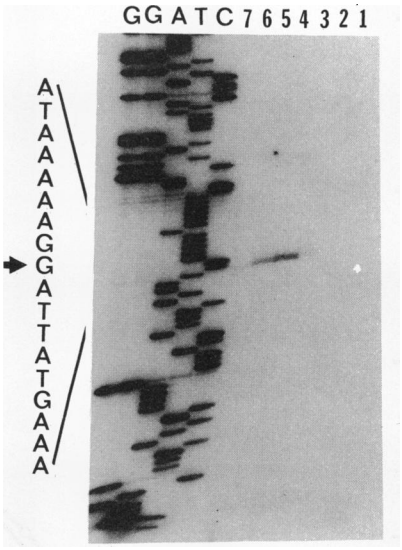

FIG. 5. Determination of the 5′ end of *dacF* mRNA by primer extension analysis with a 17-mer primer. RNA was extracted from MB24 at hourly intervals (lanes 1 through 7) after the start of sporulation. A sequencing ladder using the same primer is also shown. The letters above the lanes indicate which dideoxynucleotide was used to terminate the sequencing reaction. The sequence indicated is of the nontranscribed strand and is the complement of the sequence that can be read from the sequencing ladder. The transcription start site is indicated with an arrow.

shown). Strain MB24::pPP[illegible] was induced to sporulate in MSSM containing 3 μg of [illegible]hloramphenicol per ml, and samples were assayed for β-galactosidase activity. Enzyme synthesis began about 3.5 h after the start of sporulation (Fig. 7), and enzyme activity increased for about 3 h. This was in agreement with Northern blot analysis (Fig. 6). Repeated exp[illegible]riments suggested that *dacF-lacZ* was transcribed at a[illegible]out the same time as, or slightly before, *sspA-lacZ* a[illegible] *gdh*. However, the variability was such that firm conclusions cannot be drawn. An in-frame translational fusion of *dacF* to *lacZ* was constructed by inserting an *Eco*RI-*Aha*III fragment into pJF751. Chloramphenicol-resistant transformants of *B. subtilis* MB24 by pPP217 were selected, and an isolate was used for fur[illegible]her study. Southern hybridization confirmed that a single copy of the plasmid had integrated by a Campbell-like mechanism. Very little enzyme was produced by MB24::pPP217 (Fig. 7).

A substantial difference in the sporulation frequency between the strains with the transcriptional and translational *dacF-lacZ* fusions was seen. Strain MB24::pPP211 showed 86% sporulation in the experiment described above. This is similar to the frequency obtained with the parent strain, MB24. However, strain MB24::pPP217, containing the translational fusion, gave 33% sporulation under the same conditions. To see whether this effect on sporulation was enhanced by increased copy numbers of the fusion, strains MB24::pPP211 and MB24::pPP217 were subcultured in the presence of 20 μg of chloramphenicol per ml to select for derivatives with tandem repeats of the integrated plasmids. A clone of MB24::pPP211 shown to contain three copies of the integrated plasmid by the method of Piggot and Curtis

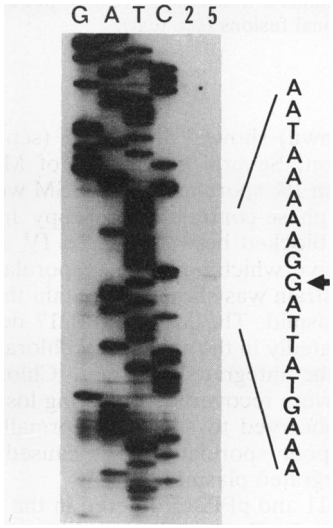

FIG. 6. Determination of the 5′ end of *dacF* mRNA by primer extension analysis with a 16-mer primer. RNA was extracted from MB24 at 2 and 5 h after the start of sporulation. A sequencing ladder using the same primer is also shown. The letters above the lanes indicate which dideoxynucleotide was used to terminate the sequencing reaction. The sequence indicated is of the nontranscribed strand and is the complement of the sequence that can be read from the sequencing ladder. The transcription start site is indicated with an arrow.

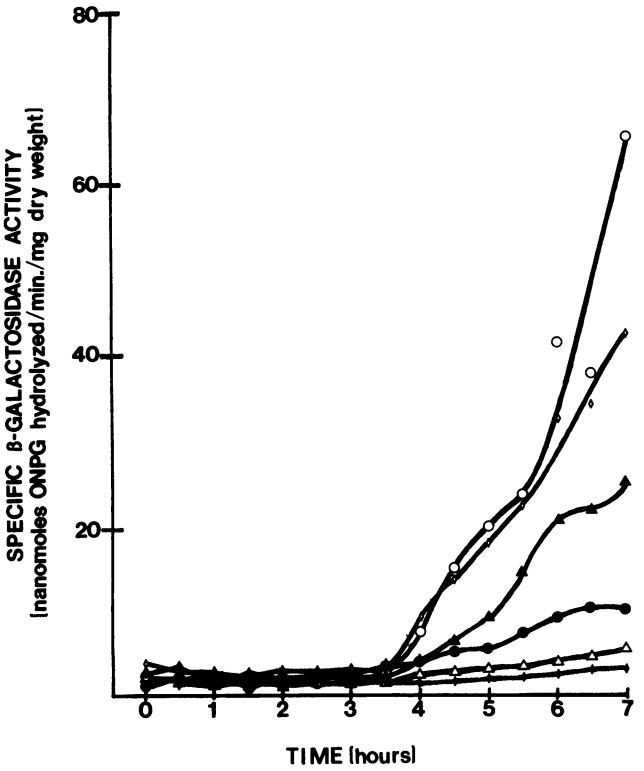

FIG. 7. Formation of β-galactosidase by strain MB24 containing integrated plasmids with *dacF-lacZ* fusions. Specific β-galactosidase activity is expressed as nanomoles of ONPG hydrolyzed per minute per milligram (dry weight) of bacteria. The time is the hour after the start of sporulation, which is defined as the end of exponential growth in MSSM containing, except for MB24, 3 μg of chloramphenicol per ml. Symbols: ●, MB24::pPP211 (one copy); ○, MB24::pPP211 (three copies); △, MB24::pPP217 (one copy); ▲, MB24::pPP217 (three copies); ◇, MB24::pPP293 (three copies); ◆, MB24. pPP217 contains a translational fusion; pPP211 and pPP293 contain transcriptional fusions (see text).

(26; data not shown) showed 55 to 65% (separate experiments) sporulation. Several derivatives of MB24::pPP217 that gave less than 1% sporulation in MSSM were obtained; examination by phase-contrast microscopy indicated that sporulation was blocked between stages IV and V. DNA from one derivative which gave 0.3% sporulation was extracted, and the strain was shown to contain three copies of the integrated plasmid. The MB24::pPP217 derivative was subcultured repeatedly in the absence of chloramphenicol to permit loss of the integrated plasmid. Chloramphenicol-sensitive clones were recovered, indicating loss of the plasmid, and were observed to sporulate normally. This confirmed that the poor sporulation was caused by multiple copies of the integrated plasmid.

Plasmids pPP211 and pPP217 differed in the extent of the cloned region (Fig. 1). To test whether this was the cause of the different effects on sporulation, plasmid pPP293 was constructed. This plasmid contained a *dacF-lacZ* transcriptional fusion in which the fused *dacF* fragment was the same *Eco*RI-*Aha*III fragment that was present in the translational fusion in pPP217. A clone of MB24::pPP293 that contained three copies of the integrated plasmid was obtained. This clone sporulated efficiently (68%), so that the poor sporulation of MB24::pPP217 could be ascribed to the fact that it contained a translational fusion to *lacZ*.

TABLE 1. β-Galactosidase activities of germinated spores of strains containing different *lacZ* fusions

Germinant sample (min)[a]	β-Galactosidase sp act (nmol of ONPG hydrolyzed/min per mg [dry wt] of bacteria)				
	MB24::pPP293 (*dacF-lacZ*)	SC262 (*sspA-lacZ*)[b]	MB24::pPP153 (*spoVA-lacZ*)[c]	MB24::SPβ:: *cotA-lacZ*[d]	No fusion
10	69	105	35	0.3	1.0
20	68	107	32	0.6	1.0
30	59	107	28	0.5	1.1
40	53	ND[e]	16	0.4	1.0
Control	3	13	3	1.0	1.0

[a] Samples were taken at various times after the addition of germinants or from cultures with no germinants.
[b] Strain SC262 containing the *sspA-lacZ* fusion was provided by Simon Cutting.
[c] Strain MB24::pPP153 containing a *spoVA-lacZ* fusion was provided by Brian Moldover.
[d] Strain MB24::SPβ::*cotA-lacZ* was provided by Adriano Henriques.
[e] ND, not determined.

The timing of *dacF-lacZ* expression was not affected by having tandem repeats of transcriptional or translational fusions (Fig. 7). The amount of enzyme produced was substantially increased compared with production by strains with single copies. The increased production was particularly noticeable for the strain (MB24::pPP217) containing the translational fusion.

Location of *dacF* transcription. To test the location of *dacF* transcription, spores were prepared from a strain containing a *dacF-lacZ* transcriptional fusion (three copies of the integrated plasmid pPP293 in strain MB24). Spores were heat activated and germinated by the addition of L-alanine, L-asparagine, D-glucose, D-fructose, and KCl. β-Galactosidase was assayed at intervals after the addition of germinants. Substantial β-galactosidase activity was detected (Table 1). This was also the case for germinated spores of strains containing *lacZ* fused to *sspA* and to *spoVA*, which are known to be expressed in the forespore (7, 37). In contrast, germinated spores from a strain containing *lacZ* fused to *cotA*, which is expressed in the mother cell (14, 17), displayed insignificant β-galactosidase activity. In each case, germination was largely complete within 40 min as indicated by a fall in OD_{580} of about 50% following the addition of germinants. The presence of the *cotA-lacZ* fusion was confirmed by showing β-galactosidase activity in cultures of sporulating bacteria derived from the germinated spores. Although the various *lacZ* fusions are in strains of disparate genetic backgrounds, it seems reasonable to conclude that *dacF-lacZ* is expressed in the forespore.

Integrative disruption of *dacF*. To test for a possible role of *dacF*, it was disrupted by the integration of plasmids pPP209 and pPP214. These plasmids were transformed into strain MB24, selecting for chloramphenicol resistance. The resulting strains MB24::pPP209 and MB24::pPP214 gave 78 and 72% sporulation, respectively, in MSSM containing 3 μg of chloramphenicol per ml, while MB24 gave 82% sporulation in MSSM in a parallel experiment. Germination rates were measured by monitoring the fall in the OD_{580} of spore suspensions upon the addition of germinants. There was no indication that disruption of *dacF* impaired germination. Southern analysis confirmed that the strains contained a

single copy of the plasmid integrated by a Campbell-like mechanism.

DISCUSSION

Transcription of the *spoIIA* locus is first induced about 1 h after the start of sporulation (25, 29, 30, 40). At that time, it is transcribed as a tricistronic operon (11, 27, 29, 30). A second period of *spoIIA* transcription is initiated 2 to 3 h later (7). The transcript during this second period is substantially larger than during the first period (29, 30). We show here that, in addition to the tricistronic region, it encodes a large ORF located immediately upstream from the tricistronic region. This ORF is preceded by a putative ribosome binding site, and it is 389 residues long. The predicted protein product shares 30 to 35% identity with DD-carboxypeptidases from *E. coli* and *B. subtilis* (Fig. 4). This is similar to the degree of identity shared by the different DD-carboxypeptidases. The ORF contains the amino acid sequence motifs that are characteristic of all penicillin-interactive proteins. Consequently, we think it likely that the ORF also codes for a DD-carboxypeptidase that will bind penicillin, although we have not established that this is the case; we designate the gene *dacF*. *dacF* is clearly distinct in nucleotide sequence from the two genes (*dacA* and *dacB*) that code for PBPs 5 and 5a (or 5*), respectively, the known DD-carboxypeptidases of *B. subtilis* (4, 39). Analysis of a translational *lacZ* fusion indicates that *dacF* is translated. The location of *dacF-lacZ* transcription in the forespore would suggest that the DacF protein is involved in primordial germ cell wall synthesis. This is in contradistinction to DacB, which is located in the mother cell and is thought to be involved in cortex synthesis (4). However, strains with *dacF* disrupted showed no obvious phenotypic changes, and, in particular, the formation and germination of heat-resistant spores appeared normal. Consequently, the role of the DacF protein is unclear. It may be that it causes some subtle change in peptidoglycan structure that is not detected by testing the heat resistance or germination of spores. Alternatively, its function may only become apparent when *dacA* and/or *dacB* is inactivated. It should be noted that a strain of *E. coli* with *dacA* and *dacC* deleted shows no obvious morphological difference when compared with the parent strain (2).

The predicted amino acid sequence of the protein product of *dacF* suggests a signal peptide for membrane translocation. Indirect evidence of the export of the DacF protein into or across a membrane comes from the analysis of a translational *dacF-lacZ* fusion. Elevated expression of such a fusion, obtained with a strain containing tandem repeats of an integrated plasmid, blocked sporulation, whereas elevated expression of a transcriptional *dacF-lacZ* fusion did not. In addition, sporulation was not blocked by the much higher levels of β-galactosidase production obtained with either transcriptional or translational *spoIIAA-lacZ* fusions (7). This would suggest that it was the fused DacF-LacZ protein that blocked sporulation. It is notable that, when the background activity is subtracted, the strain with three copies of the translational *lacZ* fusion produced much more than three times the amount of β-galactosidase than the strain with a single copy. This would be consistent with disruption of the protein export machinery rendering β-galactosidase accessible to its substrate. These results are reminiscent of those obtained with *E. coli*, in which high-level expression of exported LacZ hybrid proteins was lethal (33).

```
                                                +1
                                                *
dacF            TTTCCATCAGTGGCGTATAAAA--CCATCACGCTTGG-AAAAAATAAAAAGG
                                                *
0.3 Kb Gene     TTTCCAATAATTTTAACGAAAGTTCCATCTGATTTAACAAAAGATAAAACA
```

FIG. 8. Comparison of the sequences upstream from the transcription start point for *dacF* and for the 0.3-kb gene (34). The transcription start point is indicated with an asterisk.

A single 5′ end of the *dacF-spoIIA* mRNA sequence was identified by primer extension analysis. It is tentatively assumed to identify the transcription start point, although the possibility of processing has not been excluded. The sequence immediately upstream from this start point shows extensive homology with the sequence upstream of the 0.3-kb gene of *B. subtilis* (34) (Fig. 8). The 0.3-kb gene is also transcribed late in sporulation, is expressed in the forespore, and has no known function (23). The promoter sequence shows no homology to promoters recognized by σ^G or σ^F, the σ factors known to function in the forespore (37), or to promoters recognized by any other known σ factor. The time and location of *dacF* transcription are consistent with transcription by E-σ^G. However, preliminary experiments indicate that a *dacF-lacZ* transcriptional fusion in the *amy* locus is expressed in a strain containing the *spoIIIGΔ1* mutation (42), suggesting that σ^G is not required for *dacF* transcription. Thus, the factors controlling transcription are unknown; it is intriguing that transcription of these genes begins at about the time that forespore DNA is changing from the B to the A form (21).

Cotranscription of *dacF* with the genes of the tricistronic *spoIIA* locus potentially provides a mechanism to coordinate modulation of transcription (by production of σ^F) with morphological changes (facilitated by DacF). This differs from other types of coordination of morphological events with transcription regulation, for example, activation of σ factors E and K by proteolysis of pro forms, which is thought to be triggered by morphological events (15, 17, 35). The actual significance of the cotranscription of *dacF* and *spoIIA* is unclear, as there is no obvious sporulation phenotype resulting from disruption of the transcript. The formation of distinct *spoIIA* transcripts, one of which is made before spore septum formation (12) and one of which is made well after the time of spore septum formation, is intriguing. The significance of this observation is also uncertain, because of the lack of a clear phenotype resulting from disruption of the late transcript.

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