Characterization of *cotJ*, a σ^E -Controlled Operon Affecting the Polypeptide Composition of the Coat of *Bacillus subtilis* Spores

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The outermost protective structure found in endospores of *Bacillus subtilis* is a thick protein shell known as the coat, which makes a key contribution to the resistance properties of the mature spore and also plays a role in its interaction with compounds able to trigger germination. The coat is organized as a lamellar inner layer and an electron-dense outer layer and has a complex polypeptide composition. Here we report the cloning and characterization of an operon, *cotJ*, located at about 62° on the *B. subtilis* genetic map, whose inactivation results in the production of spores with an altered pattern of coat polypeptides. The *cotJ* operon was identified by screening a random library of *lacZ* transcriptional fusions for a conditional (inducer-dependent) Lac⁺ phenotype in cells of a strain in which the structural gene (*spoIIGB*) for the early-acting, mother-cell-specific transcriptional factor σ^E was placed under the control of the IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible *P_{spac}* promoter. Sequence analysis of cloned DNA from the *cotJ* region complemented by genetic experiments revealed a tricistronic operon preceded by a strong σ^E -like promoter. Expression of an SP β -borne *cotJ-lacZ* fusion commences at around h 2 of sporulation, as does expression of other σ^E -dependent genes, and shows an absolute requirement for σ^E . Studies with double-reporter strains bearing a *cotJ-gusA* fusion and *lacZ* fusions to other *cot* genes confirmed that expression of *cotJ* is initiated during sporulation prior to activation of genes known to encode coat structural proteins (with the sole exception of *cotE*). An in vitro-constructed insertion-deletion mutation in *cotJ* resulted in the formation of spores with no detectable morphological or resistance deficiency. However, examination of the profile of electrophoretically separated spore coat proteins from the null mutant revealed a pattern that was essentially identical to that of a wild-type strain in the range of 12 to 65 kDa, except for polypeptides of 17 and 24 kDa, the putative products of the second (*cotJB*) and third (*cotJC*) cistrons of the operon, that were missing or reduced in amount in the coat of the mutant. Polypeptides of the same apparent sizes are detected in spores of a *cotE* null mutant, on which basis we infer that the products of the *cotJ* operon are required for the normal formation of the inner layers of the coat or are themselves structural components of the coat. Because the onset of *cotJ* transcription is temporally coincident with the appearance of active σ^E , we speculate that *cotJ*-encoded products may be involved in an early stage of coat assembly.

A response to nutritional stress of cells of the gram-positive soil bacterium *Bacillus subtilis* is a differentiation process that results in the formation of endospores, a dormant cell type with unique resistance properties (16, 36, 43). Endospore development requires a series of complex morphological changes. These begin with an asymmetric division producing two cellular compartments of very different sizes. The two differentiated cell types formed after septation, each of which contains one complete copy of the chromosome, exhibit different programs of gene expression and differ in their developmental fates (16, 36, 43). The smaller cell type, the prespore, is engulfed by the larger one, the mother cell, and ultimately becomes the spore. The engulfed prespore, or forespore, exists as a free protoplast delimited by a double membrane system

inside the mother-cell compartment. During later stages of sporulation, the forespore cytoplasm becomes surrounded by two major protective layers, both of which are assembled from components present or synthesized in the mother-cell type (7, 16, 43). The more internal of these structures is a thick layer of a modified form of peptidoglycan of chemotype I known as the cortex (7, 43). The outer structure, known as the coat, is formed by the assembly of different polypeptides (2, 16, 43). In *B. subtilis* spores, the coat is differentiated into a lamella-like inner layer and an electron-dense outer-layer (2) and is composed of more than 15 different polypeptides ranging in size from 5 to 65 kDa (2, 16). Although it has always been difficult to implicate discrete structures in different resistance properties of spores, it is generally assumed that the cortex participates mainly in maintaining the dehydrated state of the spore thought to be responsible for its heat resistance (16, 43), whereas the coat, and in particular its outer layer, has been implicated in conferring resistance to lysozyme (2, 16, 43, 60). In addition, the coat may play a role in the initial interaction of the spore with germinants and therefore in the shaping of the germination response (2, 16).

Coat assembly in *B. subtilis* is a complex process that extends for a long time in the mother cell and involves at least two

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distinct classes of genes. One class is defined by genes whose products need not be major components of the spore coat but rather act as morphogenetic proteins governing the assembly of spore structural components. The *spoIVA* (8, 43, 44, 50), *cotE* (60), and *spoVID* (5) genes are in this class. In addition to its morphogenetic role, CotE is also a prominent coat component (60), whereas the SpoIVA and SpoVID proteins are unlikely to have a structural role (5, 8, 15, 43, 44, 50). A second class is defined by a growing group of genes encoding coat structural components. So far, genes for 13 coat proteins have been identified: *cotA*, *cotB*, *cotC*, *cotD* (13), *cotF* (11), *cotG* (45), *cotT* (3), *cotX*, *cotV*, *cotW*, *cotY*, *cotZ* (58), and *spoVIA* (22). Mutations in genes grouped in the first class have dramatic effects on coat morphology and resistance and germination properties (5, 8, 15, 43, 44, 50, 60). In contrast, null mutations in any of the structural genes belonging to the second class have almost no effect on ultrastructure or spore resistance or germination properties, a result that indicates either modest roles for individual components or extensive functional redundancy among them (3, 11, 13, 45, 58).

The expression of genes required for coat synthesis and assembly is regulated directly by at least two different RNA polymerase sigma factors (σ^E and σ^K) and two DNA binding proteins, SpoIID and GerE (19, 61, 62). The developmental program of gene expression in the mother cell is initiated with the activation of pro- σ^E to its transcriptionally competent form, σ^E (16, 36, 62), which then directs the transcription of the *spoIVA* (44, 50), *spoVID* (5), and *cotE* (from its p_1 promoter) (62) genes, all of which belong to the morphogenetic class, and the *spoIID* gene, which encodes a transcription regulator (19, 31, 33, 51). The product of the *spoIID* gene acts in conjunction with σ^E -containing RNA polymerase ($E\sigma^E$) to direct transcription of genes in the next temporal class, including *cotE* (from its P_2 promoter) (62) and *sigK* (32), which encodes σ^K (31). The bulk of the expression of *cot* structural genes is dependent on σ^K activity (11, 45, 59, 62). For example, *cotA* and *cotD* are transcribed by the σ^K form of RNA polymerase ($E\sigma^K$). σ^K also directs transcription of the regulatory gene *gerE* (10), whose product acts together with $E\sigma^K$ to direct expression of *cotB* and *cotC* (61, 62).

In order to identify additional genes whose products may play roles in morphogenetic processes, this laboratory started a systematic search for σ^E -dependent genes. Our strategy involved screening of a random library of *lacZ* transcriptional fusions carried in the temperate SP β bacteriophage, in lysogens of a strain in which the structural gene (*spoIIGB*) for the early-acting, mother cell σ^E factor was placed under the control of the IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible P_{spac} promoter. This search previously led to the identification, cloning and analysis of *spoVID*, required for normal coat assembly (5), and *spoVR*, involved in spore cortex synthesis (6). Here we report the cloning and characterization of an operon, *cotJ*, that encodes products required for the normal assembly of the spore coat. We mapped *cotJ* to the 62° region of the *B. subtilis* chromosome, a location distinct from that of previously characterized *cot* genes. We show that expression of *cotJ* during development is absolutely dependent on *spoIIGB*, encoding σ^E , but not on *spoIIG* (encoding σ^G) or *spoIVCB* (encoding the N-terminal moiety of the late-acting mother cell factor σ^K), and that it precedes expression of *cotA*, but not *cotE*, by at least 1 h. Because the onset of *cotJ* transcription during sporulation coincides temporally with the appearance of the active form of σ^E , we speculate that the products of the *cotJ* operon may play a role at an early stage of coat assembly.

MATERIALS AND METHODS

Bacterial strains, media, and general techniques. The *B. subtilis* strains constructed in this study are listed in Table 1. All other strains are referenced in the text. The collection of strains bearing different *spo* mutations are all isogenic with the Spo⁺ strain MB24 (42), except for the indicated markers. The *Escherichia coli* strain DH5 α (Bethesda Research Laboratories [BRL]) was used for all plasmid constructions, and strain DH5 α F' (BRL) was used for the construction and amplification of derivatives of phages M13mp18 and M13mp19 essentially as described previously (46). Luria-Bertani medium in its liquid or solid version (solidified with 1.6% agar) was routinely used for growth and maintenance of *B. subtilis* and *E. coli*. Difco sporulation medium (DSM) was used to induce sporulation of *B. subtilis* by nutrient exhaustion in liquid culture or on plates (41). The chromogenic substrates 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) for β -galactosidase and 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc) for β -glucuronidase were used on plates at a concentration of 40 μ g/ml. Detection of β -galactosidase or β -glucuronidase synthesis was also accomplished by spraying colonies of *B. subtilis* with a solution of the fluorescent substrate 4-methylumbelliferyl- β -D-galactopyranoside (MUG) or 4-methylumbelliferyl- β -D-glucuronide (MUGluc), respectively, and then examining the plates with a short-wavelength UV lamp. Whenever required, IPTG was added to liquid or solid growth medium to a final concentration of 1 mM. Antibiotic-resistant transformants of *E. coli* were selected or screened for at the following antibiotic concentrations: ampicillin (Ap^r), 75 μ g/ml; kanamycin (Km^r), 25 μ g/ml; and spectinomycin (Sp^r), 50 μ g/ml. Antibiotic resistance phenotypes of *B. subtilis* transformants or transductants were selected or screened for at the following concentrations: chloramphenicol (Cm^r), 5 μ g/ml; macrolides-lincosamide-streptogramin B (MLS^r), 1 μ g per ml; erythromycin, 25 μ g/ml; lincomycin; spectinomycin (Sp^r), 50 μ g/ml; kanamycin (Km^r), 10 μ g/ml; phleomycin (Pm^r), 2 μ g/ml; and neomycin (Nm^r), 3 μ g/ml on plates and 1 μ g/ml on liquid media. All DNA manipulations and transformation in *E. coli* were performed essentially as described by Sambrook et al. (46). DNA fragments for cloning or labeling were purified from agarose gels by using the GeneClean II kit from Bio 101, Inc.

Strain constructions. In strain EU8701 the chromosomal allele of the *spoIIGB* gene (encoding σ^E) has been replaced by an in vitro-constructed insertion-deletion of an erythromycin resistance cassette (29). Strain EU8702 (5) carries a deletion of the *spoIIGB* gene and plasmid pDG180 (a gift from P. Stragier), which carries a P_{spac} (57) fusion to a truncated form of the *spoIIGB* gene. Strain EU101 (Table 1) contains the *spoIIGB*:*erm* marker and a fusion of P_{spac} to a full-length copy of the *spoIIGB* gene ectopically inserted into the chromosome (54). The *spoIIGB*:*erm* allele was introduced into MB24 by transforming this strain to Em^r with chromosomal DNA prepared from strain EU8701. Likewise, chromosomal DNA from strains BK541 (*spoIIGB*:*erm*) (33), PY403 (*spoIIM*:Tn917 Ω HU287) (48), MO1027 (*spoIVCB*:*erm*) (obtained from P. Stragier), and AD28 (*cotE*:*cat*) (15) was used to construct congenic derivatives of strain MB24 containing the indicated alleles by transformation followed by selection for the associated drug resistance markers. The *spoIVA* mutation, obtained from the *Bacillus* genetic stock center strain 1S46, was introduced into MB24 by cotransformation with the Trp⁺ marker (approximately 40% cotransformation between the *spoIVA*178 and *trpC2* markers was found). Transduction and transformation crosses were essentially as described before (12).

Cloning of the *cotJ* operon. A phage, SP β E13, carrying a transcriptional fusion of *cotJ* to *lacZ* was identified, and the chromosomal DNA insert was cloned by previously described methods (5, 6). The procedure included transducing strain EU101 (Table 1) to chloramphenicol resistance with an SP β library (provided by P. Zuber) containing random 1- to 5-kb *Sau*3AI fragments of the *B. subtilis* chromosome cloned in front of the *lacZ* gene. Transductants were selected on DSM agar plates containing 40 μ g of X-Gal per ml, 1 mM IPTG, and 5 μ g of chloramphenicol per ml. We first prepared chromosomal DNA from a transductant, AH692 [EU101 (SP β E13)] (Table 1), and used it to transform competent cells of strain AH680, carrying plasmid pTV17, to Cm^r, screening for a Lac⁺ phenotype. Because strain AH680 is SP β ^r, transformants were expected to arise by a double-crossover event between Tn917 sequences in the plasmid and homologous sequences in the transforming DNA. Such a recombinational event would transfer the DNA derived from the *cotJ* region, flanked by *lacZ* sequences at its 3' end and by the *cat* gene and pBR322 sequences at its 5' end, to the pTV17 plasmid, via the Tn917 sequences present on both recombining molecules (5). This double crossover produced plasmid pTVE13, carried in strain AH694 (Table 1). The plasmid was then purified and used as a template in supercoil sequencing reactions primed from a position within the *lacZ* gene (see below). This allowed us to quickly obtain the sequence across the library fusion site, which revealed that the *lacZ* proximal *Sau*3AI site (at position 386 in the sequence represented in Fig. 2) was within a previously unidentified open reading frame (ORF), later shown to be the first ORF in the *cotJ* operon and designated *cotA*. The plasmid initially used to construct the library, pZ Δ 328 (63), has *Sal*I sites on both sides of the *Bam*HI site used for cloning the *Sau*3AI genomic fragments. Therefore, the size of the chromosomal DNA insert in pTVE13 could be estimated to be about 1.8 kb by digestion of the plasmid with *Sal*I.

The cloning of DNA downstream of the library fusion site was accomplished in three steps. Because the 1.8-kb *Sal*I fragment proved difficult to clone in *E. coli*, an observation that suggests that sequences upstream of *cotJ* are not well tolerated in this host when present in multiple copies, we first cloned a 4.2-kb

TABLE 1. *Bacillus subtilis* strains used in this study^a

Strain	Genotype and phenotype and/or description	Source (reference)
MB24	<i>trpC2 metC3</i> Spo ⁺	P. Piggot (42)
EU8701	<i>trpC2 pheA1 spoIIGBΔ::erm</i> Em ^r	Laboratory stock (29)
EU8702	<i>trpC2 pheA1 spoIIGBΔ::erm</i> (pDG180) Pm ^r Em ^r	Laboratory stock (5)
EU101	<i>spoIIGBΔ::erm chr::Tn917ΩHU160::P_{spac-spoIIGB}</i> Pm ^r Em ^r	Laboratory stock (54)
AH680	SPβ ^s (pTV17) Tc ^r Em ^r ; prototroph	Laboratory stock; P. Youngman
ZB307	SPβ <i>c2del2::Tn917::pSK10Δ6</i> MLS ^r	Laboratory stock; P. Zuber
1A5	<i>glyB133 metC3 tre-12 trpC2</i>	BGSC ^b
1A628	<i>trpC2 zbj-82::Tn917</i> (SPβ <i>c2del2</i>)	BGSC
AH64	<i>trpC2 metC3 cotEΔ::cat</i> Cm ^r	This study
AH72	<i>trpC2 metC3 spoIVA178</i>	This study
AH74	<i>trpC2 metC3 spoIIGBΔ::erm</i> Em ^r	This study
AH75	<i>trpC2 metC3 spoIIM::Tn917ΩHU287</i> MLS ^r	This study
AH76	<i>trpC2 metC3 spoIIDΔ::erm</i> Em ^r	This study
AH77	<i>trpC2 metC3 spoIVCBΔ::erm</i> Em ^r	This study
AH394	<i>trpC2 metC3 SPβ::pTKlac</i> (SPβ <i>lacZ</i>) Cm ^r MLS ^r	This study
AH692	EU101 (SPβE13) Em ^r Pm ^r Cm ^r	This study
AH694	PY222(pTVE13) Cm ^r	This study
AH695	SPβ <i>c2 del2::Tn917::SPβ spoIID-lacZ</i> Cm ^r MLS ^r	This study
AH739	SPβ <i>c2 del2::Tn917::pSK10Δ6::pCotE_{p2}</i> (SP β <i>cotE-lacZ</i>) Cm ^r MLS ^r	This study
AH760	ZB307 (SPβ <i>cotJ-lacZ</i>) Cm ^r MLS ^r	This study
AH770	<i>trpC2 metC3 SPβ cotJ-lacZ</i> Cm ^r MLS ^r	This study
AH775	<i>trpC2 metC3 spoIIGB55 SPβ cotJ-lacZ</i> Cm ^r MLS ^r	This study
AH776	<i>trpC2 metC3 spoIIGBΔ::erm SPβ cotJ-lacZ</i> Cm ^r MLS ^r	This study
AH777	<i>trpC2 metC3 spoIIM::Tn917ΩHU287 SPβ cotJ-lacZ</i> Cm ^r MLS ^r	This study
AH780	<i>trpC2 metC3 spoIID83 SPβ cotJ-lacZ</i> Cm ^r MLS ^r	This study
AH781	<i>trpC2 metC3 spoIIDΔ::erm SPβ cotJ-lacZ</i> Cm ^r MLS ^r	This study
AH782	<i>trpC2 spoIIGΔ1 SPβ cotJ-lacZ</i> Cm ^r MLS ^r	This study
AH783	<i>trpC2 metC3 spoIVA178 SPβ cotJ-lacZ</i> Cm ^r MLS ^r	This study
AH784	<i>trpC2 metC3 spoIVCB23 SPβ cotJ-lacZ</i> Cm ^r MLS ^r	This study
AH785	<i>trpC2 metC3 spoIVCBΔ::erm SPβ cotJ-lacZ</i> Cm ^r MLS ^r	This study
AH925	<i>trpC2 metC3 cotJΔ::km</i> Sp ^s Km ^r	This study
AH1037	<i>trpC2 metC3 cotJΔ::gusA neo</i> Nm ^r	This study
AH1038	<i>trpC2 metC3 cotJΔ::neo gusA</i> Nm ^r	This study
AH1058	<i>trpC2 metC3 cotJ::pAH238 (cotJ-gusA neo)</i> Nm ^r	This study
AH1059	<i>trpC2 metC3 cotJ::pAH239 (cotJ-neo gusA)</i> Nm ^r	This study
AH1060	<i>trpC2 metC3 cotJΔ::gusA neo SPβ spoIID-lacZ cat</i> Nm ^r Cm ^r	This study
AH1061	<i>trpC2 metC3 cotJΔ::gusA neo SPβ cotA-lacZ cat</i> Nm ^r Cm ^r	This study
AH1062	<i>trpC2 metC3 cotJΔ::gusA neo SPβ cotE-lacZ cat</i> Nm ^r Cm ^r	This study
AH1074	<i>trpC2 metC3 orf5::pAH194</i> Sp ^r	This study
AH1075	<i>trpC2 metC3 cotJ::pAH188</i> Sp ^r	This study
AH1076	<i>trpC2 metC3 cotJ::pAH189</i> Sp ^r	This study

^a Strains bearing the *spoIIGB55*, *spoIID83*, *spoIIGΔ1*, and *spoIVCB23* mutations were obtained from P. J. Piggot and lysogenized with SPβ *cotJ-lacZ*. They are all isogenic with the Spo⁺ strain MB24, except for the indicated markers. The source of all other developmental mutations and strains used in cloning of the *cotJ* locus is indicated in the text.

^b BGSC, *Bacillus* Genetic Stock Center.

StuI-SstI fragment between the *HincII* and *SstI* sites of M13mp19 (this fragment also includes about 2 kb of *lacZ* DNA downstream of the library junction). Next, the replicative-form DNA of the resulting hybrid phage was digested with *SphI* and *BamHI* to release the 1.2-kb chromosomal insert, which was cloned into *SphI*- and *BamHI*-digested pUS19 to create plasmid pUS13BS. Plasmid pUS19 is a pUC19 derivative that contains a spectinomycin resistance gene cloned between its *ClaI* and *NdeI* sites (35). Finally, pUS13BS was used to transform *B. subtilis* MB24 (Table 1) to Sp^r. DNA from one of the resulting transformants in which plasmid pUS13BS had integrated into the *cotJ* region of the chromosome by a single-reciprocal-crossover (Campbell-type) recombinational event, was digested to completion with *EcoRI* and religated at a low DNA concentration, and the mixture was used to transform *E. coli* to Ap^r and Sp^r. A plasmid, pUS13E, prepared from one such transformant was shown to contain, in addition to the initial 1.2-kb insert from the *StuI* site to the *Sau3AI* junction, a fragment extending from this junction to an *EcoRI* site located 1.7 kb further downstream (Fig. 1). The total size of the *B. subtilis* chromosomal DNA insert present in plasmid pUS13E is 2.9 kb, of which only the last 2 kb is represented in Fig. 1. Southern blot analysis revealed that the region cloned in pUS13E has a structure similar to that of the corresponding chromosomal locus and therefore that no detectable rearrangement occurred during cloning of the *cotJ* operon. All other plasmids described in this work are derivatives of pUS13E.

Regeneration of an SPβ*cotJ-lacZ* phage and construction of a *cotJ-gusA* transcriptional fusion. To recreate an SPβ*cotJ-lacZ* specialized transducing bacteriophage, plasmid pTVE13 was digested with *ScaI* and the digested DNA was

used to transform competent cells of strain ZB307 (64) to Cm^r. Transformants were expected to be generated by a double crossover between *lacZ* and pBR322 sequences in the resident prophage and homologous sequences in the invading DNA. Transformants were then screened for a Lac⁺ phenotype on DSM plates containing X-Gal. Under these conditions all the Cm^r transformants were found to have a Lac⁺ phenotype. An SPβ*cotJ-lacZ* transducing lysate was prepared from one such transformant (strain AH760) by heat induction as described previously (64) and used to transduce the wild-type strain MB24 to Cm^r, creating strain AH770. A transcriptional fusion of *cotJ* to the *E. coli gusA* gene (21) was made by inserting a *gusA-neo* cassette obtained by digestion of plasmid pMLK113 (28) with *SmaI* between the Klenow fragment-filled in *EcoNI* sites of plasmid pUS13E. The cassette was inserted in both orientations, thereby creating plasmids pAH238 (in which the *gusA* and *neo* genes are in the same orientation as the *cotJ* sequences) and plasmid pAH239 (*cotJ* and *gusA-neo* in opposite orientations).

Other plasmid constructions. Plasmid pUS13HK was constructed by replacing the 500-bp *HincII* fragment of pUS13E with a kanamycin resistance determinant (55) released from pKD102 (obtained from W. Haldenwang) with *SmaI*. Plasmid pAH187 contains the 402-bp *EcoRI-ClaI* fragment from pUS13E cloned between the *EcoRI* and *ClaI* sites of pBluescript IISK(+) (Stratagene). To construct pAH194, we digested pAH187 with *EcoRI* and *SaII* and cloned a purified fragment of about 420 bp between the *EcoRI* and *SaII* sites of pUS19. Subcloning of the 691-bp *EcoRV* fragment from pUS13E into the *SmaI* site of plasmid pMLK113 (28) yielded plasmids pAH188 (orientation of *cotJ* sequences similar

FIG. 1. Physical and genetic map of the *cotJ* region of the chromosome. The positions and lengths of six ORFs predicted from the analysis of the nucleotide sequence are indicated by boxes below a partial restriction map of the region (only relevant sites are indicated). All the ORFs indicated are transcribed in the same direction, from left to right. The region downstream of the library fusion site (the *Sau3AI* site represented in the *cotJA* coding sequence) was obtained by a chromosome walking step. The *Sau3AI* site that defines the upstream boundary of the insert cloned in front of *lacZ* in phage SP β is just to the right of the *StuI* site at the leftmost end of the 2.9-kb fragment of *B. subtilis* DNA cloned in pUS13E. The *StuI* site in pUS13E and its derivatives was destroyed during cloning (asterisk). The region to the left of the *PstI* site is not drawn to scale. The *cotJ* promoter (arrow), defined by primer extension, is located downstream of a region of dyad symmetry that could represent a terminator for the *orf1* transcriptional unit. The lines below the restriction map represent the inserts in the indicated plasmids. The striped area represents the *HindIII* fragment that was used as a probe for the physical mapping of the *cotJ* locus. The sites of different insertion-deletion mutations resulting from replacement of wild-type sequences (by double-crossover events) with in vitro-engineered alleles of *cotJ* (present in plasmids pUS13HK, pAH238, and pAH239) are also shown. The insertion elements present in pAH238 and pAH239 contain the *gusA* gene in addition to the *neo* gene, and their orientations are indicated. Nonlinearized DNA of the two plasmids was also used to transfer the fusion to the chromosome by means of a single-crossover (Campbell-type) recombinational event that occurred in the region of homology upstream of the *gusA-neo* insertion. Plasmids pAH188, pAH189, and pAH194 were also integrated into the host chromosome by a Campbell-type mechanism.

to that of *gusA*) and pAH199 (*cotJ* and *gusA* sequences in opposite orientations) (Fig. 1).

Construction of *gusA/lacZ* double-reporter strains. Strain AH1037 (bearing a *cotJ-gusA* transcriptional fusion) was lysogenized with SP β phages carrying *lacZ* transcriptional fusions to *spoIID* or different *cot* genes (Table 1). Lysogenized cells were plated on Luria-Bertani plates containing neomycin and chloramphenicol. Transductants were then transferred to DSM plates containing X-Gluc (in addition to the appropriate antibiotics) to verify expression of the *gusA* fusion, and the colonies were sprayed with MUG to verify expression of the *lacZ* fusion. The sources of the *spoIID*- and *cotA-lacZ*-transducing phages were strains AH695 and SC34 (9), respectively. Strain AH695 (Table 1) is a ZB307 derivative carrying an SP β *spoIID-lacZ* *cat* specialized transducing phage identified in a screening similar to that described in this paper for σ^E -dependent genes. Transductants of strain AH1037 with SP β *spoIID-lacZ* or SP β *cotA-lacZ* were checked for a Lac⁺ Gus⁺ Nm^r Cm^r phenotype, and appropriate transductants were selected and named AH1060 and AH1061, respectively (Table 1). An SP β *cotE-lacZ* transducing phage was obtained by transforming strain ZB307 to Cm^r with the *cotE-lacZ* fusion plasmid pCotEp₂ (obtained from E. M. Bryan), which had been linearized with *ScaI*. A Cm^r MLS^r Lac⁺ transformant (strain AH739) was then used to prepare an SP β *cotE-lacZ* *cat* lysate by heat induction as described previously (64), and the lysate was used to transduce strain AH1037 to Cm^r, producing strain AH1062. Plasmid pCotEp₂ carries a 790-bp *HindIII*-to-*SphI* fragment from the *cotE* region (60) cloned between the *HindIII* and *BamHI* sites of pZAB/HNB (30) (a *BamHI* site was introduced downstream of the *SphI* site in the *cotE* fragment by a subcloning step into *HindIII*-*SphI*-cleaved M13mp19

replicative-form DNA). Both the *cotEp*₁ and *cotEp*₂ promoters are present in front of the *lacZ* gene and drive expression of the *cotE-lacZ* fusion in pCotEp₂.

Physical and genetic mapping of the *cotJ::gusA neo* marker. The 1.4-kb *HindIII* fragment represented in Fig. 1 was gel purified and labeled by using the Multi-prime random oligonucleotide DNA labeling system obtained from Amersham and [α -³²P]dATP (3,000 Ci/mmol). The labeled fragment was used to probe a mapping membrane (kindly provided by P. Serron) containing an ordered collection of *B. subtilis* chromosomal inserts made in pYAC4 (4). The deduced location of the *cotJ* locus was then confirmed by appropriate transduction and transformation crosses (12).

Growth and sporulation of *B. subtilis*. Growth and sporulation of *B. subtilis* cultures were essentially as previously described (20). Sporulation of 50-ml cultures (in 500-ml Erlenmeyer flasks), grown at 37°C and 120 rpm, was induced by nutrient depletion in DSM. Samples (0.6 ml) to be analyzed for β -galactosidase or β -glucuronidase activity were collected at 30-min intervals during vegetative growth and until 7 h after the onset of sporulation (defined as the end of the logarithmic phase of growth and designated here *T*₀). The samples were frozen rapidly at -70°C and stored at this temperature until the time of assaying. To measure the extent of sporulation, samples (1 ml) were removed from sporulating cultures at *T*₁₈ (that is, 18 h after the onset of sporulation) and serial dilutions were made. Dilutions were plated before and after heat (80°C, 20 min), lysozyme (125 μ g/ml, 10 min at 37°C), or chloroform (1 ml, 20 min) treatment, and the numbers of CFU developing per ml after overnight growth at 37°C on Luria-Bertani plates were counted (41). Germination assays on plates with 2,3,5-

triphenyltetrazolium chloride as an indicator were conducted as previously described (41).

Purification of spores. Spores were purified essentially as described before (41). Cultures in DSM supplemented with the appropriate antibiotic were inoculated in a volume of 50 ml and grown to an A_{600} of about 0.9 (late log phase). They were then diluted 1:10 in 500 ml of prewarmed DSM, and incubation continued for 2 days at 37°C. After this period, if more than 90% of the cells in the culture formed spores (as determined by phase-contrast microscopy), the culture was transferred to a centrifuge tube and centrifuged at $4,000 \times g$ for 30 min. The spore pellet was washed with 200 ml of cold distilled water, and the spore suspension was incubated at 4°C overnight. Two more washes were made, and finally the spore pellet was resuspended in 2 ml of a 2% solution of Renografin-76 (Squibb). This suspension was layered on top of 15 ml of a 50% solution of Renografin and centrifuged at $12,000 \times g$ for 30 min. The spore pellet was resuspended in 1 ml of cold distilled water. After six washes with 1 ml of cold distilled water, the purified spores were stored at 4°C in an A_{580} of about 20.

Spore coat protein profiles. Samples (100 μ l) of purified spores of strains MB24 (Spo^+), AH64 (*cotE* Δ :*cat*), and AH1037 [*cotJ* Δ :*gusA neo*] (Table 1) were then transferred to a new tube, and the spores were harvested by centrifugation. The spore pellet was then resuspended in 100 μ l of sodium dodecyl sulfate (SDS) loading dye (4% SDS, 10% 2-mercaptoethanol, 1 mM dithiothreitol, 0.125 M Tris-HCl [pH 6.8], 10% glycerol, 0.05% bromophenol blue), and the samples were boiled for 8 min. Samples were briefly centrifuged, and 25 μ l (approximately 40 μ g of total protein) was fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 15% gel. Gels were stained with Coomassie brilliant blue R-250 and destained. Computer images of the gels were generated with a video camera and analyzed by using the Image software (version 1.55) of the National Institutes of Health.

Enzyme assays. The specific activity of β -galactosidase was determined as described by Miller (39) with the substrate *o*-nitrophenyl- β -D-galactopyranoside (ONPG). β -Glucuronidase specific activity was determined by using a similar protocol (39) with the substrate *p*-nitrophenyl- β -D-glucopyranoside (PNPG). In both cases, cells were permeabilized by treatment with toluene. Enzyme specific activity is expressed in nanomoles of substrate (ONPG or PNPG) hydrolyzed per mg (dry weight) per min.

Isolation and primer extension analysis of mRNA. mRNA for primer extension experiments was prepared from cells grown in DSM at various times as previously described (5, 6). The primer 5'-GCGGACAGGGATCAAACG-3', complementary to nucleotides 380 to 398 of the *cotJ* sequence represented in Fig. 2, was end labeled with [γ - 32 P]ATP by using T4 polynucleotide kinase. Approximately 1 ng of primer was annealed to 40 μ g of total RNA from *B. subtilis* at 60°C, and primer extension reactions were carried out with avian myeloblastosis virus reverse transcriptase (BRL). The same oligonucleotide was used in parallel to prime sequencing reactions from the pUSE13 double-stranded template, so that the size of the transcript could be read directly from the sequencing ladder.

DNA sequencing. The sequence across the library fusion site was determined by using plasmid pTVE13 as the template (purified from the corresponding PY222 derivative) according to the Sequenase (U.S. Biochemical Corporation) protocol for double-stranded DNA with a primer to the 5' end of the *lacZ* gene (5'-GCGCTATGTCGTAGGTCT-3'). DNA restriction fragments encompassing the entire region cloned (Fig. 2), obtained from plasmid pUS13E, were then subcloned into M13mp18 and M13mp19, and the sequences for both strands were determined according to the dideoxy chain termination method and the Sequenase protocol for single-stranded DNA. The DNA sequences and the deduced protein sequences corresponding to the six ORFs determined to be present in the *cotJ* region were analyzed by using the programs in the Genetics Computer Group package (17).

Nucleotide sequence accession number. The sequence of the *cotJ* operon reported in this paper has been deposited in the GenBank database and assigned accession number L38014.

RESULTS

Cloning, nucleotide sequence, and organization of the *cotJ* region of the chromosome. The general strategy devised to identify and clone *B. subtilis* genes whose expression is under the control of the sporulation transcription factor σ^E has been described previously (5, 6). A random library of *B. subtilis* chromosomal DNA fragments obtained by digestion with *Sau*3AI was made in the temperate bacteriophage SP β , and a high-titer lysate was prepared from a *c2* strain and used to transduce cells of strain EU101 (Table 1) to Cm^r . Colonies of the Cm^r transductants were then screened for a conditional (inducer-dependent) Lac^+ phenotype. In strain EU101, the P_{spac} promoter (57) drives expression of a full-length copy of the *spoIIGB* gene, encoding σ^E . The chromosomal copy of *spoIIGB* in strain EU101 has been deleted and replaced with an *erm* marker obtained from strain EU8701 (29). The allele of

the *spoIIGB* gene under P_{spac} control has the sequence that encodes the amino acid residues proteolytically removed from pro- σ^E , in a reaction that produces transcriptionally active σ^E (26, 34, 52). As a result the pro form of σ^E is made when expression of *spoIIGB* is induced with IPTG, and its processing to the active form is under sporulation control. Transduction of EU101 with the SP β -borne *lacZ* fusion library resulted in the identification of several lysogens carrying fusions to different σ^E -controlled genes (5, 6), including one, bearing SP β E13 that contained a fusion to a gene, the subject of the present report, that we named *cotJ*. The putative σ^E -dependent promoter in SP β E13 and chromosomal DNA located downstream of this promoter were cloned by using strategies described previously (5, 6) and outlined in Materials and Methods. The procedure involved transferring the *B. subtilis* genomic DNA insert present in the SP β E13 prophage to a Tn917-containing plasmid (pTV17) by homologous recombination in vivo, thus creating plasmid pTV13E. This plasmid, which contained a 1.8-kb chromosomal insert, was then used as a template for sequencing reactions primed from a position within the *lacZ* gene, which revealed the presence of a previously uncharacterized ORF (later named *cotJA*). Next, a 1.2-kb piece of *B. subtilis* DNA together with about 2 kb of *lacZ* sequences was moved to the replicative form of phage M13mp19, from which a 1.2-kb *Sph*I-*Bam*HI fragment was released and cloned into appropriately cleaved pUS19 (see Materials and Methods). The resulting plasmid, pUS13BS, was finally used in a chromosome walking step that resulted in the cloning of an additional 1.7 kb of DNA downstream of the initial fusion junction (the *Sau*3AI site in Fig. 1), a procedure that created plasmid pUS13E (Fig. 1).

The DNA sequence of the *cotJ* operon was determined for both strands by subcloning appropriate restriction fragments obtained from plasmid pUS13E into M13mp18 and M13mp19 (see Materials and Methods). Sequence analysis revealed the presence of six ORFs in the region, one of them (*orfI* in Fig. 1) ending upstream of the deduced transcriptional start site of the *cotJ* operon (described below) and followed by a possible transcription terminator (Fig. 1 and 2). Comparison of the last 64 codons of this ORF (*orfI* in Fig. 1) with sequences present in the GenBank database did reveal a weak similarity to the putative 223-amino-acid product of ORF *ipa-33d*, of unknown function, located in the *sacT* region of the chromosome (at about 330°) (18). The *cotJ* operon was found to be composed of at least three cistrons, here named *cotJA*, *-B*, and *-C*, of 83, 100, and 190 codons, capable of encoding putative products of 9.7, 11.7, and 21.7 kDa, with predicted isoelectric points of 9.8, 6.1, and 4.5, respectively. An interesting feature of the putative CotJC protein is the density of negatively charged amino acids found in its C-terminal one-third: 13 of 50 residues (or 30%) are aspartic acid or glutamic acid (these two amino acids, in a total number of 32 residues, make up 18% of the total mass of the 190-residue CotJC protein). The initiation codon of *cotJA* is separated from the stop codon of *orfI* by a 130-bp interval that accommodates the *cotJ* promoter (described below) and ribosome binding site (GGAGG, at positions 314 to 318 in Fig. 2). Extensive overlapping was observed between the *cotJA* and *cotJB* coding sequences (suggesting translational coupling), but not between *cotJB* and *cotJC* (Fig. 1 and 2). Beginning 124 bp downstream from the *cotJC* stop codon (TAA at position 1403 in the sequence shown in Fig. 2) is another ORF (*orf5* in Fig. 1), which is preceded by a strong ribosome binding site (GG GAGG, at positions 1519 to 1524 in Fig. 2) and predicted to encode a short polypeptide of 81 amino acids (8.9 kDa) with a calculated isoelectric point of 4.3. *orf5* is followed by an incomplete ORF (designated *orf6* in Fig. 1) whose translation could

FIG. 2. Nucleotide sequence and translation of the *cotJ* region of the chromosome. The nucleotide sequence of the nontranscribed strand of the chromosome is shown. The -10 and -35 sequences of the *cotJ* promoter are indicated by different lettering upstream of the transcriptional start site (+1), a G residue at position 296 (underlined). A possible SpoIID-binding box that partially overlaps the -35 region of the *cotJ* promoter is indicated by a line above the sequence. Potential ribosome-binding sites are indicated by double underlines. Possible start codons are represented in bold type, and asterisks indicate stop codons. An inverted repeat thought to act as a transcriptional terminator for the ORF immediately upstream of *cotJ* is indicated by arrows below the sequence. The primary structure of the polypeptides encoded by the *cotJ* region of the chromosome was deduced from the nucleotide sequence and is indicated in single letter code. Only relevant restriction sites are shown, including the *Sau*3AI library fusion site at positions 385 to 388 of the sequence shown.

be coupled to that of the preceding one. Comparison of the primary structures of the products predicted to be encoded by any of the ORFs downstream of the *cotJ* promoter with sequences in the GenBank database did not reveal any significant similarity.

***cotJ* is located at about 62° on the *B. subtilis* genetic map.** The map position of the *cotJ* operon was determined by a combination of physical and genetic methods. First, the 1.4-kb *Hind*III fragment totally derived from the *cotJ* region of the chromosome (Fig. 1) was purified and labeled to a high specific activity by a random priming oligonucleotide labeling method (see Materials and Methods) with [α -³²P]dATP and the Klenow fragment. The labeled fragment was then used to probe a mapping membrane spotted with samples of total DNA prepared from yeast strains containing an ordered collection of *B. subtilis* chromosomal inserts made in pYAC4 (4). The probe

hybridized to YAC clone 10-201 (4), which carries an 85-kb insert, therefore placing *cotJ* at about 62° on the genetic map (1). We then verified linkage between *cotJ*Δ:*gusA neo* and other genetic markers in the region by performing transduction and transformation crosses. A PBS1 lysate made in strain AH1037 was used to transduce strain 1A5 (*tre-12 glyB133*) to Nm^r, and the numbers of Tre⁺ and Gly⁺ recombinants were independently scored. We found *cotJ*Δ:*gusA neo* to be 88% cotransduced with *tre*⁺, and linkage between both markers (about 30%) was also detected by cotransformation (Fig. 3A). Because the *cotJ*-derived probe did not hybridize to YAC clone 11-15, which contains the *tre* gene, we infer that *cotJ* is located to the left of this marker, proximal to the origin of chromosome replication (Fig. 3A). No linkage was detected by PBS1 transduction between *cotJ*Δ:*gusA neo* and *glyB133* or *zjb-82*::Tn917 (strain 1A628), in spite of the fact that this latter

FIG. 3. Position of the *cotJ* locus on the *B. subtilis* genetic map and locations of other *cot* genes. (A) The upper line represents the genetic map of the *cotJ* region of the *B. subtilis* chromosome (not drawn to scale). The broad lines below the genetic map represent the regions of the chromosome contained in YAC clones. The map position of the *cotJ* operon was deduced from the hybridization signal obtained with the probe to YAC clone 10-201, which carries an 85-kb insert centered at 62° on the map (4). This position was confirmed by the transduction and transformation crosses involving the indicated markers in the region. (B) Comparison of the map position of *cotJ* with those of other *cot* genes. The numbers in parentheses represent the positions of the indicated loci on the *B. subtilis* genetic linkage map (scale of 0 to 360°) (1).

marker, previously mapped to 65°, was shown to be 38% linked by cotransformation to *tre-12* (56). The only other *cot* gene in the region, *cotA* (Fig. 3B), previously mapped to 52° (13), is not present in the same YAC clone as *cotJ*.

***cotJ* mutations affect proteins in the inner layer of the spore coat.** We constructed insertion-deletion mutations in *cotJ* by replacing wild-type sequences with antibiotic resistance cassettes in vitro and used them to replace the corresponding chromosomal sequences, thereby creating null mutant strains. Plasmids pAH238 and pAH239 (Fig. 1) each have insertions of a *gusA-neo* cassette in opposite orientations relative to the direction of transcription of *cotJ*, between the two *Eco*NI sites in plasmid pUS13E. Additionally a kanamycin resistance gene was inserted between both *Hinc*II sites of pUS13E, a manipulation that produced plasmid pUS13HK (Fig. 1). Plasmids pAH238, pAH239 linearized with *Sca*I, and pUS13HK linearized with *Eco*RI were used to transform competent cells of strain MB24 to *Nm*^r or *Km*^r. Transformants were then screened for their sensitivity to spectinomycin (*Sp*^s), a characteristic indicating that the antibiotic resistance phenotype

(*Nm*^r or *Km*^r) was the result of a double-crossover event (marker replacement) that occurred on both sides of the cassette inserted within *cotJ* sequences on the plasmid (Fig. 1). The resulting strains (Table 1) carry insertion-deletions of part of *cotJA* and *cotJB* (strains AH1037 and AH1038) and of *cotJB* and *cotJC* (strain AH925). In addition, strains AH1075 and AH1076 have Campbell insertions of plasmids pAH188 and pAH189, respectively, that separate *cotJABC* from *orf5* and *orf6* (Table 1 and Fig. 1), and strain AH1072 has a Campbell insertion of plasmid pAH194 that should disrupt the downstream *orf5-orf6* unit. Cultures of the strains bearing insertions in the *cotJ* region (AH925, AH1037, AH1038, AH1072, AH1075, and AH1076; Table 1) were induced to sporulate and assayed for heat, lysozyme, or chloroform resistance at *T*₁₈ (that is, 18 h after the onset of sporulation). All of these strains exhibited essentially wild-type sporulation phenotypes (results not shown). The *cotJ* null mutant strains were also assayed for a possible germination-deficient phenotype (*Ger*⁻) by the tetrazolium overlay method and were shown not to differ from the isogenic wild-type (*Ger*⁺) strain MB24. Electron microscopy of samples prepared at h 18 of sporulation and assayed in parallel for their resistance properties did not reveal any major morphological differences between the *cotJ* null mutant AH1037 and the wild-type strain MB24 (results not shown).

Since disruption of several genes encoding spore coat components had been shown to have no effect or little effect on spore morphology and resistance properties (3, 11, 13, 45, 58), and because the relative strength of the *cotJ* promoter (see below) suggested that large amounts of the *cotJ*-encoded products could be synthesized in the mother cell during sporulation, we suspected that the *cotJ* genes could encode components of the spore coat. We compared the coat protein profile of the *cotJ* null mutant strain AH1037 (harboring the most upstream insertional mutation of *cotJ*) with that of the wild-type strain MB24 and that of a strain, AH64, bearing a *cotEΔ::cat* allele (Fig. 4). Cultures of the indicated strains were induced to sporulate, and the spores were purified by centrifugation through Renografin gradients as described in Materials and Methods. Equivalent amounts of spores were pelleted and resuspended in SDS-dithiothreitol sample buffer, the samples were boiled for 8 min, and about 40 μg of total protein was fractionated by SDS-PAGE in a Tris-glycine buffer system. The pattern of protein bands found for the wild-type strain (Fig. 4A, lane 2, and Fig. 4B) was similar to those reported previously by Jenkinson and Sawyer (23) and Jenkinson et al. (24). The pattern of proteins from the *cotJ* mutant was similar to that of the wild type in the range of 12 to 65 kDa, except for a polypeptide with an apparent size of 24 kDa clearly seen for the wild-type strain (indicated by the arrowhead in Fig. 4A) but missing from the spores of the *cotJ* mutant (Fig. 4A, lane 3). Because the size predicted for the product of *cotJC* (21.7 kDa) is close to that estimated for the missing polypeptide, this polypeptide could be the *cotJC* product. Alternatively, it could be a polypeptide whose incorporation into the coat depends on the *cotJ* products. In either event, a mutation in *cotJ* affects the pattern of spore coat proteins.

A previous report estimated the size of the CotE polypeptide to be about 24 kDa (60), but under the conditions used here, the CotE species migrated with an apparent size of 26 kDa. That the 26-kDa species could be the CotE protein is supported by the fact that the corresponding band was missing from the coat of a *cotE* null mutant (Fig. 4A, lane 1), whereas the 24-kDa species was still observable. Thus, the behavior of the 26-kDa species thought to be CotE and that of the 24-kDa product believed to be CotJC are quite distinct. Incorporation of the former polypeptide into the coat is *cotE* dependent,

FIG. 4. Coat protein profiles of spores produced by a *cotE*Δ::cat null mutant, a wild-type strain, and a *cotJ*Δ::gusA neo null mutant. (A) Spores of the indicated strains were purified by centrifugation in a 20 to 50% step gradient of Renografin-76 (Squibb), washed, and resuspended in distilled H₂O to an A_{580} of about 20. Spores in 100 μ l of each suspension were collected by centrifugation and resuspended in 100 μ l of SDS loading dye. The samples were boiled for 8 min, and 25 μ l (corresponding to about 40 μ g of total protein) was resolved by SDS-PAGE on a 15% gel. The gel was stained with Coomassie brilliant blue (R-250) and

while assembly of the latter requires the *cotJ* products. Previous work showed that *cotE* mutants fail to assemble the outer coat (60). Analysis of Fig. 4A (lane 1) reveals that bands believed to correspond to CotA, CotB, and CotC are clearly seen for the wild-type strain, but are missing from the mutant, together with the 26-kDa polypeptide which we think is the product of the *cotE* gene. All these polypeptides were expected to be missing in the *cotE* mutant, since they are components of the outer coat (60). The 24-kDa protein whose assembly into the coat depends on *cotJ* was present in the *cotE* null mutant. Therefore, the 24-kDa polypeptide is likely to be an inner-coat protein.

Additionally, a small decrease in the relative amount of a band believed to represent the 65-kDa CotA polypeptide (Fig. 4) was observed when the samples were processed and resolved as described above. The effect was reproducible, although not always as pronounced as that shown in Fig. 4A. We found that transcription of the *cotA* gene is not affected by the presence of a null allele of *cotJ* (data not shown). It is possible that the absence of the *cotJ* products induces a subtle alteration in coat structure that leads to a smaller representation of the CotA protein in the coat or to a lower level of accessibility of this component to our extraction procedure.

To further extend the analysis, a computer image of the SDS gel shown in Fig. 4A was generated with a video camera and a densitometric profile obtained for each lane with the Image software (National Institutes of Health) (Fig. 4B). Because the amounts of total protein loaded in the lanes are equivalent, and because the relative amounts of most species common to the three lanes are equivalent (suggesting that the extraction procedure worked very similarly in all three cases), differences in the amplitude of a specific peak reflect differences in the relative amounts of specific proteins present in the coats of different strains. The absence of the 24-kDa component and the reduced amount of CotA are easily seen in the densitometric profiles obtained for the *cotJ* mutant (Fig. 4B, panel 3). This analysis also shows that the amount of a species of about 17 kDa (the position of which is shown for the wild-type strain by an asterisk in Fig. 4A, lane 2) is reduced in the *cotJ* mutant (Fig. 4B, panel 3) and that the intensity of a corresponding band is increased for the *cotE* mutant (Fig. 4B, panel 1). This type of behavior is that expected for an inner coat component encoded by the *cotJ* operon or an inner coat component whose assembly depends on the *cotJ* products: the polypeptide is reduced in quantity or absent in the *cotJ* mutant, but because of its possible inner coat location, it becomes more accessible in the *cotE* mutant, which lacks the outer coat layer. The size predicted for the product of *cotJB* (12 kDa) does not exactly match that of the 17-kDa component; however, abnormal migration of coat polypeptides has been observed before for the

destained, and a computer image was obtained by using a video camera. Lane numbers correspond to the following strains: 1, AH64 (*cotE*Δ::cat); 2, MB24 (wild type, Spo⁺); 3, AH1037 (*cotJ*Δ::gusA neo). The asterisk and arrowhead indicate putative *cotJ*-encoded products that are absent or reduced in amount in the *cotJ* mutant. (B) The protein profile for each lane was then obtained by analysis of the gel image with the Image software (version 1.55) (National Institutes of Health). The horizontal axis represents distance migrated, as indicated by the positions of the molecular weight (MW) markers (panel 4). The peaks represent relative intensities of different bands. The positions of the proposed *cotJ*-encoded products are indicated as follows: an arrowhead (A) indicates the 24-kDa species that is missing in the *cotJ* null mutant, and an asterisk (A and B) indicates the 17-kDa band whose intensity is increased in the *cotE* mutant. The bands labeled CotA, CotB, CotE, CotC, and CotD in panels A and/or B were assigned on the basis of their apparent molecular weights and also, in the case of CotE, on the basis of absence from the coat of the corresponding null mutant.

FIG. 5. Mapping of the transcription start site of the *cotJ* operon. Shown is an autoradiograph of *cotJ*-specific primer extension products after electrophoresis in a polyacrylamide gel. An oligonucleotide complementary to the 5' end of the nontranscribed strand of the *cotJ* cistron was used to direct cDNA synthesis of total RNA purified from cultures of *B. subtilis* under different growing conditions. RNA from mid-log-phase cultures of strain EU8702 (*sigEΔ::erm* [pDG180]) growing in Luria-Bertani broth was used in lanes i (1 mM IPTG added to the culture) and j (no IPTG added). Plasmid pDG180 carries a truncated form of the *spoIIIG* gene under the control of the inducible *P_{spac}* promoter. RNA was also purified from cultures of a wild-type *Spo*⁺ strain in sporulation medium (DSM) at the indicated times (in hours) after *T*₀, defined as the end of the exponential phase of growth and the onset of sporulation (lanes e through h). The same oligonucleotide used for the primer extension experiments was also used in parallel to prime sequencing reactions (lanes a to d) from an appropriate M13 single-stranded template, so that the size of the transcript could be read directly from the sequencing ladder. The arrow indicates the position of the *cotJ*-specific primer extension product. The asterisk indicates the deduced start site of transcription, a G residue at position 296 in the *cotJ* sequence shown in Fig. 2.

cotT (3), *cotV*, and *cotW* (58) products, perhaps as a consequence of a peculiar amino acid composition. Presumably, the component of about 17 kDa believed to correspond to CotJB is not entirely missing in the *cotJ* mutant, because of comigration with an unrelated polypeptide. Therefore, it is possible that this species corresponds to the CotJB polypeptide. With the buffer system used here, we were not able to resolve polypeptides of less than 12 kDa, and therefore we failed to detect any band that could correspond to the product of *cotJ*, whose predicted size is of about 9 kDa.

The *cotJ* promoter is used by σ^E -containing RNA polymerase. To determine the starting point of *cotJ* transcription in vivo, total RNA was isolated from sporulating cultures of a wild-type strain at the indicated times after the cessation of exponential growth and used for primer extension analysis with an end-labeled primer (Fig. 5). No extension product was detected at significant levels at *T*₀ (Fig. 5, lane h), and a single extension product was seen with RNA isolated at *T*₂, *T*₄, and *T*_{6.5} (Fig. 5, lanes e, f, and g), the size of which suggests that transcription of *cotJ* starts at a G residue at position 296 in the *cotJ* sequence (Fig. 2). Synthesis of the *cotJ* message is driven by a strong promoter, as judged from the intensity of the primer extension signal obtained, and is maximal around 4 h of sporulation (Fig. 5; also see below). Centered 10 and 32 bp upstream of the apparent start site of *cotJ* transcription are sequences that share a strong degree of conservation with the -10 and -35 regions, respectively, of promoters utilized by σ^E -containing RNA polymerase (Fig. 2) (40, 44). An equivalent species was detected, although at a weaker intensity, when RNA was prepared from vegetatively growing cells of strain EU8702 [*sigEΔ::erm* (pDG180)] in the presence (Fig. 5, lane i) but not in the absence (Fig. 5, lane j) of the inducer IPTG. Induction of active σ^E production in vegetative cells is evi-

FIG. 6. Expression of *cotJ-lacZ* and *cotJ-gusA* transcriptional fusions during growth and sporulation of a wild-type *Spo*⁺ strain. An SP β *cotJ-lacZ* phage was introduced into cells of the wild-type *Spo*⁺ strain MB24 by specialized transduction, resulting in strain AH770 (*Lac*⁺; squares). Plasmids pAH238 and pAH239 (Fig. 1) were linearized with *Eco*RI and used to transform strain MB24 to *Nm*^r, resulting in the *CotJ*⁻ strains AH1037 (*Gus*⁺; triangles) and AH1038 (*Gus*⁻; diamonds), the latter of which was used to estimate background levels of β -glucuronidase activity. Background levels of β -galactosidase activity were measured in strain AH394 (circles) (Table 1), containing an SP β phage that carries a promoterless *lacZ* gene. The specific activities of β -galactosidase and β -glucuronidase are expressed as nanomoles of the substrates ONPG and PNP hydrolyzed per mg (dry weight [dw]) per min, respectively. The end of the exponential phase of growth, or *T*₀, is defined as the onset of sporulation.

dently sufficient to activate the *cotJ* promoter in vivo. Also, both the temporal window of activity of the *cotJ* promoter and its deduced -10 and -35 regions are consistent with the regulation and structures of other well-characterized σ^E -dependent promoters (16, 40, 44). These results, together with those described below, lead us to suggest that the *cotJ* promoter is used by RNA polymerase containing σ^E .

Transcription of *cotJ-gusA* precedes that of *cotA-lacZ*, but not that of *spoIID*- or *cotE-lacZ*. Expression of an SP β -borne *cotJ-lacZ* fusion was examined throughout growth and sporulation of strain AH770 (Table 1). Expression of *cotJ-lacZ* remained undetected during vegetative growth and early stationary phase. However, it was strongly induced around 2 h of sporulation, a temporal pattern shared by many σ^E -directed genes (16, 40, 44), and transcriptional activity from the *cotJ* promoter was still detected 6 h after the onset of sporulation (Fig. 6). A similar result was also found for strain AH1037 (Fig. 6), which harbors a single copy of a *cotJ-gusA* transcriptional fusion at the *cotJ* locus. Strain AH1037 was isolated after the sporulation-proficient strain MB24 was transformed with *Eco*RI-linearized pAH238, with selection to *Nm*^r. This resulted in the integration of a *gusA-neo* cassette at the region of homology with the chromosome (the exact position of which is depicted in Fig. 1), by a marker replacement event that created a *cotJ* null mutant in the process. Introducing intact pAH238 into strain MB24 by transformation followed by selection for *Nm*^r yielded strain AH1058, in which integration of the plasmid into the *cotJ* region of homology occurred by a Campbell-type recombinational event (single crossover). Because plasmid pAH238 (a pUS13E derivative) contains the 5' end of the *cotJ* transcriptional unit, its integration into the chromosome was nondestructive. We used strain AH1058 to monitor *cotJ-gusA* expression in a *CotJ*⁺ background and found it not to differ in any significant way from that found in the *CotJ*⁻ strain

AH1037 (results not shown). Background levels of β -glucuronidase activity were found in strain AH1059, which resulted from Campbell integration of plasmid pAH239 (results not shown and Fig. 1). The results presented in Fig. 6 demonstrated that the time of induction of *cotJ* expression was the same whether expression of the operon was monitored by the *cotJ*-driven production of β -galactosidase or β -glucuronidase and that it was not influenced by the nature of the *cotJ* allele. In both cases a marked induction occurred around the second hour of sporulation (Fig. 6).

We then made use of double-reporter strains to examine more precisely the temporal relationships existing between the expression of *cotJ-gusA* and that of other genes in the σ^E and σ^K regulons. SP β -borne fusions of *spoIID*, *cotE*, or *cotA* to *lacZ* were introduced by specialized transduction into a strain (AH1037) bearing a *cotJ-gusA* fusion integrated at the *cotJ* locus in a single copy. Cultures of the resulting strains, AH1060 (*cotJ-gusA spoIID-lacZ*), AH1061 (*cotJ-gusA cotA-lacZ*), and AH1062 (*cotJ-gusA cotE-lacZ*) (Table 1), were allowed to sporulate, and the same samples collected from each culture at various times were independently assayed for β -galactosidase and β -glucuronidase activity. Time courses for β -galactosidase and β -glucuronidase production by these strains are shown in Fig. 7. We found the onset of *cotJ-gusA* expression to be temporally coincident with the induction of *spoIID-lacZ* and to precede the activation of *cotA-lacZ* by at least 1 h (Fig. 7A and C), a result that strongly suggests that *cotJ* expression is activated soon after the appearance of the active form of σ^E and well before the expression of σ^K -controlled genes is switched on. Interestingly, *cotJ-gusA* seems to be induced at a rate slower than that of *spoIID-lacZ* (Fig. 7A), a difference that cannot be attributed to the nature of the reporter gene used to monitor *cotJ* promoter activity, since a similar rate was also found when *cotJ* expression in an SP β *cotJ-lacZ* lysogen was monitored (Fig. 6). This suggested that an additional factor, such as the SpoIID protein (19, 31, 33, 51, 62), could be acting together with σ^E to transcribe *cotJ*. The overall pattern of expression of *cotJ-gusA* was also found to follow a kinetics close to that of *cotE-lacZ* (Fig. 7B).

That expression of *cotJ* is driven from a strong promoter, a prediction made on the basis of the intensity of the labeled cDNA product observed in primer extension experiments, was confirmed when β -galactosidase activity was measured in SP β *cotJ-lacZ* or SP β *spoIID-lacZ* Spo⁺ lysogens (Fig. 7A). The *spoIID* gene, a paradigm for σ^E -directed gene expression, is expressed from a particularly strong promoter (14, 16, 40). When activity was expressed in the more familiar Miller units, maximum levels of *cotJ*- or *spoIID*-driven β -galactosidase activity were found to be about 200 and 400 U, respectively (data not shown).

***cotJ* transcription is dependent on σ^E but not σ^K .** The fact that expression of *cotJ-lacZ* was totally dependent on induction of σ^E production from the *P_{spac}* promoter (which allowed us to identify it as a σ^E -dependent transcription unit in the first place) and the identification of both the 5' end of the *cotJ* message and the temporal window of activity of the *cotJ* promoter (by primer extension as well as by studies with *lac* and *gusA* fusions) provided strong evidence to support the notion that *cotJ* expression during sporulation is directly controlled by σ^E -containing RNA polymerase. We then wanted to examine more closely the genetic requirements for *cotJ* expression during sporulation. An SP β *cotJ-lacZ* fusion was introduced by specialized transduction into a collection of otherwise isogenic strains bearing different *spo* mutations, and the pattern of β -galactosidase formation during growth and sporulation was compared with that observed in the congenic Spo⁺ strain

FIG. 7. Comparison of the time of *cotJ-gusA* expression with those of *spoIID*-, *cotE*-, and *cotA-lacZ* expression. Strain AH1037 (*cotJ::gusA*) was lysogenized with phage SP β *spoIID-lacZ* (A), SP β *cotE-lacZ* (B), or SP β *cotA-lacZ* (C), resulting in the double-reporter strains AH1060 (*cotJ-gusA spoIID-lacZ*), AH1061 (*cotJ-gusA cotA-lacZ*), and AH1062 (*cotJ-gusA cotE-lacZ*). These were used to determine the exact temporal relationship between induction of *cotJ* and *spoIID*, *cotE*, or *cotA* expression. Samples were taken at 30-min intervals during growth and sporulation and assayed for both β -galactosidase (squares) and β -glucuronidase (circles) activities with the substrates ONPG and PNPG, respectively (see Materials and Methods). Enzyme specific activity was converted to a percentage of the maximum activity observed for each enzyme in each strain. *T*₀ is defined as the onset of sporulation.

AH770 (Table 1). Figure 8 shows the dependence pattern observed for *cotJ-lacZ* expression during sporulation. Mutations in the σ^E -encoding gene, such as *spoIIGB55* or *spoIIGBΔ::erm* (Fig. 8A) (26, 29), totally prevented expression of *cotJ-lacZ*, a result in conformation with the suspected role of σ^E in *cotJ* regulation. Formation of β -galactosidase was also totally prevented by mutations in *spoIIAA* and *spoIIAC*, whose products are required in a complex series of events leading to the activation of pro- σ^E (25, 26, 34, 52) (data not shown). Mutations in genes known to arrest the developmental process at stages later than that of pro- σ^E activation and mutations that interfere with the activation pathways for the late-acting sigma factors σ^G and σ^K (9, 16, 36, 37), such as those in the *spoIIM* (49) and *spoIIID* (31, 33, 51, 62) loci (Fig. 8B and C, respectively), did not prevent expression of *cotJ-lacZ*. However, expression of *cotJ*, as is that of *cotE* (62), was found to be at least partially dependent on the product of *spoIIID* (Fig. 8C). The *spoIIID* gene encodes a small DNA-binding protein that is made in the mother cell under σ^E control and is able to act together with σ^E or σ^K to activate or repress transcription of genes expressed in this compartment (19, 31, 33, 51, 62). One of the σ^E -transcribed genes which requires SpoIID as an

FIG. 8. Epistasis analysis of *cotJ-lacZ* expression. The formation of β -galactosidase was monitored throughout growth and sporulation of strain AH770, a Spo^+ lysogen of $SP\beta$ *cotJ-lacZ* (squares). The profile obtained was then compared with those observed for the following isogenic Spo^+ lysogens bearing developmental mutations: strains AH775 (*spoIIGB55*; circles) and AH776 (*spoIIGBΔ::erm*; diamonds) (A), strain AH777 (*spoIIGBΔ::erm*; circles) (B), strains AH780 (*spoIIGBΔ::erm*; circles) and AH781 (*spoIIGBΔ::erm*; diamonds) (C), strain AH782 (*spoIIGBΔ::erm*; circles) (D), strain AH783 (*spoIIGBΔ::erm*; circles) (E), and strains AH784 (*spoIIGBΔ::erm*; circles) and AH785 (*spoIIGBΔ::erm*; diamonds) (F). Background levels of β -galactosidase production, similar to those estimated with the isogenic strain AH394 and shown in Fig. 6, are not represented. Specific activity of β -galactosidase was determined as described in Materials and Methods. The end of the exponential phase of growth, or T_0 , is defined as the onset of sporulation.

auxiliary positively acting factor is *sigK*, encoding σ^K , the late-acting mother cell sigma factor (19, 31, 32). The finding that mutations in *spoIIGB*, but not in σ^G (*spoIIGBΔ*; Fig. 8D) (27, 38, 53) or σ^K (*spoIIGBΔ* and *spoIIGBΔ::erm*; Fig. 8E) (31), significantly diminished the total level of *cotJ-lacZ*-driven β -galactosidase formed is taken as an indication that the *SpoIIGB* protein is required in conjunction with $E\sigma^E$ for maximal expression of the *cotJ* operon. Thus, the partial dependence of *cotJ* on *spoIIGB* does not seem to represent an indirect effect of the requirement of *spoIIGB* for *sigK* transcription.

Finally, we note that expression of *cotJ-lacZ* during sporulation is not affected by the *spoIIV172* mutation, known to cause aberrant deposition of coat material as swirls in the mother-cell cytoplasm (8, 43, 44, 50), as judged from the nearly wild-type levels of β -galactosidase activity detected in strain AH783 (Fig. 8E). Expression of *cotJ-gusA* is also not affected by its own product (data not shown).

DISCUSSION

The present work describes a new operon involved in the formation of the coat of *B. subtilis* spores, which we designated

cotJ. The spores formed by a *cotJ* mutant have an essentially normal ultrastructure, as judged by electron microscopic analysis, and nearly normal resistance properties. However, they exhibit an altered pattern of coat polypeptides. Spores of the mutant seem to lack two polypeptides which normally form part of the inner layer of the coat, both of which could be encoded by the *cotJ* operon, and they also exhibit altered deposition of the CotA protein. We propose that *cotJ* encodes structural components of the coat of *B. subtilis* spores or is otherwise required for the deposition of a complete collection of spore coat components.

The coat becomes evident at a late stage of sporulation, when the activation of σ^K triggers the transcription of most of the *cot* structural genes, including the well-characterized *cotA*, *-B*, *-C*, *-D* (13), *-F* (11), and *-T* (3) genes and the *cotXVWYZ* cluster (58, 59). However, several lines of evidence suggested that the events leading to coat formation are initiated well before the onset of σ^K -directed *cot* gene expression. First, the transcription of a second class of genes, including *cotE*, *spoIVA*, and *spoVID*, phenotypically implicated in coat formation, occurs under the control of the early mother-cell-specific sigma factor σ^E (5, 8, 15, 43, 44, 50, 60, 62). The products of the genes grouped in this class have a notorious morphogenetic role in coat assembly (although, with the exception of CotE, a very important player at both the morphogenetic and structural levels, they are not prominent structural components of the coat), and their mutational inactivation has a profound impact on coat assembly and spore morphology and resistance properties (5, 8, 15, 43, 44, 50, 60). Second, studies with cells pulse-labeled at intermediate stages of development indicated that proteins later found in the spore coat layers were being synthesized at early stages of sporulation (23, 24), in a temporal frame consistent with the appearance of active σ^E . However, the genes encoding these early coat structural components have not been found, and therefore no example of early *cot* structural gene expression was known (with the exception of the *cotE* gene). Transcription of the *cotJ* operon is activated by σ^E ; therefore, *cotJ* could be an early-expressed *cot* locus. The *cotJ* products may correspond to at least two of the coat proteins originally identified in the pulse-labeling experiments of Jenkinson and Sawyer (23) and Jenkinson et al. (24), whose synthesis was shown to commence early in sporulation.

That *cotJ* is controlled by σ^E is supported by several lines of evidence. Induction of σ^E production in vegetatively growing cells of *B. subtilis* was shown to be sufficient to activate transcription from the *cotJ* promoter as monitored by primer extension experiments (Fig. 5). Studies of the temporal control of *cotJ* expression in Spo^+ strains using transcriptional fusions of the *cotJ* promoter to the *E. coli lacZ* or *gusA* genes (21, 28) revealed a temporal pattern of expression also found for many genes known to be under σ^E control (16, 40). Examination of the *cotJ* promoter region reveals putative -10 and -35 sequences, relative to its transcriptional start site (shown in Fig. 1), separated by a spacing of 14 bp that conform well to corresponding consensus sequences derived from the analysis of many σ^E -dependent promoters (40, 44). In the sequence centered at position -35 of the *cotJ* promoter, TCATAAT, four of seven positions match the consensus (KMATATT, where K represents G or T and M is C or A), and in the -10 sequence (CATACCT), 5 of 7 bases are identical to those in equivalent positions of the consensus sequence (CATACA-T) (40, 44). These sequences were shown to be important for the interaction of σ^E with their cognate promoters (reviewed in reference 40). Finally, we showed that expression of *cotJ* during sporulation is totally dependent on the *spoIIGB* gene, encoding σ^E (26, 29), but not on *spoIIG* or *spoIVCB*, encoding the late

forespore and mother-cell regulators σ^G (27, 38, 53) and σ^K (31), respectively.

Interestingly however, mutations in *spoIIID* appear to reduce the expression of *cotJ-lacZ* by two- to threefold (Fig. 8C). As already mentioned, the *spoIIID* gene encodes a small DNA-binding protein that can act to activate or repress the transcription of certain mother cell genes in the σ^E or σ^K regulons (19, 31, 62). The lower rate of induction of *cotJ-gusA* compared with *spoIID-lacZ* in the same strain could reflect the requirement for the SpoIIID protein. Conceivably, transcription of *cotJ* is initiated by $E\sigma^E$ but is not maximally induced until the cellular levels of the SpoIIID regulator reach a certain threshold point, a regulatory pattern also proposed for other genes, including *spoIIID* (33, 51), *cotE* (62), and *sigK* (19, 32). In support of the proposed role of SpoIIID in *cotJ* regulation, we note that a sequence with some similarity to a consensus SpoIIID binding box derived from the analysis of SpoIIID binding sites in the regulatory regions and coding sequences of four genes (19) is found in the *cotJ* regulatory region. This sequence, TTAGTCATAA, which overlaps the -35 region of the promoter (Fig. 1), can be aligned with the proposed consensus for SpoIIID binding (WRRACAR-Y, where W represents A or T, R means purine, and Y is pyrimidine) (19). The partial requirement for the SpoIIID protein in *cotJ* transcription does not reflect dependency on *spoIIID* for the transcription of *sigK*, since nearly normal expression of *cotJ* was found in *spoIVCB23* and *spoIVCBΔ::erm* mutant backgrounds (Fig. 8F).

Studies with double-reporter strains clearly showed that transcription of *cotJ-gusA* (as monitored by the production of β -glucuronidase) is induced early during sporulation, at about the same time as that of other σ^E -dependent genes such as *spoIID* and *cotE*, and at least 1 h earlier than that of other *cot* genes, an example being *cotA*. Because *cotA* belongs to the first temporal class of the σ^K regulon (47, 62) and all other *cot* genes, with the exception of *cotE* (62), are controlled by σ^K (3, 11, 45, 59, 62), we infer that expression of *cotJ* precedes that of other *cot* genes (with the mentioned exception). Because the transcription of *cotJ* is under $E\sigma^E$ control, we predict that its products are present in the mother cell cytoplasm at an early stage in development, together with the products of the *spoIVA*, *cotE*, and *spoVID* genes (5, 44, 50, 62). The role of the SpoIVA, CotE, and SpoVID proteins has been recently addressed by Driks and coauthors (15). The evidence presented by these authors suggested both a role for the members of the early morphogenetic class and the necessity for an early structural class of *cot* gene expression. The SpoIVA protein is believed to form a basal layer around the outer surface of the forespore, which is required for the formation at intermediate stages of sporulation of a CotE ring that surrounds the forespore at a small distance from it (15). Subsequent positioning of the CotE ring is dependent on the SpoVID protein (15). The ring of CotE is presumably held in place by a scaffold, which becomes the site of assembly of the inner coat, whereas assembly of the outer coat takes place on the outside of the CotE structure (15). Because (with the exception of *cotE* and now of *cotJ*) they are controlled by σ^K , no *cot* structural genes whose temporal pattern of expression could implicate them as components of the scaffold were known. An interesting possibility is that the *cotJ* products may play a role in an early phase of coat morphogenesis, perhaps as components of the scaffold. If that is in fact the case, then more scaffold genes or operons are expected to be found, since the lack of an obvious phenotype of *cotJ* null mutants indicates only a minor function or redundancy of its encoded products.

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