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CotM of *Bacillus subtilis*, a Member of the α-Crystallin Family of Stress Proteins, Is Induced during Development and Participates in Spore Outer Coat Formation

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We cloned and characterized a gene, *cotM*, that resides in the 173° region of the *Bacillus subtilis* chromosome and is involved in spore outer coat assembly. We found that expression of the *cotM* gene is induced during development under σ^K control and is negatively regulated by the GerE transcription factor. Disruption of the *cotM* gene resulted in spores with an abnormal pattern of coat proteins. Electron microscopy revealed that the outer coat in *cotM* mutant spores had lost its multilayered type of organization, presenting a diffuse appearance. In particular, significant amounts of material were absent from the outer coat layers, which in some areas had a lamellar structure more typical of the inner coat. Occasionally, a pattern of closely spaced ridges protruding from its surface was observed. No deficiency associated with the inner coat or any other spore structure was found. CotM is related to the α-crystallin family of low-molecular-weight heat shock proteins, members of which can be substrates for transglutaminase-mediated protein cross-linking. CotM was not detected among the extractable spore coat proteins. These observations are consistent with a model according to which CotM is part of a cross-linked insoluble skeleton that surrounds the spore, serves as a matrix for the assembly of additional outer coat material, and confers structural stability to the final structure.

Endospores of the gram-positive soil bacterium *Bacillus subtilis* are morphologically complex structures in which the spore core, containing the nucleoid confined within a membrane, is surrounded by a primordial germ cell wall, closely apposed to the spore membrane, and a wide cortical layer made of a modified form of peptidoglycan. This cortical layer is surrounded by a proteinaceous coat consisting of an electron-dense thick outer layer and a thinner, lamella-like inner layer (2, 16). The core and the primordial germ cell wall define the basic unit for the formation of a viable cell upon spore germination, whereas the cortex and coat layers are essential for the spore's remarkable environmental resistance properties. The coat, in particular, confers protection against lysozyme, solvents, and other harsh chemicals and is in part responsible for the prompt response of spores to compounds capable of triggering germination (2, 5, 13, 53, 56).

Biogenesis of the spore coat requires the production and assembly of over two dozen different structural components (13, 22, 38, 44, 53, 56). During an early stage of sporulation, an asymmetric division creates a larger mother cell compartment and a smaller prespore, which will develop into the mature spore (16, 32). Expression of all presently known 18 genes encoding some 21 coat polypeptides (or *cot* genes) (6, 13, 22, 38, 44, 52, 54, 56), and of other genes that control the assembly of the structural proteins (5, 14, 43, 49, 54), occurs in the mother cell under control of the mother cell-specific transcription factors σ^E, SpoIIID, σ^K, and GerE (14, 16, 32, 37). Expression of genes that govern early events in coat morphogenesis is directed by σ^E and SpoIIID (5, 19, 22, 38, 43, 49, 54), whereas the production and assembly of most of the coat structural components is controlled by σ^K and GerE (44, 52, 54, 55).

About 70% of the total coat protein can be solubilized by a variety of conditions (13, 22, 53) and resolved by gel electrophoresis. The remaining 30% is refractory to solubilization, presumably because of extensive cross-linking, and constitutes an insoluble fraction which is not amenable to electrophoretic resolution (53). Most of the *cot* genes that have been characterized encode proteins that can be extracted, resolved, and detected by gel electrophoresis (13, 22, 38, 44, 56). Yet the number of known genes cannot account for the totality of polypeptides represented in a coat protein gel, raising the expectation that other *cot* genes are to be found. Moreover, only the *cotVWXYZ* genes are known to participate in the building of the insoluble fraction (53), but deletion of *cotX*, a key member of the cluster, reduces the fraction of insoluble material by only about 30%. Undoubtedly, additional genes in this group also remain to be discovered and characterized.

To identify additional genes contributing to the assembly of the spore coat, we screened a *B. subtilis* genomic library for promoters dependent on σ^E or σ^K. In this report, we describe the *cotM* gene, found to be under σ^K control and to be negatively regulated by GerE. The *cotM* gene is located at about 173° on the *B. subtilis* chromosome and encodes a product related to the α-crystallin family of stress proteins. We show that CotM is required for the faithful assembly of the surface layers of the spore outer coat.

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* strains used in this study are listed in Table 1 or described in the text. The *B. subtilis* strains used in the functional analysis of the *cotM* locus or its regulation are congenic derivatives of the Spo$^+$ strain MB24. *Escherichia coli* DH5α and DH5αF′IQ (Bethesda Research Laboratories) were used for routine molecular cloning work. Genetic manipulations of *B. subtilis* were as previously described (9, 22). Antibiotic resistance, Amy, or Lac phenotypes of transformants, transfectants, or transductants were selected or

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TABLE 1. *B. subtilis* strains used

Strain	Genotype/phenotype	Source and/or reference
MB24	*trpC2 metC3*/Spo$^+$	P. Piggot, laboratory stock
EU8702	*trpC2 pheA1* Δ*spoIIGB*::*erm* (pDG180)/Pm^r Em^r	Laboratory stock 5
EU101	Δ*spoIIGB*::*erm chr*::Tn*917*wHU160::$P_{spac\text{-}spoIIGB}$/Pm^r Em^r	Laboratory stock 4
AH680	SPβ^S (pTV17)/Tc^r Em^r	Laboratory stock 4
ZB307	SPβ*c2del2*::Tn*917*::pSK10Δ6 prototroph/MLS^r	P. Zuber, laboratory stock
VO558	*trpC2 sigK gerE36*/Ger$^-$	Laboratory stock[a]
AH95	*trpC2 gerE36*/Ger$^-$	MB24 × VO558 (→Met$^+$)[b]
AH98	*trpC2 metC3* Δ*spoIIGB*::*sp*/Sp^r Em^s	MB24 × *Spe*I linearized pAH267 (→Sp^r)
AH99	*trpC2 metC3* Δ*spoIIID*::*sp*/Sp^r Em^s	MB24 × *Spe*I linearized pAH267 (→Sp^r)
AH100	*trpC2 metC3* Δ*spoIVCB*::*sp*/Sp^r Em^s	MB24 × *Spe*I linearized pAH267 (→Sp^r)
AH410	*trpC2 metC3* SPβ*cotA-lacZ*/Cm^r MLS^r	MB24 lysogen of SPβ*cotA-lacZ*
AH698	EU101 (SPβ*cotM-lacZ*)/Em^r Pm^r Cm^r	EU101 lysogen of SPβ*cotM-lacZ*
AH699	AH680 (pTVK24)/Tc^r Cm^r	AH680 × AH698 (→Cm^r)
AH670	*trpC2 metC3 cotM*::*sp*/Sp^r	MB24 × *Spe*I linearized pAH211 and pAH212 (→Sp^r)
AH770	*trpC2 metC3* SPβ*cotJ-lacZ*/Cm^r MLS^r	Laboratory stock 22
AH764	*trpC2 metC3* SPβ*cotC-lacZ*/Cm^r MLS^r	Laboratory stock
AH946	*trpC2 metC3* SPβ*cotM-lacZ*/Cm^r MLS^r Spo$^+$	MB24 lysogen of SPβ*cotM-lacZ*
AH1175	*trpC2 metC3* Δ*spoIIIG::sp*/Sp^r	MB24 × *Bgl*II linearized pAH254 (→Sp^r) 22
AH1245	*trpC2 metC3 cotM*::*sp* Δ*amyE*::*cotM*$^+$/Sp^r Cm^r	AH670 × *Pst*I linearized pAH349 (→Cm^r)

[a] The *gerE36* mutation was moved to MB24 by congression with the *met*$^+$ allele. Strain VO558 was the source of the *gerE36* mutation and was obtained from R. Losick. The origins of all other strains used in the analysis of the *cotM* locus are indicated in the text.

[b] In the crosses represented, the recipient strain is indicated first. Arrows denote selection for the indicated marker.

screened for as described previously (4, 5, 22). Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at 1 mM (final concentration) (4, 5, 22).

Integrational vectors. Plasmid pAH250 was constructed by inserting a 1.2-kb *Eco*RI-*Hin*dIII spectinomycin resistance gene (*sp*) from pGem7zf(+)-*sp* (a gift from D. LeBlanc) (31) between the equivalent sites of pBluescript II KS(+) (Stratagene). Digestion of pAH250 with *Pst*I released the *sp* gene, which we transferred to the *Pst*I site of plasmid pLITMUS 28 (New England Biolabs) to create pAH256. The *sp* gene in pAH256 points toward the *Spe*I site in the vector. Derivatives of plasmid pAH256 were then designed to change the antibiotic resistance associated with Tn*917* (macrolide-lincosamide-streptogramin B resistant [MLS^r]), or its erythromycin resistance gene (*erm*), to Sp^r. A fragment of 707 bp encompassing the 5′ end of the *erm* gene was amplified by using ON40 [5′-d(GTTATGGAAATAAGCTTAGAAGC)-3′ (an A at position 16 was deleted)] and ON41 [5′-d(CAATCAGATAGATC**C**TCAGACGCATG)-3′ (in which a G-to-**C** transversion was forced)]. The PCR product is delimited by positions 381 and 1088 in GenBank sequence M11180. The fragment, flanked by *Hin*dIII and *Bgl*II sites, was introduced between the *Hin*dIII and *Bam*HI sites of pAH256 to produce pAH265. A second plasmid, called pAH267, resulted from insertion of a 398-bp segment, flanked by *Sal*I and *Spe*I sites, between the compatible *Xho*I and *Spe*I sites of plasmid pAH265. This later section contains the 3′ end of the Tn*917 erm* gene (positions 1144 to 1542 in GenBank sequence M11180) and was obtained by using primers ON42 [5′-d(CTTGCACACTCAAGTC**GA**CGATTC AGC)-3′ (the **GA** pair replaces a T)] and ON43 [5′-d(CCTTGGAAGCTGTCA CTA**G**TATACC)-3′ (which incorporates a T-to-**G** transition)].

B. subtilis **developmental mutants.** Strains EU101, EU8702, AH74 (Δ*spoIIGB*::*erm*), AH76 (Δ*spoIIID*Δ::*erm*), and AH77 (Δ*spoIVCB*::*erm*) were described elsewhere (4, 5, 22). To change the specificity of the antibiotic resistance marker (*erm*) associated with the *spo* mutation in AH74 and AH76, competent cells of the two strains were transformed to Sp^r with *Spe*I-linearized pAH267 (see above). Conversely, AH77 was converted to Sp^r with *Eco*RI-linearized pEm::Sp (48). The erythromycin-sensitive (Em^s) phenotype of chosen transformants was verified, and the Sp^r marker was shown to be linked to the Spo$^-$ trait. The resulting strains were named AH98 (Δ*spoIIGB*::*sp*), AH99 (Δ*spoIIID*::*sp*), and AH100 (Δ*spoIVCB*::*sp*) (Table 1). The Δ*spoIIIG*::*sp* mutant AH1175 was obtained by transforming the wild-type strain MB24 to Sp^r with *Bgl*II-linearized pAH254 (21). Strain AH95 is a Ger$^-$ congressant produced by crossing the *met*$^+$ allele of VO558 (Table 1) into MB24. Its phenotype was scored by the tetrazolium overlay method (see below) and further confirmed by the Lac$^-$ phenotype of SPβ*cotC-lacZ* lysogens on Difco sporulation medium (DSM) agar plates. Double congression of the *skin*-less *sigK* (39) allele into MB24 was not verified.

Cloning of the *cotM* locus. A genetic screen designed to detect σ^E-dependent promoters (outlined in Results) has been described in detail elsewhere (4, 5, 22). The SPβ prophage in strain AH698 (a EU101 descendant) contains a transcriptional fusion of *cotM* to *lacZ*. The phage insert was cloned in plasmid pTV17, yielding pTVK24, and the sequence across the library fusion site was determined, revealing a new transcription unit. Digestion of pTVK24 with *Sal*I released an insert of about 2.5 kb, which we moved to *Sal*I-cut pUK19 (a gift from W. Haldenwang) (22) to create pUK24 (Fig. 1). In pUK24, the *lac*-proximal *Sau*3AI site is adjacent to the kanamycin resistance determinant of the plasmid. Chromosomal DNA from a strain bearing a Campbell-integrated copy of pUK24 was digested with *Kpn*I, religated, and used to transform *E. coli* to ampicillin resistant (Ap^r). This produced pUK24K, which carries the intact *cotM* locus flanked on its 5′ end by 2.5 kb of DNA (to the original *lacZ* distal *Sau*3AI site) and extending some 2.8 kb downstream of the *Sau*3AI site within *cotM*, as depicted in Fig. 1.

Creation of a MalE-CotM fusion. An *Nsi*I-to-*Msc*I fragment carrying the *cotM* gene was first cloned between the *Pst*I and *Sma*I sites of phage M13mp18. Second, a mutagenic oligomer, OM93 [5′-d(GAATGCTTCCATGAA**TT**CCAT TCAAAACGCAA)-3′], was used to insert the bases shown in boldface, two nucleotides downstream of the *cotM* start codon (underlined), creating an *Eco*RI site (30). A 450-bp *Eco*RI fragment was then released from this construct and inserted at the *Eco*RI site of pMal-c2 (New England Biolabs), in the proper orientation, to create plasmid pMalE-CotM. The presence of an in-frame fusion of MalE to CotM was verified by double-stranded sequencing using the *malE*-F primer. Induction of MalE-CotM production with IPTG, purification of the fusion protein on amylose columns, and its cleavage with factor Xa were done essentially as described by the manufacturer (New England Biolabs). The N-terminal sequence of the CotM moiety, ISEFHSK, where the underlined histidine corresponds to the second residue in the wild-type sequence, revealed the expected structure for the factor Xa-cleaved MalE-CotM fusion protein.

Insertional inactivation of *cotM*. The *cotM* gene was disrupted by inclusion of a Sp^r cassette into its unique *Sau*3AI site. First, primers ON94 [5′-d(ACGACA**A** AGCTTGTACTGACTGA)-3′ (a C-to-**A** transition creates a *Hin*dIII site)] and ON95 were used in a PCR to produce a fragment of 502 bp upstream of the *Sau*3AI in Fig. 1. The PCR product was cleaved with *Hin*dIII and *Sau*3AI and inserted between the *Hin*dIII and *Bam*HI sites of plasmid pAH256 (see above). The new plasmid, pAH211, carries *cotM* upstream of and in the same orientation as the *sp* gene. Second, a 235-bp PCR fragment carrying the 3′ moiety of the *cotM* gene was produced with primers ON96 [5′-d(GAAGCGGATCTGACTT TTTTAC)-3′] and ON97 [5′-d(GTTTAGCTGAGA**TCT**GAAATTGAAGAG)-3′ (engineered to carry a *Bgl*II site, in boldface)]. This product was digested with *Sau*3AI and *Bgl*II and ligated to *Bgl*II-digested pAH256, downstream of the *sp* gene and similarly oriented, to create pAH212. Finally, plasmids pAH211 and pAH212 were linearized with *Sca*I and used to convert *B. subtilis* MB24 (Table 1) to Sp^r. Transformants were the result of a triple-crossover event that transferred the in vivo-generated insertional mutation to the homologous chromosomal locus. Their chromosomal structure in the vicinity of the *cotM* locus was verified by lysing individual colonies (40) and performing PCR as described below. One appropriate transformant was selected and named AH670 (Table 1).

Complementation analysis. To conduct complementation tests, we constructed a plasmid capable of transferring a single copy of the wild-type *cotM* gene to the *amyE* locus. First, a 1.3-kb *Nsi*I-to-*Pst*I fragment obtained from pK25K was inserted at the *Pst*I site of pLITMUS 28 (the *Nsi*I site is internal to *cotL*, whereas the *Pst*I site is just downstream of the *yneK* stop codon [Fig. 1]). The resulting plasmid, pAH348, was then cut with *Nco*I and treated with the Klenow fragment of DNA polymerase in the presence of 0.2 mM each of the four nucleotides. Next, pAH348 was cut with *Bam*HI, and a 676-bp fragment was purified. This fragment, carrying the 3′ end of *cotL* and the intact *cotM* gene (Fig. 1), was inserted between the *Eco*RI (made blunt as described above) and *Bam*HI sites of the *amyE* integrational vector pDG364 (9). The resulting plasmid, called

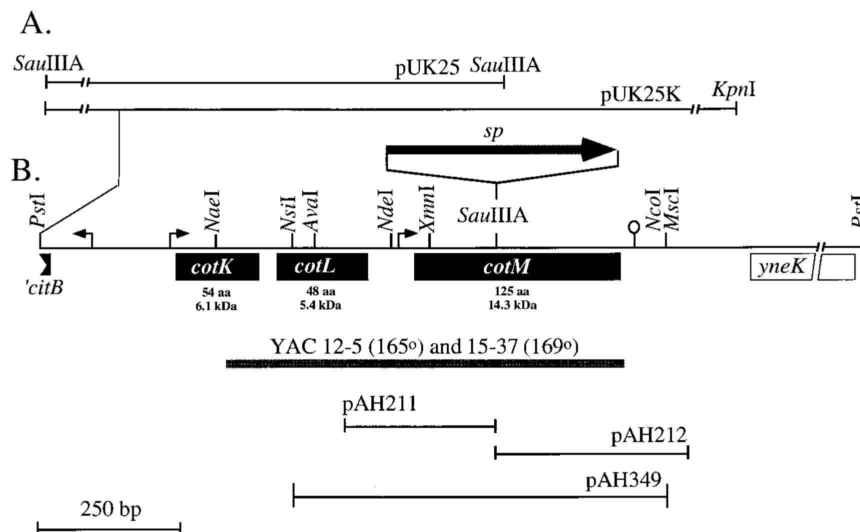

FIG. 1. The *citB-cotM* region of the *B. subtilis* chromosome. (A) The diagram represents the original 2.5-kb *Sau*3AI insert in the SPβ*cotM-lacZ* phage, after the in vivo recombinational transfer to pTV17 and subsequent insertion into pUK19 to create pUK25 (Materials and Methods). The *Sau*3AI site at its left end is the library fusion point. Plasmid pUK25K results from a chromosome walking step involving *Kpn*I digestion of DNA from a Campbell integrant of pUK25. Plasmid pUK25K carries some 2.8 kb of sequences downstream of the library fusion site. (B) Plasmids pAH211 and pAH212 provided homology regions for the introduction of a Sp^r cassette at the *Sau*3AI site in *cotM* (Materials and Methods). Plasmid pAH349 was a vehicle for the introduction of the indicated insert, carrying the wild-type *cotM* gene, at the *amyE* locus. Boxes below the partial restriction map indicate the positions and lengths of the predicted coding sequences in the region. With the exception of *citB*, they all are transcribed from left to right. The region represented by a thick line is totally contained within the indicated YAC clones (3). Promoter elements are represented by arrows. Also indicated (by a stem-loop structure) is a possible terminator immediately downstream of the *cotM* gene. aa, amino acids.

pAH349, was cut with *Pst*I and used to transform the *cotM* mutant AH670 (Table 1) to chloramphenicol resistance (Cm^r). That the integration had occurred by a marker replacement event (double crossover) was verified by the Amy^- phenotype of appropriate transformants (9). The merodiploid strain AH1245 resulted from this cross (Table 1).

Physical and genetic mapping of the *cotM* locus. Total yeast genomic DNA was prepared from the yeast artificial chromosome (YAC) collection of *B. subtilis* chromosomal inserts (3) as described previously (24), with minor modifications. Samples were organized in eight different pools, and approximately 100 ng was used in PCRs with the *cotM* primers ON94 and ON97 (see above). The PCR mixes were subjected to 30 cycles of incubation at 94°C for 60 s, 55°C for 90 s, and 72°C for 120 s in the presence of 0.05 U of *Taq* DNA polymerase (Boehringer Mannheim) per μl and 0.5 mM each primer. A pool yielding a product of the correct size was then broken down into its elements, and the PCR screen was repeated. The position of the *cotM* locus on the *B. subtilis* genetic map was deduced from the approximate location of the YAC clone(s) that produced a positive result.

Growth, sporulation, and germination of *B. subtilis*. Growth and sporulation of *B. subtilis* cultures were done as previously described (22) at 37 or 48°C. CFU per milliliter after heat, chloroform, or lysozyme treatment was measured in samples collected 18 h after the onset of sporulation (22). The use of 2,3,5-triphenyltetrazolium chloride as a germination indicator on plates was described before, as was the purification of spores on Renografin gradients (22).

Extraction and analysis of spore coat proteins. Coat proteins were extracted from Renografin-purified spores as described before (22) except that equivalent amounts of spores (2 A_{580} units) were mixed with the extraction buffer and subjected to the extraction procedure. After the extraction, the samples were briefly centrifuged and the supernatant was collected. The electrophoretic resolution of the sample was accomplished in 15% Tris-glycine polyacrylamide gels containing sodium dodecyl sulfate (SDS). Polypeptide SDS-polyacrylamide gel electrophoresis (PAGE) molecular weight markers (1.4 to 26.6 kDa) were from Bio-Rad; colored markers in different ranges were obtained from Amersham. Following electrophoresis, the gels were stained with Coomassie brilliant blue R-250 and destained. Electrotransfer of polypeptides from SDS-polyacrylamide gels to polyvinyl difluoride membranes (0.2-μm pore size) and N-terminal sequence analysis (at the Emory Microchemical Facility) were done as described before (28).

Isolation and primer extension analysis of mRNA. Total *B. subtilis* RNA was prepared at the times indicated in Fig. 6 from cells grown in DSM or Luria-Bertani medium as previously described (5). Primer extension reactions were carried out with OM92, an 18-mer oligomer [5′-d(TCATATAAATCAATAGG C)-3′] complementary to nucleotides 777 to 794 of the *cotM* sequence (Fig. 2). The primer extension reactions were as described previously (4, 5). The same primer was also used to create a sequencing ladder with an appropriate double-stranded template, so that the initiation nucleotide in the *cotM* message could be determined by inspection of the autoradiographs.

Enzyme assays. Samples (0.3 ml) were harvested every 30 min throughout growth and sporulation and briefly centrifuged, and the pellets were stored at −70°C until the time of assay. The activity of β-galactosidase was determined with the substrate *o*-nitrophenol-β-D-galactopyranoside. Enzyme specific activity is expressed in Miller units (36).

Sequence analysis. A *lacZ*-specific primer (22) was used to obtain the sequence across the library fusion site in plasmid pTVK24. Appropriate DNA restriction fragments encompassing the *citB-cotM* region were subcloned into M13 derivatives, and the sequences for both strands were resolved by the dideoxy-chain termination method using Sequenase (U.S. Biochemical Corporation). Sequences were analyzed with the programs in the Genetics Computer Group package (17).

Nucleotide sequence accession number. The *cotM* sequence was deposited in the GenBank database under accession number U72073.

RESULTS

Cloning and sequencing of the *cotM* region. Our approach to identifying promoters that are directly or indirectly dependent on σ^E has been described previously (4, 5, 22, 23). We made use of a random library of *B. subtilis* chromosomal DNA partially digested with *Sau*3AI, fused to the *lacZ* gene, and introduced in bacteriophage SPβ. The library (provided by Peter Zuber) was used to transduce recipient strains carrying the *sigE* gene (encoding σ^E) under the control of an inducible promoter (4, 5, 22, 23), and transductants were screened for an inducer-dependent Lac^+ phenotype. The SPβ*cotM-lacZ* phage was found as an IPTG-dependent Lac^+ transductant of strain EU101 (Table 1). The putative σ^E-dependent promoter in SPβ*cotM-lacZ* was cloned in pTV17 by in vivo recombination to create pTVK24 (5), from which a 2.8-kb *Sal*I fragment was released and inserted into the *Sal*I site of pUK19, producing pUK25 (Fig. 1). Sequences downstream of the library fusion site were cloned in a chromosomal walking step which used *Sph*I-digested and religated DNA from a Campbell integrant of pUK25. This created pUK25S. Sequence analysis of the

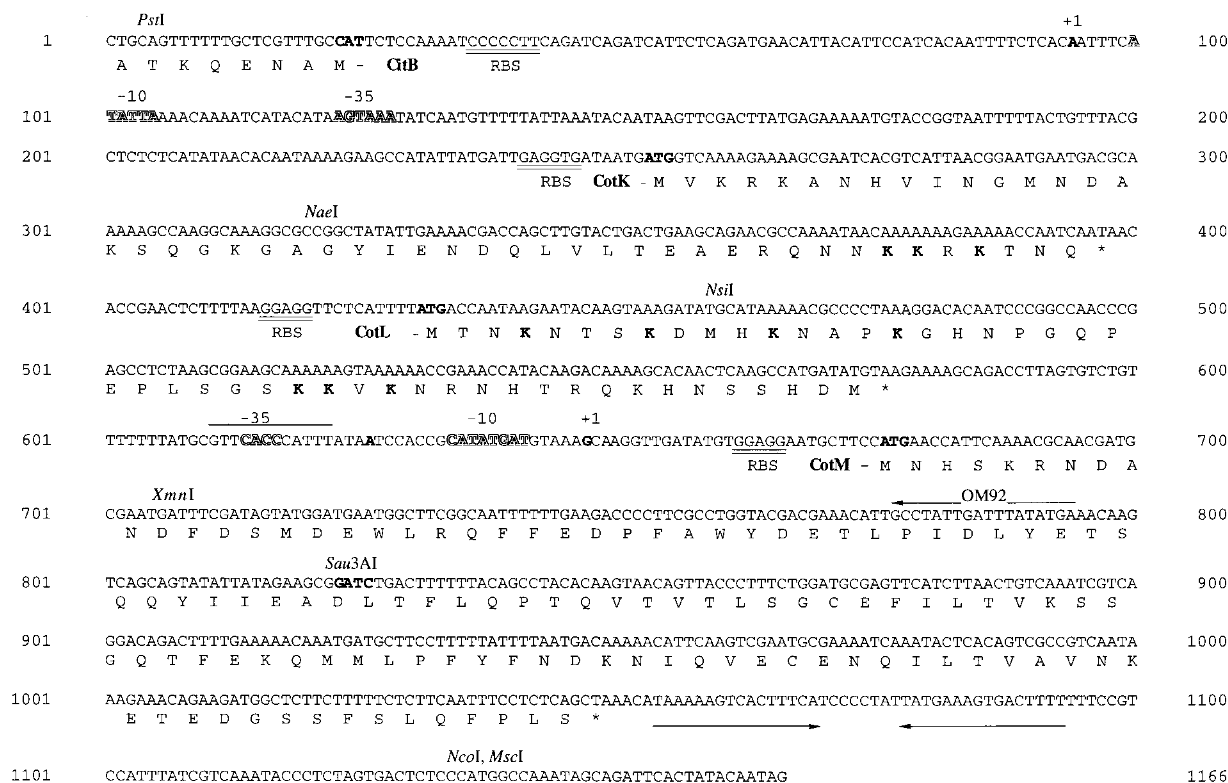

FIG. 2. Nucleotide sequence and genetic organization of the *cotM* region. The nucleotide sequence of the nontranscribed strand of the *citB-cotM* region of the chromosome is presented. Its major attributes include the −10 and −35 sequences of the *citB* and *cotM* promoters, upstream of the corresponding transcriptional start sites (+1); potential ribosome-binding sites (RBS) indicated upstream of putative start codons (in boldface); a possible GerE binding site overlapping the −35 region of the *cotM* promoter (shown by a line above the sequence); the positions and sequence of the N-terminal residues of CitB; the predicted primary structures of CotK, CotL, and CotM; and an inverted repeat thought to represent a *cotM* transcriptional terminator (arrows below the sequence). The annealing position of the oligomer (OM92) used for primer extension is also indicated. The deduced amino acid sequences are indicated in single-letter notation. Amino acids involved in motifs described in the text are shown in boldface. Only relevant restriction sites, including the original *Sau*3AI library junction site (bases 823 to 826), are depicted. An A (in boldface) at position 626 corresponds to a C in GenBank sequence Z73234.

region (Fig. 2) revealed that the fusion endpoint was within an open reading frame (ORF) (*cotM*) located upstream and divergently oriented from the *citB* gene (11), in confirmation of initial mapping results (see below). *cotM* is preceded by two similarly oriented short ORFs of equivalent sizes (54 and 48 codons), capable of encoding products of about 6 and 5.4 kDa (Fig. 1). Because they may be functionally related to *cotM* (see Discussion), these ORFs were provisionally called *cotK* and *cotL* (Fig. 1). A 209-bp-long interval separates the presumptive start codon of *cotK* from the *citB* translational start site (Fig. 1 and 2). Two transcripts are known to originate from this region (11, 12). One is produced from the *citB* promoter (11, 12), and its start point corresponds to position 94 in the sequence shown in Fig. 2. A second, sporulation-specific transcript originates about 140 bp downstream of the *citB* transcriptional start point and proceeds in the opposite direction (12). Thus, it could originate from a promoter just upstream of *cotK*. Interestingly, this transcript accumulates at intermediate stages of sporulation (12).

During the preparation of this report, the sequence of a 26.2-kb contig encompassing the *citB-cotM* region was deposited in the GenBank database under accession number Z73234; in this sequence, *cotM* corresponds to *yneL*. For the region of overlap, the sequence of the *cotM* locus here reported and that available under accession number Z73234 are in complete agreement except for an A at position 626 (Fig. 2), which is occupied by a C in sequence Z73234. A reexamination of the DNA sequence around this region confirms our assignment of an A base at the indicated position. In any case, this discrepancy is found in the interval between the −10 and −35 regions of the *cotM* promoter (Fig. 2) and does not affect sequences expected to be important for the regulation of *cotM* expression. The sequence downstream of *cotM* was also independently determined by Schiött et al. (47) and assigned accession number X87845. An ORF identified as *yneK* in GenBank sequence Z73234 or *yoxJ* in sequence X87845 is located 236 bp downstream of the *cotM* stop codon (Fig. 2). An inverted repeat sequence possibly acting as a factor-independent transcription terminator is found immediately downstream of the *cotM* gene (Fig. 2), suggesting that its expression is not associated with that of genes downstream. This interpretation is supported by our finding that unlike the expression of *cotM*, expression of *yneK* is not specifically induced during sporulation (not shown).

The 125-amino-acid-long CotM protein has a predicted molecular mass of about 14 kDa and is anticipated to be very acidic (pI of 3.9). This later property is thought to be due in part to the presence of 10 aspartate and 11 glutamate residues (17% of the total number of residues) and in part to a low representation of lysines (5 residues) and arginines (2 residues) in its primary structure. In spite of its overall acidity, three distinct regions can be recognized in the CotM sequence (Fig. 3); the first 40 residues of CotM define a very acidic N-terminal region (N), followed by a relatively hydrophobic

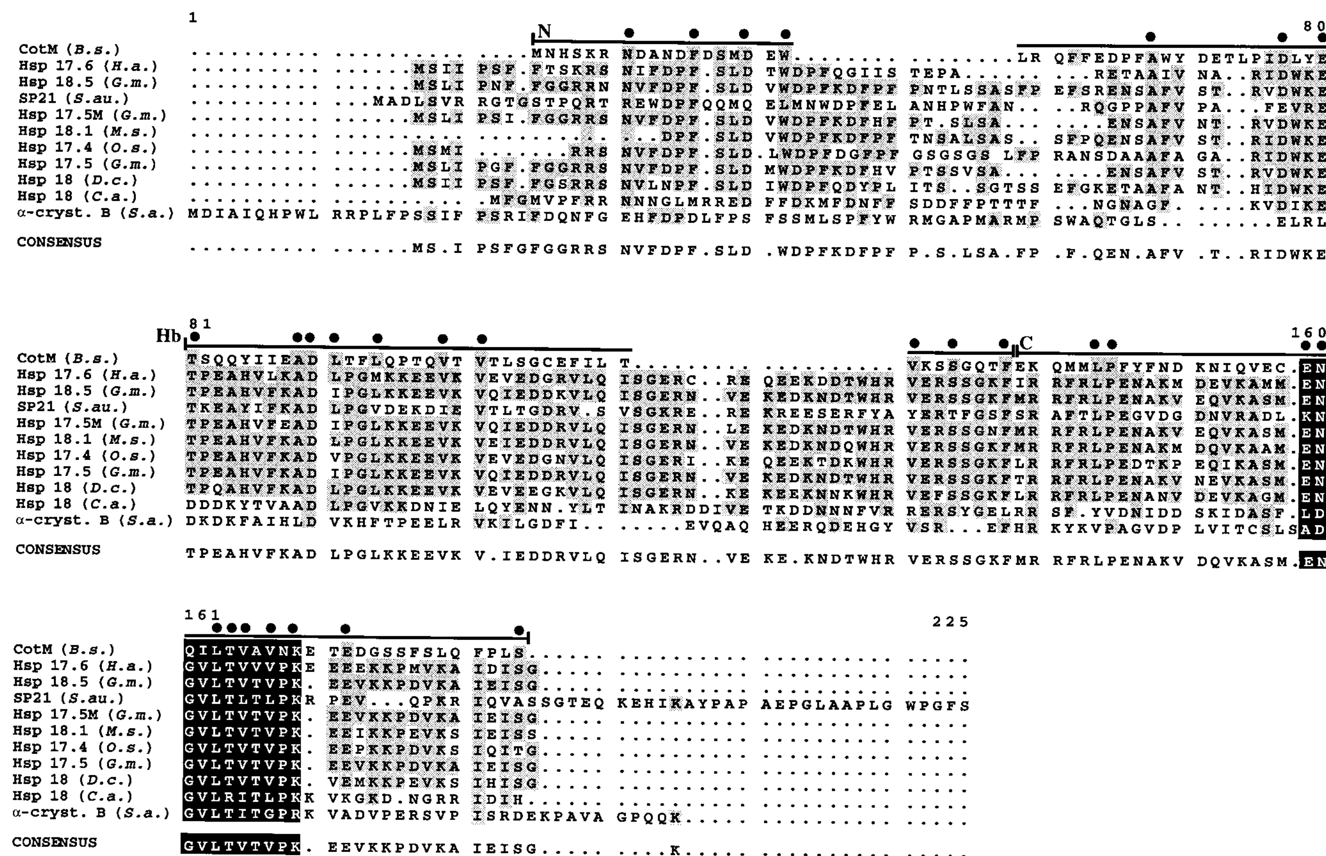

FIG. 3. CotM is related to the α-crystallin family of small HSPs. The alignment includes the *B. subtilis* (*B.s.*) CotM protein, polypeptides that were found to be most similar to CotM, and the sequence of the *Squalus acanthias* (*S.a.*) α-crystallin (α-cryst.) B as a reference (bottom line; GenBank sequence P02512). The alignment and the derived consensus sequence shown at the bottom were obtained with the PRETTY program in the Genetics Computer Group package (17). Dots represent insertions or deletions introduced to maximize the alignment. Only identical amino acids in equivalent positions of at least two-thirds of the sequences are highlighted. Highly conserved positions that include residues in the CotM sequence are indicated by closed circles. The residues shown against a black background define a highly conserved C-terminal signature shared by all members of the family. The N, Hb, and the C domains of CotM are indicated. The sequences shown correspond to the following GenBank numbers: *Helianthus annuus* (*H.a.*) Hsp17.6, P30693; *S. aurantiaca* (*S.au.*) SP21, Q06823; *Glycine max* (*G.m.*) Hsp18.5, 17.5M, and Hsp17.5, P05478, P04793, and P04794, respectively; *Oryza sativa* (*O.s.*) Hsp17.4, S24396; *Medicago sativa* (*M.s.*) Hsp18.1, P27879; *Daucus carota* (*D.c.*) Hsp18, P27397; *C. acetobutylicum* (*C.a.*) Hsp18, Q03928.

internal region (Hb; residues 41 to 79); the last 44 residues of CotM define a hydrophilic C-terminal region (C) that includes a sequence highly conserved between CotM and other related proteins (see below).

CotM is related to the α-crystallin family of stress proteins. CotM was found to have sequence similarity to a family of low-molecular-weight (LMW) heat shock proteins (HSPs), named after two of its members, the homologous subunits of the vertebrate eye lens structural protein, α-crystallin (27, 35). The alignment shown in Fig. 3 includes the nine members of the family that most resemble CotM, and the *Squalus acanthias* α-crystallin B sequence, for comparison. Among these are Hsp17.6 from the sunflower (the protein found to be most similar to CotM) and two prokaryotic representatives of the α-crystallin family (Fig. 3). The two latter are found in spore-forming organisms: a 21-kDa protein, SP21, from *Stigmatella aurantiaca* (20) and an 18-kDa protein from *Clostridium acetobutylicum* (46). Interestingly, production of the *S. aurantiaca* protein is known to be induced during fruiting-body formation and under heat shock and oxygen-limiting conditions (20) and SP21 is found in fruiting bodies and in indole-induced myxospores (34).

Two gaps were introduced into the CotM sequence during the construction of the alignment shown in Fig. 3. These gaps divide CotM into three segments of sequence similarity to members of the LMW HSPs. These segments are not related to regions N, Hb, and C, which are defined in terms of the distribution of charged and hydrophobic residues. Within each segment, identities to the corresponding segments of other members of the family range from 28 to 42%, and similarities range from 50 to 66%. The degree of sequence similarity found among the LMW HSPs increases towards the C termini of the proteins (20, 35, 46) and is maximal for a highly conserved sequence, whose consensus is ENGVLTVTVPK (Fig. 3). This sequence, which consists of a hydrophobic core flanked by charged residues, is also present in the C-terminal region of CotM.

The *cotM* locus is located at about 173° on the *B. subtilis* genetic map. We used yeast total DNA prepared from the collection of strains bearing the *B. subtilis* ordered YAC library (3) as the template in PCR with the *cotM*-specific primers ON94 and ON97 (see Materials and Methods). A single PCR product of about 700 bp (Fig. 1) was obtained with YAC clones 12-5 and 15-37, centered at about 165 and 169°, respectively, on the *B. subtilis* map (no other template in the library gave rise to a PCR product). We concluded that *cotM* is located in

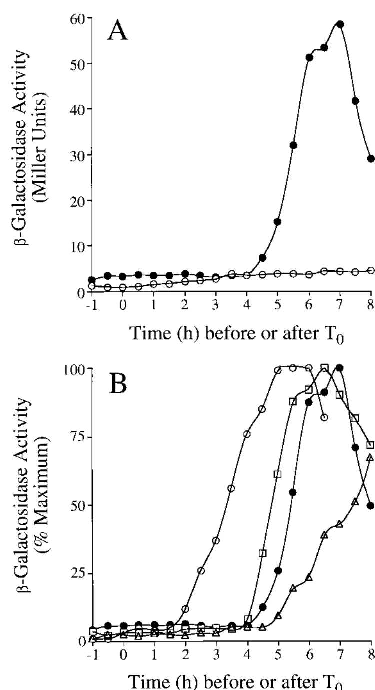

FIG. 4. Temporal analysis of *cotM-lacZ* expression. (A) Accumulation of β-galactosidase in a wild-type strain (closed circles) or a Δ*spoIIGB::sp* mutant (open circles), lysogenic for SPβ*cotM-lacZ*. (B) Comparison between the timing of *cotM-lacZ* activation and that of other genes known to be required for coat formation. The expression of the *cotM* (closed circles), *cotJ* (open circles), *cotA* (open squares), and *cotC* (triangles) was monitored in congenic strains carrying transcriptional fusions to the *lacZ* gene integrated into the SPβ prophage (Table 1).

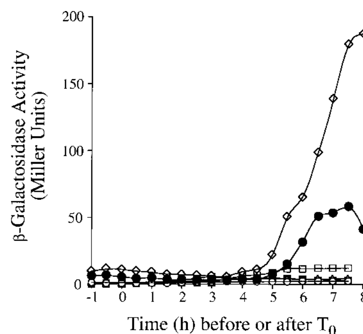

FIG. 5. Transcription of *cotM* is dependent on σ^K. Expression of an SPβ-borne *cotM-lacZ* fusion was monitored throughout growth and sporulation in a wild-type strain (closed circles) and congenic derivatives bearing the following developmental mutations: Δ*spoIIGB*Δ*::sp* (open circles), Δ*spoIIIG::sp* (closed squares), Δ*spoIIID::sp* (open squares), Δ*spoIVCB::sp* (triangles), and *gerE36* (diamonds) (Table 1). The end of the exponential phase of growth (T_0) is defined as the onset of sporulation.

the overlapping region between the two adjacent inserts in clones 12-5 and 15-37, both of which are also known to carry the *citB* locus (3). And in fact, by extending the sequence upstream of the *cotM* gene, we found *citB*, some 650 nucleotides from *cotM* (Fig. 1). *citB* is located at about 173° on the standard genetic map (1). Therefore, the same map position was adopted to the *cotM* locus.

Transcription of *cotM* is detected only during development and depends on σ^K. The original SPβ*cotM-lacZ* phage, isolated on the basis of its dependency on σ^E for expression, was introduced into wild-type strain MB24 (Table 1), and the cellular accumulation of β-galactosidase was monitored throughout growth and sporulation in DSM. Expression of the *cotM-lacZ* fusion remained undetected during exponential growth and the initial hours of development and was induced only around h 5 of sporulation (Fig. 4A). As expected, this induction was totally eliminated by a mutation in *sigE* (Fig. 5). The temporal profile of *cotM* expression however, was not that anticipated for a σ^E-controlled gene. Early mother cell-specific transcription, which relies on the activation of σ^E, is initiated at about the h 2 of sporulation (16, 32, 37), as illustrated in Fig. 4B, with a fusion of the *cotJ* promoter, a member of the σ^E regulon (22), to *lacZ*. In contrast, σ^K-controlled *cot* genes are induced after h 4 of sporulation (16, 32, 37). Expression of *cotM-lacZ* peaks slightly later than that of *cotA-lacZ* (45, 54) and precedes that of *cotC-lacZ* (Fig. 4B), a GerE-dependent gene (54). The suspicion that σ^K, and not σ^E, controlled *cotM-lacZ* expression was validated by the observation that a mutation in *sigK* (encoding σ^K) totally prevented β-galactosidase production (Fig. 5). Moreover, mutations in genes known to block transcription or activation of σ^K, such as *spoIIID* and *spoIIIG* (8, 33, 54), also prevented induction of *cotM-lacZ* (Fig. 5). In addition, we found that a mutation in *gerE* (7, 54, 55) results in a three- to fourfold increase in *cotM-lacZ* transcription (Fig. 5), making it likely that GerE is a repressor of transcription from the *cotM* promoter.

The expression of at least one bacterial member of the LMW HSP family is induced both by development and by stress conditions (20). To see if this was also true for *cotM*, we looked for but failed to find any induction of the SPβ-borne *cotM-lacZ* fusion upon heat shock of exponentially growing AH946 cells in LB medium. We have not examined accumulation of *cotM* mRNA by other methods. It has been suggested that a conserved inverted repeat found in the regulatory region of several heat-inducible genes in *B. subtilis* could have a role in their heat inducibility (51). This sequence, whose consensus has the form TTAGCACTCN$_9$GAGTGCTAA (51), is absent from the *cotM* promoter region.

Mapping of the 5′ terminus of *cotM* mRNA. The possibility that the *cotM* promoter was controlled by the σ^K-containing holoenzyme (see above) was further examined by primer extension experiments. We use the OM92 oligomer (Fig. 2) and reverse transcriptase to generate cDNA primer extension products, using RNA prepared at different times during development. Maximal accumulation of the *cotM* message was found at h 6.5 of sporulation (Fig. 6, lane e), a result consistent with the studies with *lacZ* fusions. The main start site of the *cotM* message corresponded to a G residue at position 647 (Fig. 2). Inspection of the DNA sequence upstream of this position revealed sequences centered on −10 and −35 (Fig. 2), separated by 16 nucleotides, that closely match the consensus sequence for promoter utilization by Eσ^K, recently revised by Roels and Losick (42). A shorter primer extension product corresponding to an apparent start site at a T residue, position 657, is also detected. No canonical −10 or −35 sequences were found upstream of this position, suggesting that this species may be the result of processing of the longer product or of premature pausing of the reverse transcriptase. The view that Eσ^K and not Eσ^E directs *cotM* expression is reinforced by the observation that induction of an active form of σ^E in vegetative cells in nonsporulation medium did not result in *cotM* transcription in vivo (Fig. 6, lane h).

Disruption of *cotM* affects the composition and ultrastructure of the coat. Since *cotM* is very actively transcribed during the main period of coat assembly and has sequence similarity with the α-crystallin family, we examined its role in coat as-

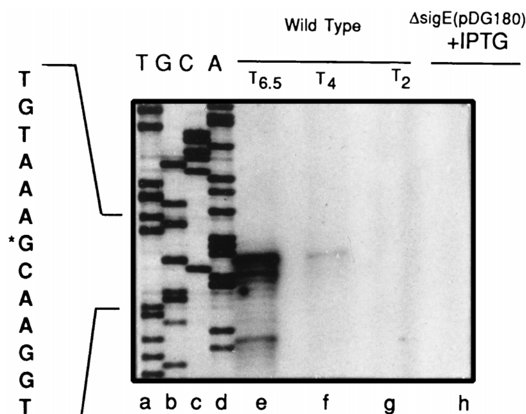

FIG. 6. Location of the 5′ terminus of *cotM* mRNA. Total RNA was purified from cultures of a Spo^+ strain in sporulation medium at the indicated times (in hours) after T_0 (the start of sporulation). Lane h represents a primer extension reaction carried out with RNA prepared from a log-phase culture in LB of a Δ*sigE*::*em* mutant (Δ*sigE*) after IPTG induction of active σ^E production from plasmid pDG180 (a gift from P. Stragier). In pDG180, an allele of *sigE* encoding an active form of σ^E is under the control of an IPTG-inducible promoter. cDNA synthesis was directed by OM92, an oligomer complementary to nucleotides 777 to 794 of the *cotM* sequence. The arrow indicates the position of the *cotM*-specific primer extension product. The same oligomer was used in sequencing reactions (lanes a to d) with an appropriate double-stranded template, allowing the position of the apparent start site of the *cotM* transcript (a G at position 647, identified by an asterisk) to be read from the sequence.

sembly. We compared the protein compositions of the coats of wild-type and *cotM* mutant spores by electrophoresis through 15% Tris-glycine polyacrylamide gels containing SDS. The pattern of polypeptides extracted from the mutant spores differed from that of wild-type spores in that several polypeptides were present in reduced amounts (Fig. 7, lanes 3 and 4). This reduction was more obvious with older spores, suggesting that

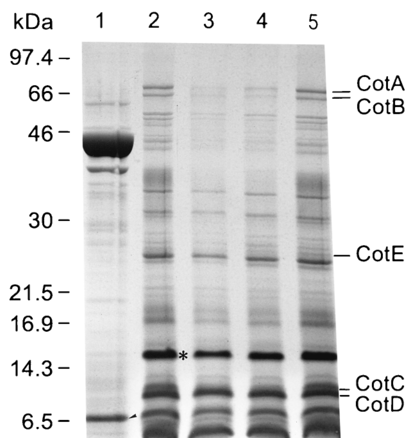

FIG. 7. A *cotM* mutant has an altered electrophoretic profile of spore coat proteins. Spores of the indicated strains were purified in gradients of Renografin, washed, and resuspended in distilled H_2O. Spore coat proteins were extracted from equivalent numbers of spores, corresponding to about 2 absorbance units, as described in Material and Methods, and the samples were resolved by SDS-PAGE (15% polyacrylamide gel). Samples were prepared from the following strains: lanes 2 and 5, MB24 (wild type); lanes 3 and 4, AH670 (*cotM*::*sp*). Lane 1 was loaded with about 20 μg of a factor X-cleaved MalE-CotM fusion protein. The position of the CotM moiety is indicated by an arrow. That equivalent amounts of spores were used in the extractions is shown by the relative concentration of the band marked with an asterisk, whose extractability is essentially independent of the status of the coat layers (19, 20). The positions of specific coat polypeptides, CotA to CotE, are indicated.

the coat integuments may be structurally unstable in the mutant (not shown). A sample of a partially purified MalE-CotM fusion protein that had been cleaved with factor Xa was also included in the gel (Fig. 7, lane 1) so that the relative mobility of the wild-type CotM protein could be estimated. As indicated (Fig. 7), the CotM protein has an apparent mobility of about 6 kDa, which may result from its extreme acidic character (see above). No species of the size expected for CotM was missing from the coats of the mutant (Fig. 7). Antibodies raised against the partially purified CotM protein were able to recognize the MalE-CotM fusion protein or its cleaved CotM moiety made in *E. coli* (not shown). They did not, however, react specifically with any CotM-dependent species present in total cell extracts prepared at different stages of sporulation or in extracts prepared from wild-type spores. However, an estimation of the level of detection of the partially purified protein suggests that CotM would have to be an abundant soluble component to be detected by the antiserum (not shown).

Thin sections of purified spores from the *cotM* mutant were then examined by transmission electron microscopy. In wild-type spores, the coat appeared composed of a thick, multilayered, dense outer coat and of a lamellar inner coat (Fig. 8A and B).. Compared to those produced by the parental strain, the mutant spores revealed some distinctive morphological features (Fig. 8C to F). The outer coat appeared to have lost most of its structural integrity, presenting a more diffuse appearance (Fig. 8C to F). In the mutant, considerably less material appeared to be assembled in the surface layers of the outer coat, and in some areas a lamella-type structure is seen. The appearance of this lamella-type structure of lower electron density is very similar to that typical of the inner coat layers (Fig. 8C to E). Occasionally, a pattern of closely aligned surface ridges (Fig. 8F) was observed. The inner coat layers, as far as can be detected by electron microscopy (Fig. 8), remained unaffected in the mutant. Thus, the effect of the *cotM*::*sp* mutation is restricted to the outer coat layers. Aronson and Fitz-James (2) observed a very similar but not identical phenotype when sodium sulfite was added at a late time to sporulating cultures of *B. subtilis*, an effect that the authors attributed to the inhibition of a cysteine exchange reaction.

To verify that the phenotypes observed for the *cotM*::*sp*-bearing strain were not due to a polar effect or to the fortuitous introduction of mutations in *cotL* or in the *cotM*-*yneK* intergenic region, a complementation test was carried out. An intact copy of the wild-type *cotM* gene was inserted into the pDG364 vector (9), which allows integration of cloned DNA at the *amyE* locus in single copy (see Material and Methods). Introduction of the wild-type *cotM* allele at *amyE* in the *cotM*::*sp* mutant AH670 produced strain AH1245 (Table 1), whose spores had apparently normal coat layers when analyzed by SDS-PAGE (not shown). This result demonstrated that $cotM^+$ is transdominant over the *cotM*::*sp* allele and that the phenotypes herein described for strain AH670 are truly due to loss of *cotM* function.

We detected no effect of the *cotM* insertional mutation on germination or resistance to heat, lysozyme, or chloroform of spores produced by cultures grown at 37 or at 48°C.

DISCUSSION

The results described in this report implicate *cotM* of *B. subtilis* in the assembly of the surface layer of the outer coat. The *cotM* gene is located in the 173° region of the chromosome, close but not adjacent to the previously characterized *cotC* locus (13). Many genes involved in coat assembly are expressed late in the mother cell, under σ^K control, and some

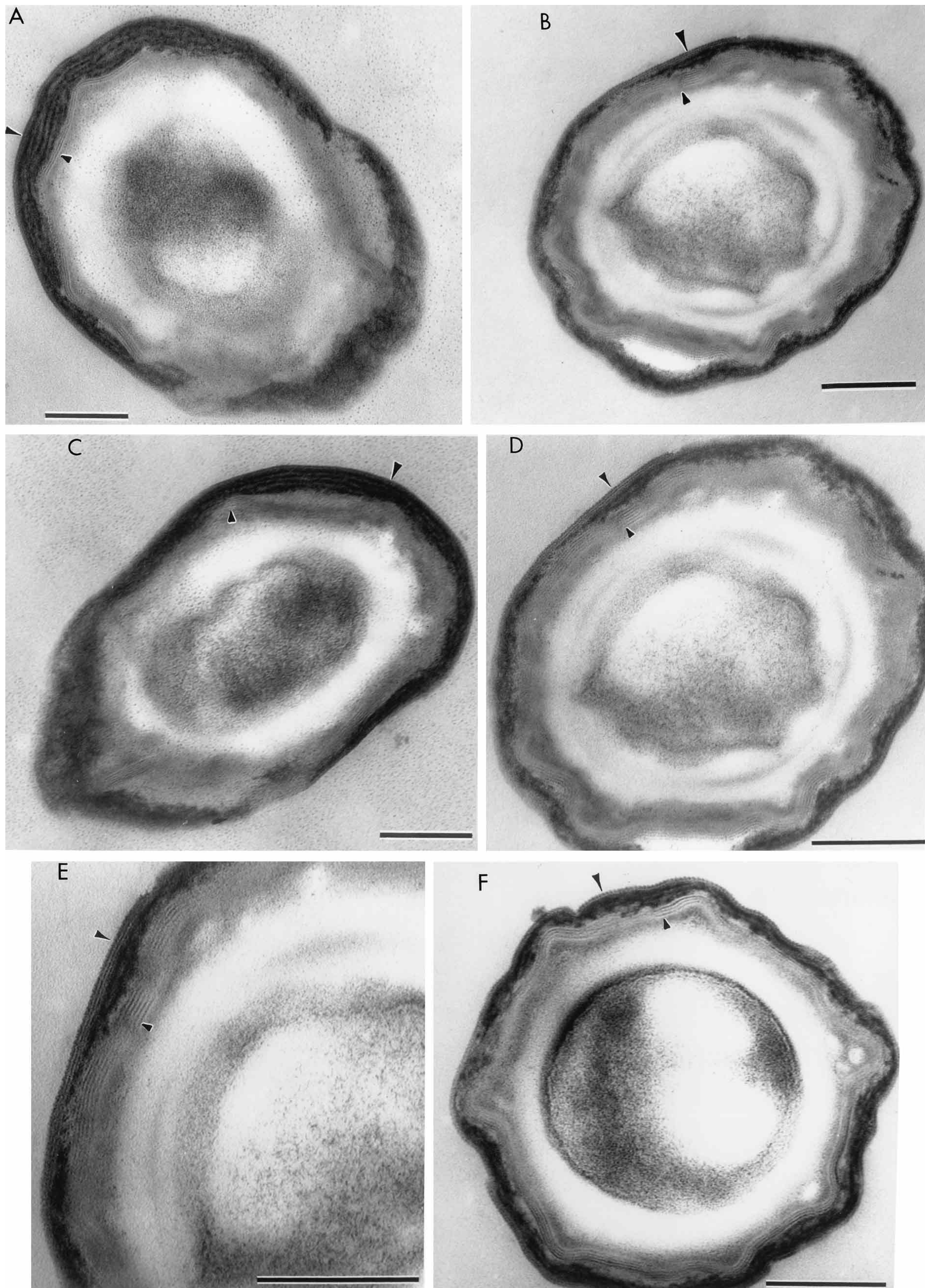

FIG. 8. *cotM* mutants spores have an anomalous morphological arrangement of the coat surface layers. Electron micrographs show sections of wild-type (A and B) and *cotM* mutant (C to F) spores. Large arrowheads indicate the outer coat; small arrowheads point to the inner coat. Samples of cultures in sporulation medium were harvested 18 h after the onset of sporulation and extensively washed with water, and the spores were purified in Renografin gradients as described in Materials and Methods. Samples of the spore suspension were then fixed and processed for electron microscopy. Bars, 0.2 μm.

are controlled by the GerE protein (52, 54, 55). The *cotM* gene is under σ^K control. First, induction of *cotM* coincides with the expression of *cotA* and precedes that of *cotC*, members of the first and last classes of σ^K-controlled genes (45, 54). Second, mutations in *sigK*, or in genes involved in its transcription (*sigE* and *spoIIID*) (54) or activation (such as *spoIIIG*) (8, 30), totally prevented expression of the *cotM* gene. That *cotM* is under σ^K control was further supported by the identification in its promoter region, as defined by primer extension, of sequences very close to those that signal promoter recognition by the σ^K-containing holoenzyme (42). Lastly, transcription from the *cotM* promoter seems to be repressed by the GerE factor, a regulatory twist that is also characteristic of the *cotA* gene (54, 55). In agreement with this observation, we note the presence of a possible GerE binding site in the *cotM* regulatory region (Fig. 2), overlapping the −35 region of the promoter (52, 53). Presumably, this putative GerE target would function in repression by blocking access of the RNA polymerase holoenzyme to the *cotM* promoter. *cotA* and *cotM* are the only two examples of negative regulation of *cot* gene expression by the GerE protein, and only two other genes, *csk22* (23) and *spoVF* (10), are thought to be negatively regulated by GerE. *gerE* is transcribed, along with *cotM* and *cotA*, during the first wave of σ^K-directed transcription (7, 54), and the GerE protein stimulates transcription by σ^K from several other *cot* promoters (52, 54, 55).

Inactivation of *cotM* results in spores with an altered profile of electrophoretically resolved coat proteins (Fig. 7) and distinctive morphological features, as revealed by electron microscopy. The outer coat appears incomplete and has a diffuse appearance. In some cases, a lamellar structure very similar to that of the inner coat or a pattern of closely spaced ridges was observed in its outermost layer. It is not known if these features represent an underlying structure of the outer coat that is uncovered by the loss of material in the mutant. Alternatively, the observed structures could represent an intermediate in the normal assembly process or even an abnormal pathway. In agreement with the idea that the outer coat is incomplete in the mutant, several polypeptides seem to be underrepresented in but not missing from the coat integuments of the *cotM* mutant. Interestingly, the level of CotD (thought to be an inner coat protein [56]), is unaffected in the mutant, whereas the level of the outer coat protein CotC is reduced. This lends further support to the idea that CotM functions mainly in outer coat assembly. In contrast to *cotM*, mutations in the *cotE* or *cotH* gene cause the complete absence of a subset of proteins from the coats (38, 56), and deletion of *cotG* entirely prevents the assembly of CotB (44). Moreover, CotE, CotH, and CotG are prominent structural components of the coats, whereas no CotM-dependent band was found to be specifically missing from the coat soluble fraction in *cotM* mutant spores.

CotM belongs to the α-crystallin family of LMW HSPs. α-Crystallin is a major structural component of the vertebrate eye lens (25, 35). Some properties of α-crystallin and other members of the LMW HSP family include the ability to form high-molecular-weight aggregates, to bind to and inhibit a family of structurally related serine proteases (25, 27, 35), and to function as molecular chaperones (25, 27, 35). In addition, some members of the family are known to be substrates for transglutaminase-mediated protein cross-linking (25, 27, 35). CotM is missing two internal regions present in other members of the family, and it is unlikely that CotM has all of the properties associated with the LMW HSPs. In addition, these proteins may perform different functions in different organisms or tissues (20). The SP21 protein of *S. aurantiaca*, for example, seems to associate mainly with the cell wall of fruiting-body-derived spores (20), and its function is probably not related to that of CotM during sporulation in *B. subtilis*. Interestingly, the chaperone-like function of the α-crystallins seems to depend on their C-terminal regions (50), which include the highly conserved sequence signature shared by CotM (Fig. 3). An interesting possibility is that CotM functions as a chaperone, preventing nonproductive interactions among coat polypeptides prior to their assembly into the coat. However, we found no evidence for a chaperone-like function. Specifically, we found no change in the resistance properties of the mutant spores at 48°C.

We favor a model according to which CotM could be a substrate for a transglutaminase involved in outer coat assembly. According to this model, CotM participates in the construction of an insoluble matrix that envelops the coat near its outermost boundary. This matrix may serve as a basal layer for the assembly of further outer coat material and to confer mechanical stability to the structure. In its absence, multiple proteins in the coat soluble fraction tend to be lost, in a nonspecific way. The reduction of several proteins in the *cotM* mutant and its altered outer coat morphology would be consistent with this view. It is known that deletion of well-characterized genes involved in the building of the insoluble fraction, such as *cotX*, *cotY*, and *cotZ*, also results in the production of spores with an incomplete outer coat (53). Moreover, the CotX protein, whose absence causes a significant reduction in the amount of total insoluble material, is barely detected in the soluble fraction of wild-type coats (53). Analogously, a CotM-dependent specific polypeptide was not detected in the soluble fraction. We have suggested that CotM could be a minor coat constituent whose level is below our detection limit. However, sequestering of CotM in a cross-linked array, via a transglutaminase-dependent formation of ε-(γ-glutamyl)lysine bonds, could also make it inaccessible to our electrophoretic analysis of the coat layers. The ε-(γ-glutamyl)lysine cross-link is known to confer a high degree of mechanical and chemical resistance (e.g., to proteolysis) to keratins or during blood clotting and is often the cause of protein insolubility (18, 26). Interestingly, 10 glutamine residues are found throughout the length of CotM, and the following suggestive lysine motifs are found in CotK and CotL (Fig. 2): a KKRK motif near the C terminus of CotK, a regular repeat of lysine residues near the N terminus of CotL, and a KKVK motif near the center of the polypeptide chain (Fig. 3). CotK and CotL could be cross-linking partners of CotM.

The involvement of ε-(γ-glutamyl)lysine isopeptide bonds in coat assembly has been previously suggested (53). A transglutaminase activity has been detected during sporulation in *B. subtilis* (29, 41). Significant levels of enzyme activity have been found at the time of coat formation (at h 6 and 10 of sporulation) but not in vegetative cells (29). More importantly, ε-(γ-glutamyl)lysine isopeptide bonds were detected in a spore coat protein fraction, establishing that this type of cross-link participates in spore coat formation (29). These findings reinforce our model that a transglutaminase-dependent layer confers structural stability to the outer coat and that CotM could be involved in its formation.

The phenotype caused by the addition of sodium sulfate to cultures of *B. subtilis* late in sporulation (2) is similar in certain aspects to the one described for the *cotM* insertional mutant. Sodium sulfite was hypothesized to inhibit a cysteine exchange reaction involved in loosening the tertiary structure of a polypeptide, facilitating its assembly. The relation of this to the phenotype of the *cotM* mutant is unclear, but cysteine residues are known to be part of the active sites of transglutaminases (18, 26). One possibility is that the sulfite is also an inhibitor of

the transglutaminase, thereby establishing a functional link between the two phenotypes.

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REFERENCES

1. **Anagnostopoulos, C., P. J. Piggot, and J. A. Hoch.** 1993. The genetic map of *Bacillus subtilis*, p. 425–461. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.
2. **Aronson, A. I., and P. Fitz-James.** 1976. Structure and morphogenesis of the bacterial spore coat. Bacteriol. Rev. **40:**360–402.
3. **Azevedo, V., E. Alvarez, E. Zumstein, G. Damiani, V. Sgaramella, S. D. Ehrlich, and P. Serror.** 1993. An ordered collection of *Bacillus subtilis* DNA segments cloned in yeast artificial chromosomes. Proc. Natl. Acad. Sci. USA **90:**6047–6051.
4. **Beall, B., and C. P. Morgan, Jr.** 1994. Cloning and characterization of *spoVR*, a gene from *Bacillus subtilis* involved in spore cortex formation. J. Bacteriol. **176:**2003–2012.
5. **Beall, B., A. Driks, R. Losick, and C. P. Moran Jr.** 1993. Cloning and characterization of a gene required for assembly of the *Bacillus subtilis* spore coat. J. Bacteriol. **175:**1705–1716.
6. **Cutting, S., L. Zheng, and R. Losick.** 1991. Gene encoding two alkali-soluble components of the spore coat from *Bacillus subtilis*. J. Bacteriol. **173:**2915–2919.
7. **Cutting, S., S. Panzer, and R. Losick.** 1989. Regulatory studies on the promoter for a gene governing synthesis and assembly of the spore coat in *Bacillus subtilis*. J. Mol. Biol. **207:**393–404.
8. **Cutting, S., V. Oke, A. Driks, R. Losick, S. Lu, and L. Kroos.** 1990. A forespore checkpoint for mother-cell gene expression during development in *Bacillus subtilis*. Cell **62:**239–250.
9. **Cutting, S. M., and P. B. Vander Horn.** 1990. Genetic analysis, p. 27–74. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Willey & Sons Ltd., Chichester, England.
10. **Daniel, R. A., and J. Errington.** 1993. Cloning, DNA sequence, functional analysis and transcriptional regulation of the genes encoding dipicolinic acid synthetase required for sporulation in *Bacillus subtilis*. J. Mol. Biol. **232:**468–483.
11. **Dingman, D. W., and A. L. Sonenshein.** 1987. Purification of aconitase from *Bacillus subtilis* and correlation of its N-terminal amino acid sequence with the sequence of the *citB* gene. J. Bacteriol. **169:**3062–3067.
12. **Dingman, D. W., M. S. Rosenkrantz, and A. L. Sonenshein.** 1987. Relationship between aconitase gene expression and sporulation in *Bacillus subtilis*. J. Bacteriol. **169:**3068–3075.
13. **Donovan, W., L. Zheng, K. Sandman, and R. Losick.** 1987. Genes encoding spore coat polypeptides from *Bacillus subtilis*. J. Mol. Biol. **196:**1–10.
14. **Driks, A., and R. Losick.** 1991. Compartmentalized expression of a gene under the control of sporulation transcription factor σ^E in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **88:**9934–9938.
15. **Driks, A., S. Roels, B. Beall, C. P. Moran, Jr., and R. Losick.** 1994. Subcellular localization of proteins involved in the assembly of the spore coat of *Bacillus subtilis*. Genes Dev. **8:**234–244.
16. **Errington, J.** 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. **57:**1–33.
17. **Genetics Computer Group.** 1994. Program manual for the Wisconsin package, version 8, September 1994. Genetics Computer Group, Madison, Wis.
18. **Greenberg, C. S., P. J. Birckbichler, and R. H. Rice.** 1991. Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. FASEB J. **5:**3071–3077.
19. **Halberg, R., and L. Kroos.** 1994. Sporulation regulatory protein SpoIIID from *Bacillus subtilis* activates and represses transcription by both mother-cell-specific forms of RNA polymerase. J. Mol. Biol. **243:**425–436.
20. **Heidelbach, M., H. Skladny, and H. U. Schairer.** 1993. Heat shock and development induce synthesis of a low-molecular-weight stress-responsive protein in the myxobacterium *Stigmatella aurantiaca*. J. Bacteriol. **175:**7479–7482.
21. **Henriques, A. O., and C. P. Moran, Jr. (Emory University).** Unpublished results.
22. **Henriques, A. O., B. W. Beall, K. Roland, and C. P. Moran Jr.** 1995. Characterization of *cotJ*, a σ^E-controlled operon affecting the polypeptide composition of the coat of *Bacillus subtilis* spores. J. Bacteriol. **177:**3394–3406.
23. **Henriques, A. O., E. M. Bryan, B. W. Beall, and C. P. Moran Jr.** *cse15*, *cse60*, and *csk22*, are new members of mother-cell-specific sporulation regulons in *Bacillus subtilis*. Submitted for publication.
24. **Hoffman, C. S., and F. Winston.** 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene **57:**267–272.
25. **Horwitz, J.** 1992. α-crystallin can function as a molecular chaperone. Proc. Natl. Acad. Sci. USA **89:**10449–10453.
26. **Ichinose, A., R. E. Bottenus, and E. W. Davie.** 1990. Structure of transglutaminases. J. Biol. Chem. **265:**13411–13414.
27. **Jakob, U., M. Gaestel, K. Engel, and J. Buchner.** 1993. Small heat shock proteins are molecular chaperones. J. Biol. Chem. **268:**1517–1520.
28. **Kellner, E. M., A. Decatur, and C. P. Moran, Jr.** 1996. Two-stage regulation of an anti-sigma factor determines developmental fate during bacterial endospore formation. Mol. Microbiol. **21:**913–924.
29. **Kobayashi, K., Y. Kumazawa, K. Miwa, and S. Yamanaka.** 1996. ε-(γ-Glutamyl)lysine cross-links of spore coat proteins and transglutaminase activity in *Bacillus subtilis*. FEMS Microbiol. Lett. **144:**157–160.
30. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
31. **LeBlanc, D. J., L. N. Lee, and J. M. Inamine.** 1991. Cloning and nucleotide base sequence analysis of a spectinomycin adenyltransferase AAD(9) determinant from *Enterococcus faecalis*. Antimicrob. Agents Chemother. **35:**1804–1810.
32. **Losick, R., and P. Stragier.** 1992. Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*. Nature **355:**601–604.
33. **Lu, S., R. Halberg, and L. Kroos.** 1990. Processing of the mother-cell σ factor, σ^K, may depend on events occurring in the forespore during *Bacillus subtilis* development. Proc. Natl. Acad. Sci. USA **87:**9722–9726.
34. **Lünsdorf, H., H. U. Schairer, and M. Heidelbach.** 1995. Localization of the stress protein SP21 in indole-induced spores, fruiting bodies, and heat-shocked cells of *Stigmatella aurantiaca*. J. Bacteriol. **177:**7092–7099.
35. **Merck, K. B., P. J. T. A. Groenen, C. E. M. Voorter, W. A. de Haard-Hoeckman, J. Horwitz, H. Blomendal, and W. W. de Jong.** 1993. Structural and functional similarities of bovine α-crystallin and mouse small heat-shock protein. A family of chaperones. J. Biol. Chem. **268:**1046–1052.
36. **Miller, J.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Moran, C. P., Jr.** 1993. RNA polymerase and sigma factors. *In* A. L. Sonenshein, J. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.
38. **Naclerio, G., L. Baccigalupi, R. Zilhão, M. de Felice, and E. Ricca.** 1996. *Bacillus subtilis* spore coat assembly requires *cotH* gene expression. J. Bacteriol. **178:**4375–4380.
39. **Oke, V., and R. Losick.** 1993. Multilevel regulation of the sporulation transcription factor σ^K in *Bacillus subtilis*. J. Bacteriol. **175:**7341–7347.
40. **Promega Corporation.** 1995. Technical manual, *fmol* DNA sequencing system. Promega Corporation, Madison, Wis.
41. **Ramanujan, M. V., and J. H. Hageman.** 1990. Intracellular transglutaminase (EC 2.3.2.13) in a procaryote: evidence from vegetative and sporulating cells of *Bacillus subtilis* 168. FASEB J. **4:**A2321.
42. **Roels, S., and R. Losick.** 1995. Adjacent and divergently oriented operons under the control of the sporulation regulatory protein GerE in *Bacillus subtilis*. J. Bacteriol. **177:**6263–6275.
43. **Roels, S., A. Driks, and R. Losick.** 1992. Characterization of *spoIVA*, a sporulation gene involved in coat morphogenesis in *Bacillus subtilis*. J. Bacteriol. **174:**575–585.
44. **Sacco, M., E. Ricca, R. Losick, and S. Cutting.** 1995. An additional GerE-controlled gene encoding an abundant spore coat protein from *Bacillus subtilis*. J. Bacteriol. **177:**372–377.
45. **Sandman, K., L. Kroos, S. Cutting, P. Youngman, and R. Losick.** 1988. Identification of a promoter for a spore coat protein gene in *Bacillus subtilis* and studies on the regulation of its induction at a late stage of sporulation. J. Mol. Biol. **200:**461–473.
46. **Sauer, U., and P. Dürre.** 1993. Sequence and molecular characterization of a DNA region encoding a small heat shock protein of *Clostridium acetobutylicum*. J. Bacteriol. **175:**3394–3400.
47. **Schiött, T., C. von Wachenfeldt, and L. Hederstedt.** Identification and characterization of the *ccdA* gene, required for multiple cytochrome C synthesis in *Bacillus subtilis*. Submitted for publication.
48. **Steinmetz, M., and R. Richter.** 1994. Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*, through in vivo recombination. Gene **142:**79–83.
49. **Stevens, C. M., R. Daniel, N. Illing, and J. Errington.** 1992. Characterization of a sporulation gene, *spoIVA*, involved in spore coat morphogenesis in *Bacillus subtilis*. J. Bacteriol. **174:**586–594.
50. **Takemoto, L., T. Emmons, and J. Horwitz.** 1993. The C-terminal region of

α-crystallin: involvement in protection against heat-induced denaturation. Biochem. J. **294:**435–438.

51. **Völker, U., S. Engelmann, B. Maul, S. Riethdorf, A. Völker, R. Smith, H. Mach, and M. Hecker.** 1994. Analysis of the induction of general stress proteins of *Bacillus subtilis*. Microbiology **140:**741–752.
52. **Zhang, J., H. Ichikawa, R. Halberg, L. Kroos, and A. I. Aronson.** 1994 Regulation of the transcription of a cluster of *Bacillus subtilis* spore coat genes. J. Mol. Biol. **240:**405–415.
53. **Zhang, J., P. Fitz-James, and A. I. Aronson.** 1993. Cloning and characterization of a cluster of genes encoding polypeptides present in the insoluble fraction of the spore coat of *Bacillus subtilis*. J. Bacteriol. **175:**3757–3766.
54. **Zheng, L., and R. Losick.** 1990. Cascade regulation of spore coat gene expression in *Bacillus subtilis*. J. Mol. Biol. **212:**645–660.
55. **Zheng, L., R. Halberg, S. Roels, H. Ichikawa, L. Kroos, and R. Losick.** 1992. Sporulation regulatory protein GerE from *Bacillus subtilis* binds to and can activate or repress transcription from promoters for mother-cell-specific genes. J. Mol. Biol. **226:**1037–1050.
56. **Zheng, L., W. P. Donovan, P. Fitz-James, and R. Losick.** 1988. Gene encoding a morphogenetic protein required in the assembly of the outer coat of the *Bacillus subtilis* endospore. Genes Dev. **2:**1047–1054.