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TRANSCRIPTIONAL REGULATION OF GENES ENCODING SPORE COAT PROTEINS BY MOTHER-CELL SPECIFIC SIGMA K RNA POLYMERASE DURING BACILLUS SUBTILIS SPORULATION

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TRANSCRIPTIONAL REGULATION OF GENES ENCODING SPORE COAT PROTEINS BY MOTHER-CELL SPECIFIC σ^K RNA POLYMERASE DURING BACILLUS SUBTILIS SPORULATION

By

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ABSTRACT

TRANSCRIPTIONAL REGULATION OF GENES ENCODING SPORE COAT PROTEINS BY MOTHER-CELL SPECIFIC σ^K RNA POLYMERASE DURING BACILLUS SUBTILIS SPORULATION

By

Hiroshi Travis Ichikawa

Sporulation of the Gram-positive bacterium *Bacillus subtilis* is a well established model system to study temporal and spatial gene regulation. Upon starvation, *B. subtilis* initiates sporulation. Asymmetrical cell division into the larger mother cell and the smaller forespore is the first easily observed morphological change. Each compartment expresses different sets of sporulation specific genes from the identical genome. As sporulation proceeds, the forespore is engulfed by the mother cell, forming a free protoplast inside the mother cell. Maturation of the forespore involves deposition of spore coat proteins, which are encoded in *cot* genes. Later, the mature spore is released by lysis of the mother cell. These interesting morphological changes are a consequence of cascade activation of RNA polymerase σ subunits, which allows each cell type to sequentially express specific genes for sporulation. In the mother cell, in addition to σ^E and σ^K, the sequential appearance of two DNA-binding proteins, SpoIIID and GerE, regulates gene expression.

The research presented in this dissertation is focused on understanding the regulatory role of GerE and SpoIIID in transcription by σ^K-containing RNA polymerase during late stages of sporulation in the mother cell. GerE was demonstrated to be a sequence-specific DNA-binding protein by DNase I footprinting, and mapping of these sites revealed a consensus GerE binding sequence. Based on *in vitro* transcription

experiments, GerE directly activates transcription of several *cot* genes by σ^K RNA polymerase, and this activation, in part, involves interaction with the C-terminal domain of the α subunit of RNA polymerase.

In addition to its positive effects, GerE also initiates a negative feedback loop that inhibits transcription of *sigK,* the gene that encodes σ^K. This was demonstrated by comparing expression of the *sigK-lacZ* fusion in wild-type cells and in a *gerE* mutant, and by comparing the SigK protein level of these strains by Western blot analysis. GerE was found to bind within the *sigK* promoter region near the transcriptional start site and repress transcription by σ^K RNA polymerase.

The combined action of GerE and SpoIIID regulates expression of some *cot* genes. Analysis of *lacZ* fusions and mRNA levels showed that *cotB* is expressed slightly earlier than *cotX*, whereas *cotC* expression lags behind that of *cotX*. This pattern of expression can be explained by different levels of GerE activation and/or SpoIIID repression of the three *cot* genes, as observed in *in vitro* transcription experiments. The results support a model in which a decreasing level of SpoIIID and an increasing level of GerE during sporulation set the timing and the level of expression of these *cot* genes, which may be important for proper assembly of the spore coat.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER V

APPENDIX A

LIST OF ABBREVIATIONS

ADP	adenosine-5'-diphosphate
AMV	avian myeloblastosis virus
ATP	adenosine-5'-triphosphate
A_{660}	absorbency at 660 nm
bp	base pair
BRL	Bethesda Research Laboratories
BSA	bovine serum albumin
C-terminus	carboxy terminus
CTP	cytosine-5'-triphosphate
Da	dalton
dATP	deoxyriboadenosine-5'-triphosphate
dCTP	deoxyribocytosine-5'-triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
αNTD	N-terminal domain of α-subunit
dNTP	deoxyribonuceotide
DPA	dipicolinic acid
DSM	Difco sporulation medium
DTT	dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	(Ethylenedinitrilo)tetraacetic acid
GDP	guanosine-5'-diphosphate

GFP	green flourescent protein
GTP	guanosine-5'-triphosphate
IPTG	isopropyl β-D thiogalactopyranoside
kb	kilobases
kDa	kilodalton
LB	Luria-Bertani
mRNA	messenger ribonucleic acid
O.D.	optical density
ONPG	*o*-nitrophenol-β-D-galactoside
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecylsulfate
SM	Sterlini-Mandelstam
T_x	x hours after the onset of sporulation
tRNA	transfer ribonucleic acid
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
UTP	uracil-5'-triphosphate
vol.	volume
v/v	volume per volume
w/v	weight per volume

INTRODUCTION

Upon starvation, *B. subtilis* initiates sporulation. Asymmetrical cell division into a larger mother cell and a smaller forespore is the first easily observed morphological change. Each compartment expresses different sets of sporulation-specific genes. As sporulation proceeds, the forespore is engulfed within the mother cell, forming a free protoplast. Maturation of the forespore involves deposition of spore coat proteins, which are encoded in *cot* genes that are expressed in the mother cell. Later, the mature spore is released by mother cell lysis. These interesting morphological changes are a consequence of cascade activation of RNA polymerase σ subunits, which allows each cell type to sequentially express specific genes for sporulation. In the mother cell, in addition to σ^E and σ^K, the sequential appearance of two DNA-binding proteins, SpoIIID and GerE, regulates gene expression.

The experiments in Chapter II demonstrate that GerE is a sequence-specific DNA-binding protein that directly activates transcription of *cotB* and *cotC* and inhibits transcription of *sigK* (encoding σ^K) and *cotA,* by σ^K-containing RNA polymerase. This work was a collaboration between L. Zhang, S. Roels and R. Losick at Harvard University and R. Halberg, L. Kroos, and myself at Michigan State University. I was responsible for constructing a plasmid containing the *cotC* promoter region and performing DNase I footprinting experiments to map a GerE binding site that is close to the *cotC* transcriptional start site. This work was published in the *Journal of Molecular Biology.*

Experiments in Chapter III demonstrate that GerE binds to and activates transcription from the promoters of the *cotVWXYZ* cluster. We also established the GerE

binding site consensus sequence from the GerE binding sites that were mapped. This work was a collaboration between J. Zhang and A. Aronson at Purdue University and R. Halberg, L. Kroos, and myself at Michigan State University. I was responsible for preparing GerE from an *E. coli* strain, performing DNase I footprinting of GerE on the *cotVWX*, *cotX*, and *cotYZ* promoter regions, and measuring GerE activation of transcription from the *cotVWX*, *cotX* and *cotYZ* promoters using *in vitro* transcription assays. This work was published in the *Journal of Molecular Biology.*

The effects of transcription inhibition by GerE are described in Chapter IV. These experiments provide evidence that the level of SigK products is negatively regulated by GerE . A feedback loop is initiated when *gerE* is transcribed by σ^K-containing RNA polymerase because GerE represses *sigK* transcription. The experiments in Chapter IV also demonstrate that GerE binding to a site centered at -25.5 relative to the *cotD* transcriptional start site is involved in repression of *cotD* transcription. This work was a collaboration with R. Halberg, a former graduate student in the Kroos lab, who contributed mapping of one of the two GerE binding sites in the *cotD* promoter region. This work was published in the *Journal of Biological Chemistry.*

The timing and level of gene expression of individual genes during sporulation is tightly controlled by transcription factors. GerE binds to two sites in each of several *cot* gene promoters. The importance of GerE binding to the upstream site in the *cotB*, *cotC*, and *cotX* promoters was examined by fusing a *lacZ* gene to promoters with or without the upstream GerE binding site. Also in Chapter V, I demonstrate that the pattern of expression from the *cotB*, *cotC*, and *cotX* promoters appears to be regulated by the combined action of SpoIIID and GerE. The order of appearance of transcription factors in the mother cell is SpoIIID first, followed by σ^K and finally GerE. *In vitro* transcription

experiments showed that, of the three genes, *cotB* is most sensitive to activation by GerE, and *cotC* is most sensitive to repression by SpoIIID. This may explain why, as the level of GerE rises, *cotB* is expressed slightly earlier than *cotX* and *cotC*, and as the level of SpoIIID falls, *cotC* is the last of the three genes to be fully expressed. This work was published in the *Journal of Biological Chemistry*.

In vitro transcription experiments in Appendix A were performed with heterologous RNA polymerase consisting of *B. subtilis* σ^K and *E. coli* core RNA polymerase with or without the C-terminal domain of the α subunit (*E. coli* core subunits were provided by A. Ishihama of the National Institute of Genetics in Japan). The levels of *cotB*, *cotC,* and *cotX* transcription in the presence of GerE were lower when heterologous RNA polymerase was missing the C-terminal domain of the α subunit, indicating that GerE transcriptional activation of these genes may involve interaction with the C-terminal domain of α. This dependency on the C-terminal domain of α was not detected when *sigK* transcription was tested in the presence of SpoIIID, suggesting that SpoIIID transcriptional activation of *sigK* involves a different mechanism.

CHAPTER I

Literature Review

Literature review

Gene regulation is one of the fundamental activities that governs life. Living organisms regulate gene expression spatially and temporally during development and in response to numerous internal and external cues. Therefore, programmed gene regulation is a subject of great interest. Sporulation of *Bacillus subtilis* is an excellent system to study spatial and temporal gene regulation because of its well-established experimental tractability and a large database of genetic information.

Overview of Morphological Changes during Sporulation.

Nutrient depletion, cell density, the Krebs cycle, DNA damage and DNA synthesis generate signals that trigger initiation of sporulation of the Gram-positive soil bacterium *Bacillus subtilis.* The first morphological change of sporulation is characterized by formation of an axial filament in which the two chromosomes from the last round of DNA replication become aligned across the long axis of the cell (Stage I) (Figure 1). Next, an asymmetrical septum divides the cell into a lager mother cell and a smaller forespore (Stage II). Each compartment receives a copy of the genome. The septum migrates toward the forespore pole to engulf and finally pinchs off the forespore as a free protoplast within the mother cell (Stage III). Maturation of the forespore occurs while it resides in the mother cell. Cell-wall like material known as cortex, which is thought to be involved in attaining or maintaining the dehydrated and heat-resistant state of the spore, is synthesized in the space between the two membranes surrounding the forespore (Stage IV). As the final step of spore maturation in the mother cell, spore coat proteins are synthesized and deposited around the surface of the forespore (Stage V). The spore coat consists of a lamellar inner layer and an electron-dense outer layer, providing a

Figure 1. The morphological stages of sporulation. The stages are designated by Roman numerals. The wavy lines are chromosomes. The sporangia are surrounded by cytoplasmic membrane (*thin line*) and a cell wall (*thick line*). The developing spore (stage IV-VI) is encased in a layer of cortex (*light stippling*) and a coat layer (*dark stippling*). The four specific sporulation sigma factors are shown in the cells where they become active.

thick, protective barrier that encases the mature spore. Maturation of the forespore also includes coating the forespore chromosome with small acid-soluble proteins and condensation of the DNA into a doughnut-like structure, conferring resistance to UV radiation (Stage VI). Finally, lysis of the mother cell releases the spore (Stage VII). The sporulation process takes six to ten hours under laboratory condition. This series of complex morphological changes is controlled by sequential activation of five sporulation-specific RNA polymerase σ factors (reviewed in Haldenwang 1995; Stragier and Losick 1996).

Initiation of Sporulation

Environmental and physiological signals culminate in phosphorylation of a key regulatory protein, Spo0A. Spo0A is a member of the response regulator family of two-component regulatory systems. There are at least three protein kinases that transfer phosphate to Spo0F. The phosphate of Spo0F~P is transferred to Spo0A *via* Spo0B. Spo0A~P can be dephosphorylated by Spo0E and Spo0F~P can be dephosphorylated by RapA (The phosphorelay is reviewed in Grossman 1995). Spo0A~P binds to various promoter regions to activate transcription of genes that are important to initiate sporulation. Activation of transcription of the *spoIIE* gene and the *spoIIA* operon leads to forespore-specific gene expression (Bramucci et al. 1995). Activation of the two-cistron operon *spoIIG* leads to mother-cell specific gene expression (Baldus et al. 1994; Bird et al. 1996). There is also one or more unknown gene(s) regulated by Spo0A~P that determines the switch of the position of cell division from medial to polar.

Asymmetric Septum Formation and Chromosome Segregation

During binary fission, daughter chromosomes first segregate and then a symmetric septum forms where FtsZ molecules assemble into a ring structure (Z-ring)

(Levin and Losick 1996). Upon entering sporulation, daughter chromosomes align along the long axis of the cell (forming the axial filament) and Z-rings are formed near both poles of the cell (Levin and Losick 1996). One or more unknown gene(s) under Spo0A control is responsible for Z-ring formation (Levin and Losick 1996). The product of *spo0H*, a sigma subunit (σ^H) controls the next set of genes, whose products make the polar septum. However, no genes in this group have yet been discovered. Several genes (*ftsZ*, *divIB*, *divIC* and *pbpB*) involved in binary fission during growth are also involved in asymmetric septum formation during sporulation (Beall and Lutkenhaus 1989; Levin and Losick 1994; Yanouri et al. 1993).

The polar sporulation septum forms around the axial filament and then one of the chromosomes is translocated across the septum into the forespore by the product of the *spoIIIE* gene, which resembles proteins that mediate conjugational plasmid transfer in *Streptomyces* (Wu et al. 1995). A chromosomal position effect on transcription by the forespore-specific σ^F RNAP in *spoIIIE* mutant cells can be explained by the finding that only 30% of the origin-proximal region of one chromosome is present in the forespore after the septum forms (Wu and Errington 1994). The product of *spo0J* may play a role in anchoring the origin region of the chromosomes to the cell poles (Sharpe and Errington 1996). In disporic mutant sporangia, there are two polar forespore-like compartments into which chromosomes are translocated, leaving an anucleate compartment in the middle (Setlow et al. 1991). During vegetative growth, polar division is prevented by the products of the last two ORFs of the *divIVB* locus that are homologous to *E. coli minC* and *minD* (Barak et al. 1998). In a *minCD* mutant, however, SpoIIIE pumps DNA out of the polar compartment, resulting in production of anucleate minicells (Sharpe and Errington 1995). During sporulation, there is a thin mid-cell septum found in *minD*

mutants (Barak et al. 1998). Therefore, MinCD complex may play an important role in preventing symmetric septum formation during sporulation.

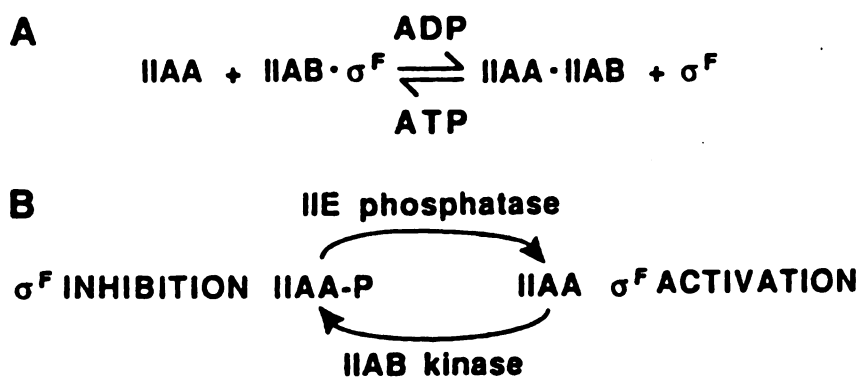
Effects of *spoIIE* on sporulation septum formation have been reported. Mutations in the extreme C-terminal portion of SpoIIE make the sporulation septum develop an aberrantly thicker layer of peptidoglycan and the time of septum formation is delayed (Feucht et al. 1996). In a *minD* mutant, the thin septum is co-localized with SpoIIE-GFP by fluorescent microscopy (Barak et al. 1998). In wild-type cells, SpoIIE is required for the Spo0A-directed formation of polar Z-rings composed of FtsZ (Khvorova et al. 1998). Likewise, FtsZ is required for formation of polar E-rings composed of SpoIIE (Levin et al. 1997). These rings form early in sporulation and one becomes the site of septum formation whereas the other ring disappears (Bouche and Pichoff 1998).

Activation of σ^F

After the asymmetric division, σ^FRNAP is responsible for transcribing genes in the forespore. Several lines of evidence indicate that σ^F is produced prior to septation (Arigoni et al. 1996; Gholamhoseinian and Piggot 1989; Lewis et al. 1994). How its activity is confined to the forespore has been a focus of great interest. The activity of σ^F is key event in σ^F activation (Garsin, Duncan et al. 1998). Once phosphorylated, SpoIIAA cannot bind to SpoIIAB; SpoIIAB remains bound to σ^F and keeps it inactive. Proteins similar to SpoIIAA and SpoIIAB regulate the stress response σ factor, σ^B, in response to cellular ATP levels (Voelker et al. 1995). It is unknown whether the ATP/ADP ratio is different in the forespore and mother cell compartment when σ^F becomes active in the forespore (Magnin et al. 1997).

Serine phosphatase activity of SpoIIE dephosphorylates SpoIIAA~P, resulting in activation of σ^F. SpoIIE has membrane spanning segments in its N-terminal domain and

Figure 2. Activation of σ^F. A. The anti-σ factor SpoIIAB(IIAB) binds to σ^F in the presence of ATP, but ADP favors binding to SpoIIAA (IIAA) and release of σ^F. B. SpoIIAA can be phosphorylated (IIAA-P) by SpoIIAB in the presence of ATP, resulting in σ^F inhibition, as SpoIIAA-P can not bind to SpoIIAB. SpoIIE (IIE) preferentially dephosphorylates SpoIIAA in the forespore, allowing it to bind to SpoIIAB, which permits σ^F activation as shown in (A). Adopted from Kroos *et al.*, 1999.


A
ADP
IIAA + IIAB·σ^F ⇌ IIAA·IIAB + σ^F
ATP
B
IIE phosphatase
σ^F INHIBITION IIAA-P
IIAA σ^F ACTIVATION
IIAB kinase

the C-terminal portion extends into the cytosol (Arigoni et al. 1999). SpoIIE-mediated dephosphorylation is the rate-limiting step in activation of σ^F. Overexpression of SpoIIE triggers premature activation of σ^F (Arigoni et al. 1996; Feucht et al. 1996). Moreover, active σ^F is found in the mother cell compartment of a *spoIIIE* null mutant in which SpoIIE persists in the mother cell longer than usual (Sun et al. 1991). One group has presented evidence that SpoIIE is sequestered to the forespore face of the sporulation septum (Feucht et al. 1996; Wu et al. 1998). This would explain why σ^F becomes active only in the forespore. However, another group has presented evidence that SpoIIE is equally distributed to both sides of the septum (Duncan et al. 1995). These investigators suggest that the smaller size of the forespore leads to a more rapid increase in the concentration of unphosphorylated SpoIIAA, allowing σ^F activation in the foresore.

SpoIIE may require two checkpoints for indirect activation of σ^F by dephosphorylating SpoIIAA~P. The first checkpoint is the formation of Z-rings at the two potential polar division sites. SpoIIE co-localizes with FtsZ, forming E-rings. SpoIIE phosphatase activity requires FtsZ and there is evidence that SpoIIAA~P is already dephosphorylated by SpoIIE at the stage of ring formation since unphosphorylated SpoIIAA accumulates in a *divIC* mutant which undergoes ring formation but not septation (King et al. 1999). However, σ^F remains inactive in the *divC* mutant, suggesting that septum formation serves as a second checkpoint. Based on the effects of modified and mutant forms of SpoIIE, it has been proposed that after SpoIIAA~P is dephosphorylated by SpoIIE, SpoIIAA is retained by SpoIIE (or by another protein in the septum) so that SpoIIAA is unable to react with the SpoIIAB-σ^F complex to activate σ^F (Kinget al. 1999). Release of SpoIIAA is proposed to be dependent on completion of the septum (King et al. 1999).

Activation of σ^E

Soon after the activation of σ^F in the forespore, σ^E becomes active only in the mother cell. Compartmentalization of σ^E activity has been demonstrated directly visualizing β-galactosidase or the green fluorescent protein expressed under the control of a σ^E dependent promoter, using immunoelectron and immunofluorescence microscopy (Driks and Losick 1991; Harry et al. 1995; Webb et al. 1995). Also, disruption of the forespore copy of σ^E controlled sporulation genes has been shown to have no effect on sporulation (Errington 1993). The inactive form of σ^E is called pro-σ^E and is 27 amino acids longer at the N-terminal end than active σ^E. Pro-σ^E is the product of *spoIIGB*, the downstream gene in the in the two-cistron *spoIIG* operon. Spo0A~P activates transcription from the *spoIIG* promoter, stimulating the vegetive RNAP containing σ^A prior to septation. Activation of pro-σ^E occurs only on the mother cell side and involves SpoIIGA, the product of the first gene in the *spoIIG* operon. SpoIIGA is believed to be a protease that processes pro-σ^E to σ^E (Stragier et al. 1988).

Some of the genes required to activate σ^E include *spoIIAA*, *spoIIAB*, *spoIIE*, *ftsZ* and *divIC*, which are also involved in the activation of σ^F. This suggested a requirement for an additional gene that is controlled by σ^F. The gene was found to be *spoIIR* (or *csfX*) (Karow et al. 1995; Londono-Vallejo and Stragier 1995). When spoIIR, spoIIGA and spoIIGB are expressed during exponential growth, efficient pro-σ^E processing is observed (Londono-Vallejo and Stragier 1995). SpoIIR has an apparent signal sequence and is secreted from *B. subtilis* if expressed during the exponential phase of growth (Hofmeister et al. 1995). When produced in the forespore, SpoIIR is thought to cross one septal membrane and act as signal molecule in the space between the two septal membranes (Hofmeister et al. 1995; Karow et al. 1995; Londono-Vallejo and Stragier 1995;

Figure 3. Processing of pro-σ^E in the mother cell depends on a signal from the forespore. The top part depicts a sporulating cell just after polar septation. Pro-σ^E and SpoIIGA (IIGA) made earlier are thought to associate with both septal membranes. The bottom part shows the cell slightly later. σ^F RNAP in the forespore transcribes the gene encoding SpoIIR (IIR). SpoIIR probably crosses the membrane surrounding the forespore and we speculate that it activates SpoIIGA by promoting dimerization, resulting in pro-σ^E processing in the mother cell. Conceivably, SpoIIR might also activate SpoIIGA in the septal membrane adjoining the forespore (not shown in the bottom part), but normally this does not occur or, if it does, another mechanism inhibits σ^E activity in the forespore. Adopted from Kroos *et al.,* 1999.

Londono-Vallejo 1997). There, it activates pro-σ^E processing by SpoIIGA, an integral membrane protein (Ju et al. 1997; Hofmeister 1998). SpoIIGA is believed to be a receptor/protease with a receptor domain that interacts with SpoIIR and a protease domain, that cleaves pro-σ^E in the mother cell (Londono-Vallejo 1997).

How is the mother-cell specific activity of σ^E established? Three patterns of subcellular localization of the *spoIIGB* product have been observed by immunofluorescence microscopy (Hofmeister 1998). Pro-σ^E is found first in association with the cytoplasmic membrane. After asymmetric division, pro-σ^E accumulates at the septum. Finally, the processed active σ^E is found only in the mother cell cytoplasm. Amino acids of the pro-sequence are responsible for membrane association (Hofmeister 1998). Pro-σ^E is in a complex with SpoIIGA or with a protein that depends on SpoIIGA; however, pro-σ^E associates with membranes even in the absence of SpoIIGA (Hofmeister 1998). Interestingly, the product of *gfp* fused to the pro-sequence of *sigE* is found to be localized on the mother-cell side of the septum (Ju and Haldenwang 1999). This localization is pro-sequence dependent as deletion of the first 15 amino acids of pro-σ^E abolishes the specific localization of the GFP fusion protein (Ju and Haldenwang 1999). Localization of pro-σ^E to the mother-cell side of the septum could explain how σ^E activity is confined to the mother cell. The SpoIIIE DNA translocase is somehow involved in confining σ^E activity to the mother cell because mutations in *spoIIIE* allow σ^E activity to also accumulate in the forespore (Pogliano et al. 1997). One hypothesis is that SpoIIIE is necessary for translocation of pro-σ^E to the mother-cell side of the septum, as well as for DNA translocation into the forespore (Hofmeister 1998; Ju and Haldenwang 1999). Alternatively or in addition, SpoIIIE mediated DNA translocation into the forespore may activate a protease that destroys pro-σ^E, SpoIIGA, and any σ^E inadvertently formed in the

forespore (Ju et al. 1998; Ju and Haldenwang 1999).

Phagocytic-like Forespore Engulfment.

Several morphological steps are observed during the process of forespore engulfment (Perez et al. 2000; Sharp and Pogliano 1999). First, peptidoglycan between the septal membranes becomes thinner or is removed. This septal thinning proceeds from the middle toward the edges of the septum. The elimination of this rigid structure leads to the bulging of the forespore compartment into the mother cell, presumably reflecting the higher osmolarity of the forespore cytoplasm. Next, the corners of the septum migrate toward the forespore pole and the membrane fuses there. This process results in a protoplast-like forespore within a double membrane which freely floats in the mother cell. Abundant *de novo* synthesis of membrane components is required for forespore engulfment by the mother cell and this is likely to be under the control of σ^E and σ^F.

The *spoIIB* and *spoVG* genes are directly or indirectly controlled by σ^H (Resnekov et al. 1995). SpoIIB is found in the sporulation septum during septal biogenesis, but is degraded once the septum is complete (Perez et al. 2000). The sporangia of *spoIIB* mutant displays a transient engulfment defect in which the forespore pushes through the septum and bulges into the mother cell, however, the sporangia completes engulfment in slower speed compared to the wild-type sporangia (Perez et al. 2000). The investigators suggest that SpoIIB facilitates the rapid and spatially regulated dissolution of septal peptidoglycan (Perez et al. 2000). SpoIIB, in conjunction with SpoVG, is required for initiation of degradation of the septal peptidoglycan. But the synergistic mechanism of SpoIIB and SpoVG action during engulfment is not clearly understood. Inactivation of *spoVS* compensates for the absence of *spoIIB* and *spoVG*, suggesting an additional level of regulation (Resnekov et al. 1995). A possible role for SpoIIB/SpoVG is to antagonize

the action of SpoVS, which may prevent cell wall degradation (Stragier and Losick 1996). Inactivation of σ^E-controlled genes such as *spoIID*, *spoIIM* and *spoIIP* leads to a blockage in the completion of cell wall dissolution at the periphery of the septum (Frandsen and Stragier 1995; Lopez-Diaz et al. 1986; Smith and Youngman 1993). The biochemistry of these gene products is not well understood, however, structural analysis predicts that these proteins are membrane-associated.

Although the initiation of migration of the mother-cell membrane around the forespore is σ^F dependent, all other genes required for degradation of septum peptidoglycan (other than *spoIIB*/*spoVG*) are controlled by σ^E (Stragier and Losick 1996). One of the genes controlled by σ^F is *spoIIQ* (Londono-Vallejo 1997). Its product is involved in completion of engulfment process. Most of the SpoIIQ protein is located outside of the forespore membrane (Londono-Vallejo 1997). SpoIIQ appears to facilitate the wrapping movement of the mother-cell membrane around the forespore (Londono-Vallejo 1997). It is also possible that SpoIIQ is involved in dissociating the connection between the forespore membrane and the polar cell wall at the initiation of engulfment (Londono-Vallejo 1997).

The final stage of engulfment is to pinch off the forespore as the migrating membrane meets and fuses at the forespore pole. SpoIIIE may be involved in this process based on its localization pattern (Sharp and Pogliano 1999). Moreover, the two functions of SpoIIIE, DNA translocation and completion of engulfment, are separable by mutational analysis (Sharp and Pogliano 1999).

Activation of σ^G

The *spoIIIG* gene encodes σ^G. σ^G controls the expression of a large set of genes in the engulfed forespore, including the *ssp* genes whose products are the so-called small

acid soluble proteins (SASP) (Cabrera-Hernandez and Setlow 2000; Sun et al. 1989). Transcription from the *spoIIIG* promoter is initially σ^F-controlled but σ^G RNAP also recognizes this promoter and thus, autoregulates its own transcription (Partridge and Errington 1993).

There is about an hour delay between activation of σ^F and initiation of *spoIIIG* expression (Partridge and Errington 1993). This time lag is not due to the weakness of the *spoIIIG* promoter. Rather, it depends on σ^E-controlled events in the mother cell (Sun et al. 1991). Although a *spoIIB spoVG* double mutant arrests sporulation immediately after polar septation, there is no effect on σ^F-dependent *spoIIIG* transcription, suggesting that no further morphological development is required for activation of *spoIIIG* transcription (Stragier and Losick 1996).

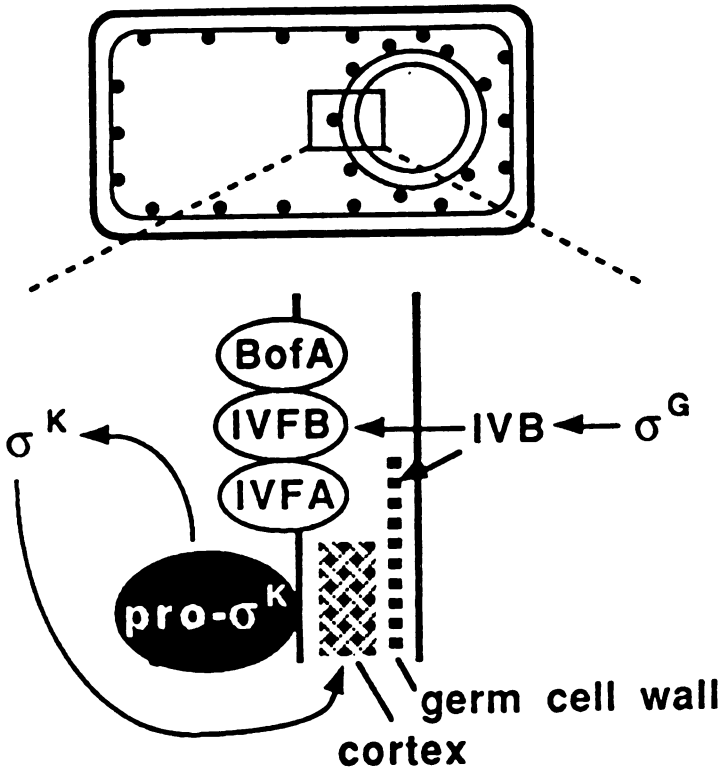
Placing the *spoIIIG* coding sequence under the control of the σ^F-controlled *spoIIQ* promoter uncouples σ^G synthesis from dependency of SpoIIIG transcription on σ^E. In this system, there was no premature σ^G activity (Stragier and Losick 1996). There is growing evidence that σ^G activity is antagonized by SpoIIAB, the same anti-σ factor that inhibits σ^F activity (Kellner et al. 1996). In addition to *spoIIAB* and completion of engulfment, the vegetatively expressed *spoIIIJ* and the eight products of the σ^E-controlled *spoIIIA* operon are required for σ^G post-transcriptional activation (Kellner et al. 1996). Some evidence suggests that SpoIIAB might be preferentially degraded in the forespore in a *spoIIIA*-dependent fashion (Kirchman et al. 1993).

σ^G RNAP transcribes *spoVT*, whose product appears to be a DNA-binding protein. SpoVT regulates transcription of genes under the control of σ^G. SpoVT also negatively regulates *spoIIIG* transcription (Bagyan et al. 1996).

Activation of σ^K.

The last sporulation-specific σ factor to be activated is σ^K, which is first expressed as an inactive precursor called pro-σ^K (Lu et al. 1990). Processing of pro-σ^K is blocked in mutants that fail to activate σ^G (Lu et al. 1990). However, expressing *spoIVB* in the forespore using a σ^F-dependent promoter eliminates the need for σ^G and is sufficient to allow processing of the N-terminal 20 amino acids of pro-σ^K to produce active σ^K (Gomez, Cutting et al. 1995). One of the products of the σ^E-controlled *spoIVF* operon, SpoIVFB, contains amino acid sequences conserved in a newly recognized family of putative metalloproteases, and mutations in these sequences inhibit pro-σ^K processing (Rudner et al. 1999; Kroos et al. 1999). SpoIVFB is believed to be a thermolabile protein as it requires the other product of the *spoIVF* operon, SpoIVFA, to function at 42°C but not at 30°C (Cutting et al. 1990). The requirement for the SpoIVB-dependent forespore signal in processing of pro-σ^K can be bypassed by some mutation in the C-terminal portion of SpoIVFA (Cutting, Oke et al. 1990). Therefore, in addition to its role in stabilizing SpoIVFB, SpoIVFA appears to inhibit SpoIVFB activity until the inhibition is released by the signal from the forespore (Resnekov and Losick 1998). Another inhibitor of SpoIVFB activity is the product of the σ^E-controlled *bofA* gene (Resnekov and Losick 1998). There is evidence that the concentration of SpoIVB is critical for releasing SpoIVFB inhibition since modification of the concentration of SpoIVB in the forespore influenced the timing of pro-σ^K processing (Stragier and Losick 1996). SpoIVFA, SpoIVFB and BofA are thought to be localized to on the membrane surrounding the forespore (Green and Cutting 2000; Kroos et al. 1999). SpoIVB might reside on the outer face of the forespore membrane, anchored by a single membrane-spanning domain. SpoIVB has a putative serine protease catalytic site and may be self-processed to smaller

Figure 4. Processing of pro-σ^K. The upper diagram show a sporangium in which engulfment of the forespore has been completed. Black dots represent pro-σ^K, which associates with the mother cell membrane and the outermost membrane surrounding the forespore. The latter membrane is thought also to contain SpoIVFB-SpoIVFA-BofA complexes, shown only in the enlarged view (lower diagram). σ^G RNAP in the forespore transcribes the gene encoding SpoIVB, a protein thought to cross the innermost membrane surrounding the forespore or, perhaps, to be anchored in this membrane. SpoIVB both signals processing of pro-σ^K to begin and plays a role in formation of the germ cell wall. SpoIVFB appears t be the protease that processes pro-σ^K. σ^K RNAP transcribes a gene(s) necessary for cortex formation and many genes encoding proteins that form the spore coat (not shown). Adopted from Kroos *et al.,* 1999.


BofA
IVFB
IVFA
IVB
σ^G
σ^K
pro-σ^K
germ cell wall
cortex

forms that freely diffuse in the space between the two membranes surrounding the forespore (Oke et al. 1997). It is possible that activation of SpoIVFB (and therefore processing of pro-σ^K) occurs as the mature SpoIVB cleaves the external domains of SpoIVFA or BofA (Stragier and Losick 1996). The coupling between σ^K activation and forespore signaling is very important because premature σ^K activation in the mother cell leads to decreased efficiency of sporulation and release of germination defective spores (Cutting et al. 1990).

Unlike SpoIVFB, a SpoIVFB-GFP fusion protein is able to accumulate in vegetatively growing cells and this fusion protein alone is sufficient to process pro-σ^K to σ^K. Expressing SpoIVFA in this system increases the SpoIVFB-GFP level, presumably by stabilizing the fusion protein. Addition of BofA to this system inhibits pro-σ^K processing and this depends upon SpoIVFA. Recent results with this system suggested that SpoIVFA is the primary inhibitor of SpoIVFB and that BofA stabilizes SpoIVFA (Resnekov 1999).

A gene expressed in the forespore called *bofC* contains a putative signal sequence and is also implicated in the regulation of pro-σ^K in the absence of σ^G, but SpoIVB is still required (Gomez and Cutting 1996). It is thought that a very small amount of SpoIVB possibly produced by σ^F RNAP in *spoIIIG* mutant cells is enough to activate pro-σ^K processing, but only if BofC is absent. Hence, BofC appears to be an inhibitor of spoIVB activity (Gomez and Cutting 1996). BofC appears to play an accessory role, at least under laboratory conditions, because mutation of *bofC* does not affect the timing of σ^K activation or the production of heat-resistant spores in cells with a functional *spoIIIG* gene (Gomez and Cutting 1996).

Hierarchical Regulatory Cascade in the Mother Cell.

Once σ^E is activated, a hierarchical regulatory cascade of gene expression is played out over the course of the next five to six hours in the mother cell. The appearance of four transcription factors in the order σ^E, SpoIIID, σ^K and finally GerE constitutes the cascade (Zheng and Losick 1990). SpoIIID and GerE are mother-cell specific DNA-binding proteins (Halberg and Kroos 1994; Zhang et al. 1997; Zheng et al. 1992; Zhang et al. 1994). The *spoIIID* gene is transcribed by σ^E RNAP (Kunkel et al. 1989; Halberg and Kroos 1994). The product, SpoIIID, recognizes specific DNA sequences in the promoter regions and open reading frames of *bofA*, *spoIVCA*, *sigK*, *cotD* and *spoVD* (Halberg and Kroos 1994). SpoIIID activates *spoIVCA* and *sigK* transcription but represses *bofA* and *spoVD* transcription by σ^E RNAP *in vitro* (Halberg and Kroos 1994). SpoIIID also activates and represses *sigK* and *cotD* transcription, respectively, by σ^K RNAP (Halberg and Kroos 1994).

Production of σ^K involves multiple steps before the forespore-dependent processing of pro-σ^K discussed earlier can occur (Kroos 1991). The *sigK* gene (encoding pro-σ^K) is generated as a consequence of a sporulation specific chromosomal rearrangement (Kunkel et al. 1990). The *sigK* gene is interrupted by an intervening sequence of 48 kb of DNA known as *skin*, which contains the *spoIVCA* gene (Stragier and Losick 1996). The product of *spoIVCA* is a site-specific recombinase that rearranges mother-cell chromosomal DNA to generate the intact *sigK* gene (Sato et al. 1994). Because of the DNA sequence similarity to bacteriophage PBSX, *skin* is thought to be a cryptic prophage (Krogh et al. 1996). Excision of this DNA sequence is mandatory in order to successfully complete sporulation (Kunkel et al. 1990). Transcription of *sigK* is initially controlled by σ^E, but active σ^K also contributes to transcription of its own gene

(setting up a positive feedback loop) (Kroos et al. 1989; Halberg and Kroos 1994).

The mother cell is responsible for the production of spore cortex and coat proteins. A model for coat morphologenesis may help explain the existence of the hierarchical regulatory cascade in the mother cell. The σ^E-controlled *spoIVA* gene product is assembled on the forespore surface and is proposed to recruit other proteins to form a scaffold structure. Another σ^E-controlled gene product, CotE, assembles on the outside surface of the scaffold. Then, other coat proteins expressed under the control of σ^K and in some cases GerE are deposited within the scaffold, forming the inner coat, or are recruited by CotE to form the outer coat (reviewed in Driks 1999).

How does the newly activated σ factor in a cascade replace the previous σ factor from the core RNA polymerase in order to redirect gene transcription? Such a problem arises only if the RNA polymerase core enzyme is in limiting supply. A possible solution is for the new σ factor to have a higher affinity for core RNA polymerase than the previous σ factor. Such competition between σ factors for a limiting amount of core RNAP has been demonstrated between the vegetative σ^A and the minor factor σ^H in growing cells and at the initiation of sporulation (Hicks and Grossman 1996). Some evidence also suggests that σ^E displaces σ^A from core RNAP and that σ^K displaces σ^E as sporulation proceeds (Ju et al. 1999). In addition, σ^K appears to initiate two negative feedback loops that limit further production of σ^E as well as its own synthesis. One feedback loop leads to inhibition of *sigE* transcription by σ^A RNA polymerase (Zhang and Kroos 1997; Zhang et al. 1999). One or more σ^K-controlled gene products are thought to be involved in this loop. The second feedback loop involves the σ^K-dependent GerE protein repressing *sigK* transcription (Zheng et al. 1992). A similar feedback loop is also found in the forespore, as the gene encoding σ^G is negatively regulated by the σ^G-

controlled *spoVT* gene product (Bagyan et al. 1996).

CHAPTER II

Sporulation Regulatory Protein GerE from *Bacillus subtilis* Binds to and Can Activate or Repress Transcription from Promoters for Mother-cell-specific Genes

Sporulation Regulatory Protein GerE from *Bacillus subtilis* Binds to and Can Activate or Repress Transcription from Promoters for Mother-cell-specific Genes

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The mother-cell line of gene expression during sporulation in *Bacillus subtilis* is a hierarchical cascade consisting of at least four temporally controlled gene sets, the first three of which each contain a regulatory gene for the next gene set in the pathway. *gerE*, a member of the penultimate gene set, is a regulatory gene whose product is required for the transcriptional activation of genes (coat protein genes *cotB* and *cotC*) in the last gene set. The *gerE* product also influences the expression of other members of the penultimate gene set (coat protein genes *cotA* and *cotD* appear to be repressed and activated, respectively). We now report that the purified product of *gerE* (GerE) is a DNA-binding protein that adheres to the promoters for *cotB* and *cotC*. We also show that GerE stimulates *cotB* and *cotC* transcription *in vitro* by RNA polymerase containing the mother-cell sigma factor σ^K. These findings support the view that GerE is a positively acting, regulatory protein whose appearance at a late stage of development directly activates the transcription of genes in the last known temporal class of mother-cell-expressed genes. In addition, GerE stimulates *cotD* transcription and inhibits *cotA* transcription *in vitro* by σ^K RNA polymerase, as expected from *in vivo* studies, and, unexpectedly, profoundly inhibits *in vitro* transcription of the gene (*sigK*) that encodes σ^K. The effects of GerE on *cotD* and *sigK* transcription are just the opposite of the effects exerted by the earlier-appearing, mother-cell regulatory protein SpoIIID, suggesting that the ordered appearance of first SpoIIID, then GerE, ensures proper flow of the regulatory cascade controlling gene expression in the mother cell.

Keywords: sporulation; sigma factor; regulatory protein; *Bacillus subtilis*

1. Introduction

Following the formation of a transverse septum at morphological stage II, gene expression during the process of sporulation in *Bacillus subtilis* is regulated differentially between the forespore and mother-cell chambers of the developing sporangium (De Lencastre & Piggot, 1979; Losick & Stragier, 1992). Thus, the transcription of certain genes is restricted to the forespore, whereas the transcription of other genes is limited to the mother cell. Although some heterogeneity in the time of induction of gene expression in the forespore has been reported (Panzer *et al.*, 1989; Sussman & Setlow, 1991), most forespore-expressed genes are switched on co-ordinately and only a single regulatory gene, *spoIIIG*, which encodes the forespore sigma factor σ^G (Karmazyn-Campelli *et al.*, 1989; Sun *et al.*, 1989), is known to be exclusively transcribed in the forespore chamber of the sporangium. Gene expression in the mother cell, in contrast, is relatively complex, involving the expression of at least four co-ordinately controlled gene sets, which are switched on successively during the course of sporulation (Cutting *et al.*, 1989; Kunkel *et al.*, 1988, 1989; Sandman *et al.*, 1988; Zheng & Losick, 1990).

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These gene sets constitute a hierarchical cascade in that the first three gene sets each contain a regulatory gene that governs the expression of the next gene set in the pathway (Zheng & Losick, 1990). *spoIIID*, a member of the earliest regulon, is a regulatory gene whose product (a small, DNA-binding protein; R.H. & L.K, unpublished results) turns on the transcription of the next set of genes (Halberg & Kroos, 1992; Kroos *et al.*, 1989; Kunkel *et al.*, 1989; Stevens & Errington, 1990). One of the genes in the *spoIIID*-dependent class of coordinately controlled genes is *sigK* (Kroos *et al.*, 1989; Kunkel *et al.*, 1988, 1989), a composite gene (generated from two truncated genes by a DNA rearrangement in the mother-cell chromosome; Kunkel *et al.*, 1990; Stragier *et al.*, 1989) that encodes the mother-cell sigma factor σ^K (Kroos *et al.*, 1989). The *sigK* gene product (after a regulatory step involving the conversion of its primary product, pro-σ^K, to the mature sigma factor; Cutting *et al.*, 1990; Lu *et al.*, 1990) then turns on the penultimate class of genes. This set of genes includes the spore coat protein genes *cotA* and *cotD* (Sandman *et al.*, 1988; Zheng & Losick, 1990) and the regulatory gene *gerE* (Cutting *et al.*, 1989; Holland *et al.*, 1987), which encodes a product (GerE) that is, in turn, required for the expression of genes in the last known gene set in the mother-cell line of gene expression (Zheng & Losick, 1990). Two members of this *gerE*-dependent gene set are the coat protein genes *cotB* and *cotC* (Donovan *et al.*, 1987).

Here we are concerned with the role of *gerE* in the hierarchical cascade of mother-cell gene expression. *gerE* is inferred to be a transcriptional regulatory gene because: (1) a *gerE* nonsense mutation (*gerE36*; Cutting, 1986) has highly pleiotropic effects on sporulation, causing the production of spores that are lysozyme-sensitive, germination-defective and aberrant in coat ultrastructure and protein composition (Feng & Aronson, 1986; Jenkinson & Lord, 1983; Moir, 1981); (2) *gerE36* partially inhibits expression of *cotD* (Zheng & Losick, 1990) and causes overexpression of *cotA* in rich sporulation medium (Cutting *et al.*, 1989; Sandman *et al.*, 1988); (3) as indicated above, *gerE36* prevents the expression of cot genes *B* and *C* (Zheng & Losick, 1990); and (4) the predicted product of *gerE* (GerE), an 8·5 kDa polypeptide (Cutting & Mandelstam, 1986; Hasnain *et al.*, 1985), contains a region of similarity to the α-helix–β-turn–α-helix motif of many procaryotic transcriptional regulatory proteins (Holland *et al.*, 1987) and exhibits high overall similarity to the COOH-terminal region of certain regulatory members (the BvgA sub-group) of the family of two-component sensor-regulator systems in bacteria (Gross *et al.*, 1989; Kahn & Ditta, 1991), which includes the *B. subtilis* regulatory proteins DegU and ComA (Henner *et al.*, 1988; Kunst *et al.*, 1988; Weinrauch *et al.*, 1989). GerE is also similar to the COOH-terminal region of the *Escherichia coli* regulatory protein MalT (Gross *et al.*, 1989) and to the COOH-terminal region of sigma factors, which is involved in the recognition of the −35 region of bacterial promoters (Kahn & Ditta, 1991).

On the basis of DNase I footprinting experiments with purified GerE, we now report that the *gerE* gene product is a DNA-binding protein that adheres to the regulatory regions for *cotB* and *cotC*. We also show that GerE greatly stimulates *cotB* and *cotC* transcription *in vitro* by σ^K RNA polymerase, a finding in support of the view that the appearance of GerE at a late stage of sporulation directly activates transcription of these genes. Moreover, we show that GerE affects the *in vitro* transcription of several other mother-cell-expressed genes, either positively or negatively, findings that suggest a functional analogy between GerE and the mother-cell regulatory protein SpoIIID (Kroos *et al.*, 1989). However, the effects of GerE on *cotD* and *sigK* transcription *in vitro* are just the opposite of the effects exerted by SpoIIID. Because production of σ^K RNA polymerase appears to cause a decrease in the level of SpoIIID (Halberg & Kroos, 1992) and is required for the transcription of *gerE* (Cutting *et al.*, 1989; and this work), we propose that a declining level of SpoIIID and a rising level of GerE produce a reinforced switch in the pattern of mother-cell gene expression during sporulation.

2. Materials and Methods

(a) *Strains and plasmids*

E. coli K38 (HfrC *trp thi* λ^+) carrying plasmid pGP1-2 (Tabor & Richardson, 1985) was maintained at 30°C in LB medium containing 25 μg of kanamycin/ml.

The source of the *gerE* open reading frame was pSGMU101 (provided by J. Errington of Oxford University; Cutting & Mandelstam, 1986). The *gerE* open reading frame was released as a 0·5 kb† *Eco*RV/*Xba*I fragment and was cloned into the *Sma*I/*Xba*I site of pT713 (Bethesda Research Laboratories) to create pLZ304. The *Eco*RV site is 67 bp upstream from the *gerE* coding sequence, whereas the *Xba*I site is a polylinker site adjacent to a *B. subtilis Mbo*I site located 227 bp downstream from the *gerE* open reading frame.

Plasmids pLZ304 and pT713 were introduced into K38 cells containing pGP1-2 by transformation and selection on LB medium containing 50 μg of ampicillin/ml and 25 μg of kanamycin/ml at the permissive temperature (30°C).

Plasmids pLRK100 and pBK16 containing the *cotD* and *sigK* promoter regions, respectively, served as templates for *in vitro* transcription and have been described previously (Kroos *et al.*, 1989). A 0·6 kb *Eco*RI/*Pvu*II fragment (Fig. 1) containing the *cotB* promoter region (Zheng & Losick, 1990) was subcloned into *Eco*RI/*Sma*I-digested replicative form M13mp18 (Yanisch-Perron *et al.*, 1985) and replicative form DNA of the recombinant phage was used for *in vitro* transcription. Plasmid pBD261 containing the *cotC* promoter region was constructed as follows: the *lacZ-cat*-containing *Bam*HI fragment of pSGMU31 (Errington, 1986) was cloned into the *Bcl*I site of pBD95 (Zheng & Losick, 1990), creating an in-frame fusion of *cotC* to *lacZ* in pBD239, then the

†Abbreviations used: kb, 10^3 bases or base-pairs; bp, base-pairs; nt, nucleotides.

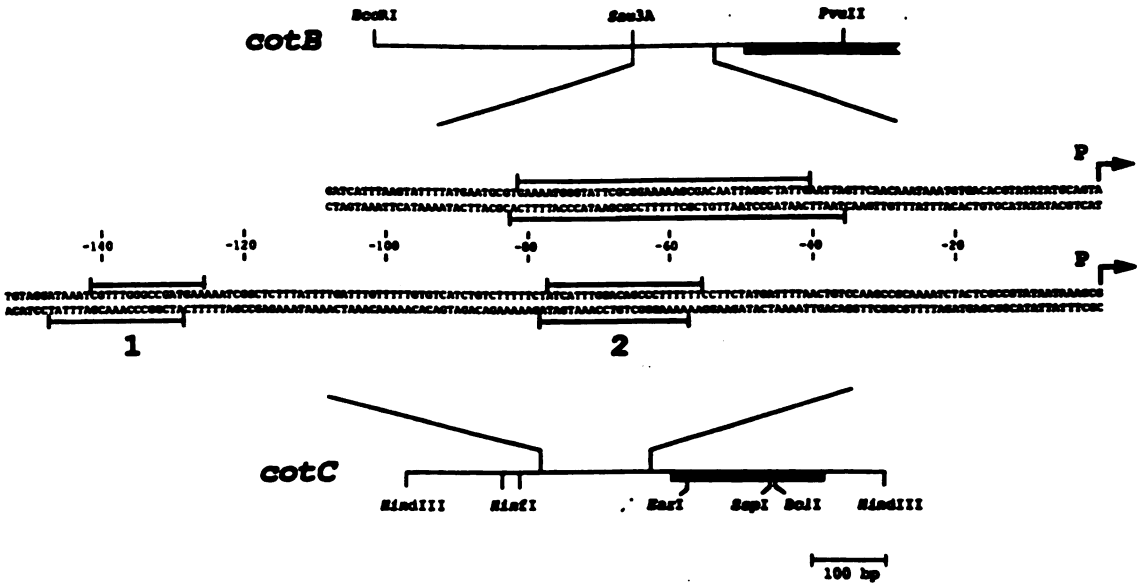

Figure 1. The GerE binding sites in the 5′ regions of *cotB* and *cotC*. The Figure shows restriction maps of DNA in the vicinity of *cotB* and *cotC*, based on a previous report (Donovan *et al.*, 1987) and other unpublished analysis. (Note that the map of *Sau*3A sites is incomplete and only a single site is shown.) The positions of the open reading frames for each gene are shown by the filled bars (the map includes only part of the *cotB* open reading frame). Also shown are the nucleotide sequences of the promoter regions of both genes. The startsites of transcription are indicated by the arrows. Regions of DNA that were protected by GerE from the action of DNase I are indicated above and below each strand. The 2 GerE binding sites in *cotC* are designated 1 and 2 in the Figure.

cotC-lacZ-cat-containing *Bam*HI/*Bgl*II fragment of pBD239 was cloned into the *Bam*HI site of pBR322 (Bolivar *et al.*, 1977) to construct pBD259 in which the *Hin*dIII site upstream from the *cotC* promoter is proximal to the *Hin*dIII site of the vector, and finally pBD259 was digested with *Hin*dIII and recircularized to construct pBD261 in which the small *Hin*dIII fragment was deleted. pHI1 was constructed by subcloning a 0·5 kb *Hin*dIII/*Xba*I fragment from pBD261 (extending from the *Hin*dIII site upstream from *cotC* to an *Xba*I site in the polylinker downstream from the former *Bcl*I site of *cotC* (Fig. 1) into *Hin*dIII/*Xba*I-digested pUC19 (Yanisch-Perron *et al.*, 1985). Restriction fragments from pHI1 were purified after electrophoresis on an agarose gel and used as templates for *in vitro* transcription of *cotC*. The *gerE* promoter-containing plasmid, pSC146, was constructed by S. Cutting as follows: the 266 bp *Alu*I fragment from pSGMU101 (encompassing the *gerE* promoter region; Cutting & Mandelstam, 1986) was subcloned into the *Sma*I site in the polylinker of pSGMU31 (Errington, 1986), then excised as a *Kpn*I/*Bam*HI fragment and inserted into *Kpn*I/*Bam*HI-digested pUC19 (Yanisch-Perron *et al.*, 1985). Three *cotA* promoter-containing plasmids were constructed by K. Sandman as follows: (1) pKS22 was constructed by ligating *Hin*dIII linkers to a 0·8 kb *Hinc*II/*Ava*I fragment from pKS11 (encompassing the *cotA* promoter region: Sandman *et al.*, 1988), cleaving the linkers with *Hin*dIII, and subcloning the fragment into *Hin*dIII-digested pIBI76 (International Biotechnologies, Inc.); (2) pKS23 was constructed by digesting pKS19 (Sandman *et al.*, 1988) with *Pst*I and ligating to delete *B. subtilis* DNA beyond 55 bp upstream from the *cotA* transcriptional startsite; (3) pKS24 was constructed by digesting pKS19 (Sandman *et al.*, 1988) with *Eco*RV and ligating to delete *B. subtilis* DNA beyond 115 bp upstream from the *cotA* transcriptional startsite.

(b) *Production of GerE in* E. coli

Cultures of K38 cells containing pGP1-2 (bearing the phage T7 RNA polymerase gene) alone, pGP1-2 and pT713, or pGP1-2 and pLZ304 were grown at 30°C to an A_{600} of 0·3. Cells were induced by a temperature shift to 42°C for 20 min. Rifampicin was added to a final concentration of 200 μg/ml, and the cells were incubated at 42°C for 10 min and then at 30°C for 30 min. Cells were collected by centrifugation and dissolved in sample buffer (Laemmli, 1970). The sample was denatured at 90°C for 2 min and fractionated by electrophoresis through an SDS/polyacrylamide gel containing 15% acrylamide.

For the preparation of GerE, protein from induced K38 cells containing pGP1-2 and pLZ304 was subjected to electrophoresis and the putative GerE band was cut from the gel. GerE was then eluted from the gel slice and renatured as described (Hager & Burgess, 1980). For the preparation of control protein, protein from induced K38 cells containing pGP1-2 and pT713 was subjected to electrophoresis. A slice from the position corresponding to that of GerE was cut from the gel. Protein was then eluted and renatured from the gel slice as described for GerE.

(c) *Preparation of DNA probes labeled at only one end*

For the preparation of radioactive *cotC* probes, a 358 bp *Hin*fI/*Bcl*I fragment (Fig. 1) whose *Hin*fI terminus had been rendered flush by use of the Klenow fragment of DNA polymerase I was cloned into *Hinc*II/*Bam*HI-digested pUC18 (Yanisch-Perron *et al.*,

1985). This created plasmid pLZ1275 in which the *Hin*dIII site of the vector was upstream from the *cotC* promoter. Plasmid pLZ1275 was linearized with *Hin*dIII and then treated with alkaline phosphatase. Next, the *cotC*-containing fragment was released from the pUC18 vector by digestion with *Eco*RI, which cuts at the end of the polylinker next to the *Bam*HI site. A probe labeled at the *Hin*dIII site in the non-transcribed strand was prepared using phage T4 polynucleotide kinase and [γ-^{32}P]ATP. To prepare a probe labeled at the *Hin*dIII site in the transcribed strand, *cotC* was released as a *Hin*dIII/*Sma*I fragment (*Sma*I also cuts in the polylinker next to *Bam*HI site) and was labeled by end-filling the *Hin*dIII terminus using the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP. Additional *cotC* probes were prepared by digesting pHI1 (see above) with *Ear*I, which cleaves in the 7th codon of *cotC* (Donovan *et al.*, 1987), labeling in the non-transcribed strand using the fill-in reaction of the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP or labeling in the transcribed strand by treatment with alkaline phosphatase followed by phage T4 polynucleotide kinase and [γ-^{32}P]ATP, then digesting with *Eco*RV and *Eco*RI, and purifying the 239 bp *Eco*RV/*Ear*I fragment encompassing the *cotC* promoter region after electrophoresis on a non-denaturing, polyacrylamide gel containing 8% acrylamide (Maniatis *et al.*, 1982).

For preparation of radioactive *cotB* probes, we took advantage of a plasmid pUC18 derivative called pBD136 (constructed by W. Donovan, unpublished results), which contains a 0·8 kb *Sau*3AI/*Sau*3AI fragment that includes the promoter and the NH_2-terminal coding region of the *cotB* open reading frame cloned into the *Bam*HI site in an orientation such that the polylinker *Eco*RI site was proximal to and upstream from the promoter. pBD136 was digested with *Eco*RI, treated with alkaline phosphatase, and the *cotB*-containing fragment was released by digestion with *Hin*dIII, which cuts at the opposite end of the polylinker. The non-transcribed strand probe was labeled at the *Eco*RI site by the T4 polynucleotide kinase reaction. To prepare the transcribed strand probe, a *cotB*-containing, *Eco*RI/*Pvu*II fragment was purified (see Fig. 1) and was then labeled by end-filling using the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP.

(d) *DNase I footprinting*

Two different methods were used to carry out the DNase I footprinting experiments. In method (1), the conditions for the binding of GerE were the same as described by Strauch *et al.* (1989), except that poly(dI-dC) was added to a final concentration of 30 μg/ml. DNA fragments labeled at one end were incubated in separate experiments without protein, with control protein, or with different amounts of GerE protein in 20 μl reaction mixtures for 15 min. Then 1 μl of a 0·01 mg/ml DNase I solution (BRL) was added to each reaction for 1 min. The digests were stopped by adding 5 μl of stop solution (0·1 M-EDTA and 0·5% SDS) and chilled on ice. The DNA in each reaction was precipitated with 1 ml of ethanol and with 0·2 μg of poly(dI-dC) as carrier. The precipitates were dissolved in formamide loading buffer (Maniatis *et al.*, 1982) and denatured at 90°C for 90 s. The samples were then subjected to electrophoresis in an 8 M-urea-containing polyacrylamide gel.

In method (2) the conditions for the binding of GerE were the same as in the *in vitro* transcription experiments (see below), except that poly(dI-dC) was added to a final concentration of 1·2 μg/ml. DNA fragments labeled at one end were incubated at 37°C in separate experiments without protein, with control protein, or with different amounts of GerE in 42 μl reaction mixtures for 10 min. Then 3 μl of 0·0004 mg DNase I/ml (Boehringer-Mannheim) solution (prepared by diluting a stock solution (Davis *et al.*, 1980) with buffer (20 mM-Tris·HCl, pH 8·0, 20 mM-$MgCl_2$, 20 mM-$CaCl_2$) was added to each reaction. After 1 min, the digests were stopped by adding 50 μl of buffer (100 mM-Tris·HCl, pH 8·0, 50 mM-EDTA, 200 μg yeast tRNA/ml) and incubating for 2 min at 65°C. The DNA in each reaction was precipitated with 250 μl ethanol. The precipitates were dissolved in formamide loading buffer (Maniatis *et al.*, 1982) and denatured by boiling. The samples were then subjected to electrophoresis in an 8 M-urea/polyacrylamide gel containing 6% acrylamide.

(e) *DNA sequencing*

End-labeled DNA probes were subjected to the chemical cleavage reactions of Maxam & Gilbert (1980) with a kit from New England Nuclear or as described previously (Maniatis *et al.*, 1982).

(f) In vitro *transcription*

σ^K RNA polymerase was partially purified from *B. subtilis* strain SC104 (*trpC2 gerE36* SPβ::*cotA-lacZ*) as described (Kroos *et al.*, 1989). This enzyme was comparable in protein composition and in *cotD*- and *sigK*-transcribing activities to fraction 24 shown in Fig. 2 of Kroos *et al.* (1989). σ^K RNA polymerase was reconstituted from gel-purified, renatured σ^K and *B. subtilis* core RNA polymerase as described previously (Kroos *et al.*, 1989). Transcription reactions were performed as described previously (Kroos *et al.*, 1989) except that heparin (6 μg) was added 2 min after the addition of nucleotides to prevent reinitiation, and after the reactions were stopped 10 μl of the reaction mixture was subjected to electrophoresis. [α-^{32}P]CTP was the labeled nucleotide unless indicated otherwise. After gel electrophoresis, transcripts were detected by autoradiography and the signals were quantitated using a Visage 110 Image Analyzer (BioImage).

(g) *Primer extension analysis*

RNA was prepared from sporulating cells as described by Cutting *et al.* (1991*a*). *In vitro* synthesized *cotC* transcripts were generated as described above (but without radiolabeled nucleotide) and then precipitated with ethanol and suspended in 25 μl of diethylpyrocarbonate-treated water. In preparation for primer extension analysis, a sample of the *in vitro* synthesized RNA (4 μl) was treated with 5 units of DNase I (Pharmacia) in buffer (20 mM-Tris·HCl, pH 7·6, 10 mM-$MgCl_2$) in a total reaction volume of 5 μl. The reaction was incubated at 37°C for 10 min and then at 90°C for 10 min.

Primer extension was carried out by use of the *cotC*-specific oligonucleotides Pr1 and Pr2 (Zheng & Losick, 1990). The oligonucleotides were 5′-end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (BRL) as described by Sambrook *et al.* (1989). For analysis of *in vivo* synthesized RNA, from 2 to 6 pmol of 5′-end labeled oligonucleotide was incubated with 5 μg of total RNA, and reactions were carried out as described by Roels *et al.* (1992). For analysis of *in vitro*-generated *cotC* transcripts, 12 pmol of 5′-end-labeled Pr2 oligonucleotide was incubated with 4 μl *in vitro* synthesized RNA (from above) in 10 μl of annealing buffer (50 mM-Tris·HCl, pH 7·6, 100 mM-KCl) at 90°C for 2 min and then at 47°C for

30 min: 5 μl of the primer:RNA hybrid solution was then incubated with 5 units of phage M-MuLV reverse transcriptase (Pharmacia) at 47°C for 45 min in a final volume of 10 μl of reverse transcriptase buffer (50 mM-Tris·HCl, pH 7·6, 60 mM-KCl, 10 mM-$MgCl_2$, 1 mM-dithiothreitol (DTT), 2·5 mM of each dNTP, 1 unit placental RNase inhibitor/μl (Pharmacia)) after which 7 μl of 95% formamide loading dye was added.

The 5′-end-labeled oligonucleotides were also used to generate sequence ladders by the dideoxy chain termination method of Sanger *et al.* (1977). The products of primer extension were subjected to electrophoresis in 6% polyacrylamide slab gels containing 8 M-urea.

3. Results

(a) *Purification of GerE*

GerE was purified by engineering *E. coli* cells to express a cloned copy of the *gerE* gene using the T7 RNA polymerase/T7 promoter system of Tabor & Richardson (1985). A DNA fragment containing the *gerE* open reading frame was placed under the control of a phage T7 promoter by inserting into the expression vector pT713 a *gerE*-containing segment of *B. subtilis* DNA that extended 67 bp upstream and 227 bp downstream from the *gerE* open reading frame to create plasmid pLZ304 (see Materials and Methods). Cells containing pLZ304, the vector pT713, or neither plasmid, were grown at 30°C and were then shifted to 42°C to induce transcription from the phage T7 promoter. Total cellular proteins from induced and uninduced cells were displayed by electrophoresis through an SDS/polyacrylamide gel (Fig. 2). In addition to normal cellular proteins, induced cells of the pLZ304-containing strain (lane F) produced a protein of 6 to 8 kDa, which was absent in induced cells that lacked the *gerE*-bearing plasmid (lane D) or that contained the plasmid vector, pT713 (lane E). This protein was also absent in cells that were not heat-induced (lanes A to C). The size of the protein was consistent with that (8·5 kDa) deduced from the nucleotide sequence of the *gerE* open reading frame (Cutting & Mandelstam, 1986). Because there was no other open reading frame in the *gerE*-bearing *B. subtilis* insert in pLZ304 that could encode a protein of this size, we presume that the 6 to 8 kDa protein was GerE.

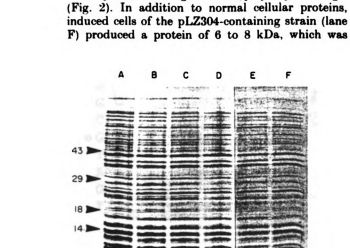

Figure 2. Production of GerE in *E. coli*. Total cellular proteins were extracted from cells grown at 30°C (lanes A to C) or from cells that had been shifted to 42°C (lanes D to F) and were then resolved by electrophoresis in a SDS/12% polyacrylamide gel. The cells were derivatives of *E. coli* strain K38 containing pGP1-2 alone (lane A and D), pGP1-2 and pT713 (lane B and E), or pGP1-2 and pLZ304 (lane C and F). (pGP1-2 contains the phage T7 RNA polymerase gene.) The positions of the molecular mass markers (in kDa) are shown on the left.

To purify GerE, the 6 to 8 kDa protein band was excised from an SDS/polyacrylamide gel displaying proteins from pLZ304-containing bacteria. Protein was eluted from the gel slice and renatured as described by Hager & Burgess (1980). As a control, a gel slice corresponding in position to GerE was cut from an SDS/polyacrylamide gel displaying total cellular proteins from induced cells of pT713-containing bacteria. Protein was eluted from the gel slice and renatured and is referred to as "control protein".

(b) *Mapping the 5′ terminus of* cotC *mRNA*

To study the interaction of GerE with *cotB* and *cotC*, it was first necessary to know the precise sites from which transcripts from these genes originate. Previously reported primer extension experiments established the location of the 5′ terminus of *cotB* mRNA (see Fig. 1) but only provided a tentative assignment for the 5′ terminus of *cotC* mRNA (Zheng & Losick, 1990); an extension product of 127 nt that would correspond to an apparent 5′ terminus located 120 bp upstream from the *cotC* initiation codon was obtained with an oligonucleotide primer called Pr1 but no extension products were observed with two other oligonucleotides called Pr2 and Pr3 (see Fig. 1 of Zheng & Losick (1990) for a description of the primers). Alerted from the results of *in vitro* transcription experiments (below) that the true-startsite of transcription was actually very close to the beginning of the *cotC* open reading frame (and hence that relatively short extension products were to be expected), we repeated the primer extension analysis and found that Pr1 and Pr2 generated extension products of 33 and 68 nt, respectively, that corresponded (as judged by electrophoresis alongside Pr1 and Pr2-generated nucleotide sequencing ladders; Fig. 3(a) and 3(b)) with a 5′ terminus that preceded the initiation codon by only 26 bp (Fig. 1). These extension products were specific to *gerE*$^+$ cells at a late stage of sporulation in that Pr1 and Pr2 generated little or no extension products with RNA from wild-type cells at an early stage of development or with RNA from *gerE* mutant cells at a late stage of development (Fig. 3(d)). Consistent with our earlier results (Zheng & Losick, 1990), Pr1 generated the previously observed 127 nt extension product in addition to the 33 nt product (Fig. 3(a)), but direct nucleotide sequencing of this extension product by the use of dideoxynucleotides in the primer exten-

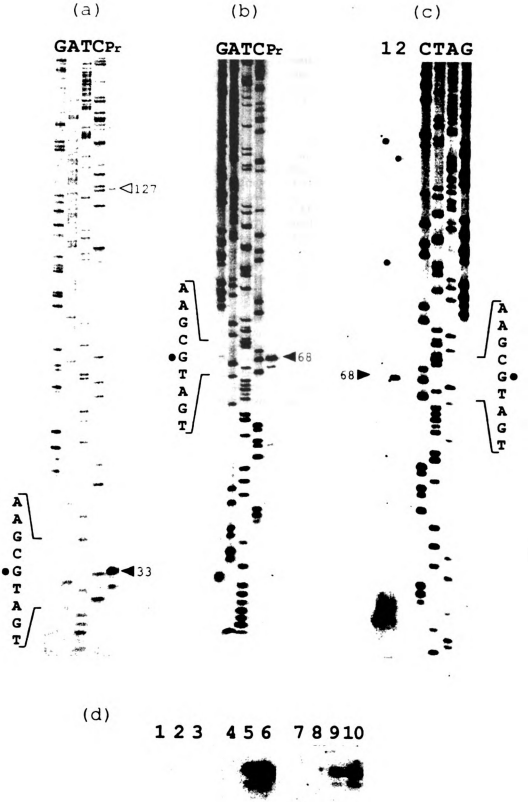
(a)
GATCPr
127
AAGCGTAGT
33
(b)
GATCPr
AAGCGTAGT
68
(c)
12 CTAG
68
AAGCGTAGT
(d)
1 2 3 4 5 6 7 8 9 10

Fig. 3.

sion reaction established that the 127 nt product was not copied from *cotC* mRNA but rather from the transcript of another, similarly regulated gene (data not shown). Finally, the previous failure to observe an extension product with Pr3 is now explained by the fact that this primer corresponds to a sequence (see Fig. 1 of Zheng & Losick, 1990) that is located upstream from the 5′ terminus of *cotC* mRNA.

(c) *GerE binds to specific sequences*

Preliminary gel retardation experiments indicated that GerE binds to a *Hin*dIII/*Ssp*I fragment of 500 bp containing the 5′ region of *cotC* and to an *Eco*RI/*Pvu*II fragment of 635 bp containing the 5′ region of *cotB* (Fig. 1; and Zheng, 1990). To localize the binding of GerE to *cotB* and *cotC* more precisely, DNase I protection experiments were carried out with radioactive DNA probes separately end-labeled on one or the other DNA strand. The radioactively labeled DNAs were incubated with GerE and then mildly treated with DNase I to generate a spectrum of fragments. After the enzyme digestion step, the DNA fragments were fractionated by gel electrophoresis. Figure 4(a) displays the pattern of fragments generated by enzyme treatment of GerE-bound *cotC* DNA that had been labeled on the non-transcribed strand (the upper strand in Fig. 1) at a *Hin*dIII site located upstream from the promoter. The Figure shows that GerE caused protection from the action of DNase I along an approximately 16 bp stretch of DNA extending from position −126 to position −141 relative to the 5′ terminus of *cotC* RNA. No protection was observed with control protein (lane 1). Likewise, Figure 4(b) shows that GerE protected a 19 bp stretch of DNA extending from position −129 to position −147 on the transcribed strand (the lower strand in Fig. 1) of *cotC* and that no protection was observed with control protein.

A second GerE binding site was mapped in the *cotC* promoter region using DNA probes labeled at the *Ear*I site located downstream from the promoter. Figures 4(c) and (d) show that GerE protected an approximately 22 bp stretch of DNA extending from position −56 to position −77 on the non-transcribed strand (the upper strand in Fig. 1) and an approximately 21 bp stretch of DNA extending from position −58 to position −78 on the transcribed strand (the lower strand in Fig. 1), respectively. In both cases, no protection was observed with control protein. The regions upstream from *cotC* that were protected from DNase I digestion by GerE binding are indicated in Figure 1.

Analogous experiments showed that GerE protected a wide region of *cotB* from DNase I digestion. Figure 4(e) shows that the GerE-protected region on the non-transcribed strand (the upper strand in Fig. 1) was 40 to 50 bp in length, with the strongest protection occurring between position −41 and position −81. Figure 4(f) shows that a similar region was protected on the transcribed strand, the protected region being 47 bp in length and extending from position −36 to position −82. The region upstream from *cotB* that was protected from DNase I digestion by the binding of GerE is indicated in Figure 1.

(d) *GerE stimulates* cotB *and* cotC *transcription* in vitro

To test for effects of GerE on transcription of *cotB* and *cotC* *in vitro*, linearized DNA templates were transcribed in the presence of GerE or control protein with σ^K RNA polymerase partially purified from a *gerE* mutant (see Materials and Methods). σ^K RNA polymerase produced run-off transcripts of the expected sizes from *cotB* in the presence of GerE (Fig. 5(a), lanes 3 and 4). The signal was sevenfold weaker in the presence of control protein (Fig. 5(a), lane 2) or with no addition (Fig. 5(a), lane 1). σ^K RNA polymerase reconstituted from gel-purified σ^K and purified *B. subtilis* core RNA polymerase was also stimulated by GerE to transcribe *cotB* (data not shown).

Partially purified σ^K RNA polymerase failed to transcribe a linearized plasmid (pBD261) bearing the *cotC* promoter, even in the presence of GerE, apparently because sequences located in the vector portion of the plasmid compete with the sequences located upstream from *cotC* for binding to GerE (data not shown). However, when a 0·5 kb restric-

Figure 3. Mapping the 5′ terminus of *cotC* mRNA. (a) and (b) Results of high-resolution mapping of the 5′ terminus of *cotC* mRNA using the oligonucleotide primers Pr1 (a) and Pr2 (b) to prime cDNA synthesis from total RNA from PY79 (*spo*$^+$) cells harvested 10 h after the onset of sporulation (t_{10}). (c) Results of high resolution mapping of the 5′ terminus of *cotC* mRNA using the oligonucleotide primer Pr2 to prime cDNA synthesis from RNA transcribed *in vitro* with σ^K-polymerase in the absence (lane 1) or presence (lane 2) of GerE protein. The products of primer extension were subjected to electrophoresis alongside nucleotide sequencing ladders generated with either the Pr1 or Pr2 primer. The arrows indicate the position and size of the principal extension product(s) obtained with each oligonucleotide primer. The open arrow in (a) indicates the position of an artifact band, seen with Pr1 but not Pr2, that is not the result of extension of *cotC* mRNA (see the text). The sequences shown in the vicinity of the 5′ terminus correspond to the non-transcribed DNA strand and the filled circles indicate the nucleotide corresponding to the 5′ terminus. (d) The time course and genetic dependence of the appearence of *cotC* mRNA. The primers Pr1 (lanes 1 to 6) and Pr2 (lanes 7 to 10) were used to generate extension products from total RNA from sporulating PY79 (*spo*$^+$) cells harvested at t_5 (lanes 4 and 8), t_8 (lanes 5 and 9), or t_{10} (lanes 6 and 10) or from KS450 (*gerE36*) cells harvested at t_5 (lane 1), t_8 (lane 2), or t_{10} (lanes 3 and 7).

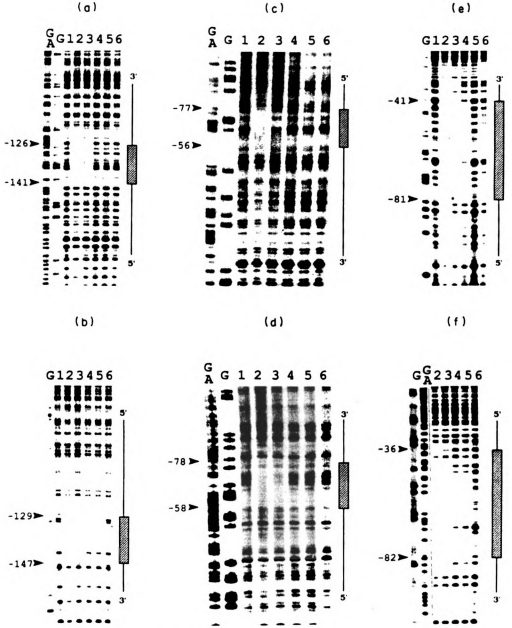

Figure 4. GerE footprints in *cotB* and *cotC* DNAs. Radioactive DNA fragments separately end-labeled on the transcribed or non-transcribed strand were incubated in separate reactions without protein (lane 6), with control protein (lane 1), or with 1 μg (lane 2), 0·5 μg (lane 3), 0·1 μg (lane 4), and 0·05 μg (lane 5) of GerE. After treatment with DNase I, the partially digested DNAs were separated by electrophoresis through an 8 M-urea/polyacrylamide gel alongside a sequencing ladder generated by chemical cleavage of the respective end-labeled DNAs. (a) and (b) Footprints of the non-transcribed (upper strand in Fig. 1) and the transcribed (lower) strands of *cotC*, respectively, using probes labeled at the *Hin*dIII site located upstream from the promoter. (c) and (d) Footprints of the non-transcribed (upper) and transcribed (lower) strands of *cotC*, respectively, using probes labeled at the *Ear*I site located downstream from the promoter. (e) and (f) Footprints of the non-transcribed (upper) and transcribed (lower) strands of *cotB*, respectively, using probes labeled at the *Eco*RI site located upstream from the promoter. The experiments of (a), (b), (e) and (f) were carried out using method (1) in Materials and Methods, and the experiments of (c) and (d) were carried out using method (2), although in other experiments (data not shown) similar footprinting results to those seen in (a) and (b) were obtained using method (2).

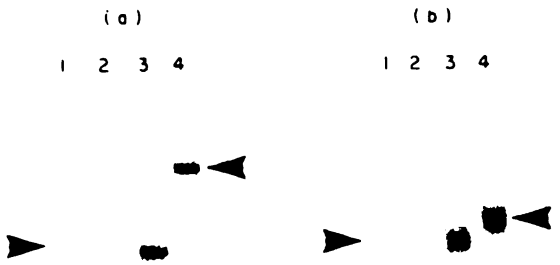

Figure 5. GerE stimulates *cotB* and *cotC* transcription *in vitro*. Template DNA (0·4 pmol) was transcribed with partially purified σ^K RNA polymerase (0·2 μg) alone, or with control protein or GerE (0.4 μg) added immediately after the addition of RNA polymerase. Run-off transcripts were electrophoresed in 5% polyacrylamide gels containing 8 M-urea and were detected by autoradiography. Arrowheads denote the positions of run-off transcripts of the expected sizes in each panel, as judged from the migration of end-labeled DNA fragments of *Msp*I-digested pBR322. (a) *cotB* transcription from replicative form DNA of a recombinant phage containing the *cotB* promoter region. The phage DNA was linearized with *Bam*HI (lanes 1 to 3, 177-base transcript) or *Hin*dIII (lane 4, 207-base transcript) and transcribed with σ^K RNA polymerase alone (lane 1), or with control protein (lane 2) or GerE (lanes 3 and 4) added. (b) *cotC* transcription from restriction fragments isolated from pHI1. The *Hin*dIII/*Xba*I fragment (lanes 1 to 3, 174-base transcript) or the *Hin*dIII/*Sal*I fragment (lane 4, 180-base transcript) were transcribed with σ^K RNA polymerase alone (lane 1), or with control protein (lane 2) or GerE (lanes 3 and 4) added.

tion fragment was used as the template for *in vitro* transcription, σ^K RNA polymerase produced run-off transcripts of the expected sizes from *cotC* in the presence of GerE (Fig. 5(b), lanes 3 and 4). A very weak signal was observed in the presence of control protein (lane 2) or with no addition (lane 1) in a longer autoradiographic exposure than that shown in Figure 5(b). Primer extension analysis of the GerE-stimulated *cotC* transcript produced by σ^K RNA polymerase *in vitro* demonstrated that it had the same 5′ terminus as *cotC* mRNA produced *in vivo* (Fig. 3(c)).

(e) *Effects of GerE on* in vitro *transcription of other mother-cell-expressed genes*

σ^K RNA polymerase partially purified from a *gerE* mutant has been shown to transcribe from the *cotD* and *sigK* (previously called *spoIVCB*) promoters *in vitro* (Kroos *et al.*, 1989). GerE stimulated *cotD* transcription two- to threefold (Fig. 6(a)) and completely inhibited *sigK* transcription (Fig. 6(b)) by partially purified σ^K RNA polymerase. Although the effect of GerE on *cotD* transcription was modest, a two- to threefold stimulation was consistently observed in four independent experiments (data not shown). The level of stimulation was not further enhanced by the use of twice as much GerE as that employed in the experiment of Figure 6(a) (data not shown).

The partially purified σ^K RNA polymerase produced run-off transcripts of the expected sizes from *gerE* (Fig. 6(c), lanes 1 and 2) and from *cotA* (Fig. 6(d), lanes 1 and 2). These transcripts were also produced by σ^K RNA polymerase reconstituted

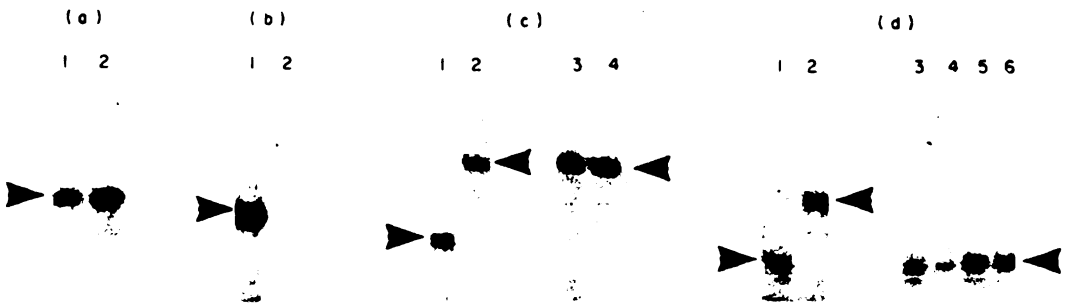

Figure 6. Effects of GerE on *cotD*, *sigK*, *gerE* and *cotA* transcription *in vitro*. Linearized plasmid DNA (2 μg) was transcribed with partially purified σ^K RNA polymerase (0·2 μg) alone, or with control protein or GerE (0·4 μg) added immediately after the addition of RNA polymerase. Run-off transcripts were electrophoresed in 5% polyacrylamide gels containing 8 M-urea and were detected by autoradiography. Arrowheads denote the positions of run-off transcripts of the expected sizes in each panel, as judged from the migration of end-labeled DNA fragments of *Msp*I-digested pBR322. (a) *cotD* transcription from *Hin*dIII-digested pLRK100 (225-base transcript) with σ^K RNA polymerase and control protein (lane 1) or GerE (lane 2). (b) *sigK* transcription from *Xba*I-digested pBK16 (170-base transcript) with control protein (lane 1) or GerE (lane 2). (c) *gerE* transcription from pSC146 digested with *Bam*HI (lane 1, 174-base transcript) or *Hin*dIII (lanes 2 to 4, 204-base transcript) with σ^K RNA polymerase alone (lanes 1 and 2), or with control protein (lane 3) or GerE (lane 4) added. (d) *cotA* transcription from pKS23 digested with *Nco*I (lane 1, 131-base transcript) or *Eco*RI (lane 2, 149-base transcript), from *Nco*I-digested pKS22 (lanes 3 and 4, 131-base transcript), and from *Nco*I-digested pKS24 (lanes 5 and 6, 131-base transcript) with σ^K RNA polymerase alone (lanes 1 and 2), or with control protein (lanes 3 and 5) or GerE (lanes 4 and 6) added. [α-^{32}P]UTP was the labeled nucleotide in the experiments shown in (c) and (d).

from gel-purified σ^K and *B. subtilis* core RNA polymerase (data not shown). GerE protein had no effect on *gerE* transcription *in vitro* by partially purified σ^K RNA polymerase (Fig. 6(c), lane 4). The effect of GerE on *cotA* transcription *in vitro* varied depending on the particular DNA template used (Fig. 6(d)). GerE had little effect on *cotA* transcription from a template containing 115 bp of DNA upstream from the *cotA* transcriptional startsite (lane 6); however, GerE inhibited *cotA* transcription approximately twofold (in 2 independent experiments) from a template containing approximately 430 bp of upstream DNA (lane 4).

Discussion

(a) *Transcriptional activation by GerE*

We have identified binding sites for GerE at the 5′ ends of *cotB* and *cotC*, coat protein genes whose transcription depends on the appearance of GerE during sporulation. We have also shown that GerE stimulates *cotB* and *cotC* transcription *in vitro* by σ^K RNA polymerase and that the *gerE* gene itself can be transcribed *in vitro* by σ^K RNA polymerase. These results support the view (Zheng & Losick, 1990) that in the mother cell, σ^K RNA polymerase first directs the transcription of *gerE*, then acts in conjunction with the product of *gerE* to direct the transcription of *cotB*, *cotC* and, perhaps, other late-activated sporulation genes.

Interestingly, the region of GerE-conferred protection from DNase I action in *cotB* (41 to 47 bp) was approximately twice the length of the two separate protected regions in *cotC* (16 to 19 bp for binding site 1 and 21 to 22 bp for binding site 2). Our interpretation of this observation is that *cotB* contains tandem GerE binding sites and that *cotC* contains two separate GerE binding sites. Inspection of the sequences in the protected regions reveals three similar 5 bp sequences, two (TGGGT and TAGGC) in *cotB* and one (TGGGC, found in binding site 1) in *cotC*. If these are recognition sequences for GerE, then a possible consensus sequence for the GerE binding site is TPuGGPy. The closest match to this consensus in *cotC* binding site 2 is the sequence TGGAC. Nevertheless, binding site 2 appears to be sufficient to mediate GerE-stimulated transcription of *cotC*, since a DNA template with less than half of binding site 1 (produced by cleavage with *Hae*III at position −133) retains the ability to be transcribed *in vitro* by σ^K RNA polymerase in the presence of GerE (R.H. & L.K., unpublished results).

The downstream boundaries of the GerE binding sites in *cotB* (position −36) and in *cotC* (position −56 for binding site 2) are immediately adjacent to or near regions of DNA that are expected to interact with RNA polymerase (that is, the promoters). By analogy with the positioning of the binding sites for several well-characterized, positively acting regulatory proteins, the DNA-bound GerE can be considered to be appropriately positioned to stimulate RNA polymerase to transcribe from the *cotB* and *cotC* promoters. For example, the binding region (the tandem operator sites O_{R2} and O_{R1}) for the phage λ repressor is located between 34 and 74 bp upstream from the transcriptional startsite for the promoter (P_{RM}) from which the repressor stimulates transcription of its own structural gene (*c*I) by *E. coli* RNA polymerase (Johnson *et al.*, 1979; Meyer & Ptashne, 1980). As another example, the catabolite gene activator protein (CAP) binds to sequences (typically protecting about 25 bp) centered from about 41 to 107 bp upstream from the transcriptional startsite of genes whose transcription it stimulates (de Crombrugghe *et al.*, 1984).

An added significance of our demonstration *in vitro* that GerE can bind to and stimulate transcription from the regulatory regions of genes under its control is that GerE is the prototypical representative of the putative regulatory domain of a large and diverse group of procaryotic transcriptional activator proteins. Thus, GerE exhibits high overall similarity to the COOH-terminal regions of certain regulator members (the BvgA sub-group, which includes DegU, ComA and FixJ) of the family of two-component sensor and response regulator systems in bacteria (Gross *et al.*, 1989; Kahn & Ditta, 1991). The NH_2-terminal region of the response regulator proteins contains a site for phosphorylation by the sensor component of the two-component system, and its phosphorylation state influences the activity of the COOH-terminal domain with respect to transcriptional activation (Kofoid & Parkinson, 1988; Nixon *et al.*, 1986). Strikingly, GerE, which lacks the NH_2-terminal domain, is highly similar along its entire 72 amino acid length to the COOH-terminal domain of the BvgA sub-group of response regulator proteins. No member of the BvgA sub-group has (to our knowledge) been shown to bind to DNA or activate transcription *in vitro*, but our results reinforce the view that the GerE-like, COOH-terminal domain of these proteins is directly responsible for the transcriptional activation of genes under the control of this sub-group of response regulator proteins. Likewise, the similarity of GerE to the COOH terminus of *E. coli* MalT, a large regulatory protein whose NH_2-terminal region is dissimilar to the phosphorylation domain of the two-component regulator proteins (Gross *et al.*, 1989), once again suggests that the GerE-like region of MalT could be responsible for DNA-binding and transcriptional activation by this regulatory protein (Richet *et al.*, 1991; Vidal-Ingigliardi *et al.*, 1991). Finally, the similarity of GerE to the COOH-terminal domain (region 4) of sigma factors (Kahn & Ditta, 1991) reinforces the view that this domain mediates the recognition of the −35 region of promoters (Gardella *et al.*, 1989; Siegele *et al.*, 1989).

(b) *Consensus sequence for promoters recognized by σ^K RNA polymerase*

σ^K RNA polymerase has been shown to transcribe from the *cotD* and *sigK* (previously called *spoIVCB*)

```
                    -35                 -10
Consensus           AC      - 17 bp -   CATA---TA

sigK      cggtacagacACagacagcctcccggtcaCATAcatTtacatatagge
cotA      atttttgtaACcatcacgtccttattgtCATtaacTAtagtaccaat
cotD      ttgcatcagaACatgtacccctttattttCATAactTAgtattgtaat
gerE      tgtaaacgtcACctcctgcgcccttcttaCATAtgaTAtctcgactat

cotB      ttgaattagttCaacaaataaatgtgacaCgTAtatatgcagtatgt
cotC      aactgtccaagCcgcaaaatc tactcgcCgTAtaaTAaagcgtagta
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Figure 7. Alignment of promoters transcribed by σ^K RNA polymerase. The nucleotide sequences of the *sigK*, *cotA*, *cotD* and *gerE* promoter regions (see the text for references) are aligned with respect to conserved nucleotides (capital letters) in the −10 and −35 regions relative to the transcriptional startsites (underlined). Shown above are the consensus −10 and −35 sequences, separated by 17 bp, and shown below are the *cotB* and *cotC* promoter regions with matches to the consensus indicated by capital letters (a 1 bp gap was introduced into the *cotC* sequence between the −10 and −35 regions).

promoters *in vitro* (Kroos *et al.*, 1989). Efficient transcription of *sigK* by σ^K RNA polymerase also required a small, DNA-binding protein that is the product of the *spoIIID* gene (Kroos *et al.*, 1989; Kunkel *et al.*, 1989). Using σ^K RNA polymerase reconstituted from gel-purified σ^K and *B. subtilis* core RNA polymerase, we find that *gerE* and *cotA*, like *cotD*, are transcribed efficiently by σ^K RNA polymerase in the absence of SpoIIID, findings that confirm and extend the results of studies on the regulation of these genes *in vivo* (Cutting *et al.*, 1989; Sandman *et al.*, 1988). As shown in Figure 7 and as noted previously (Foulger & Errington, 1991; Zheng & Losick, 1990), the promoter regions of *cotD* (Zheng & Losick, 1990), *gerE* (Cutting *et al.*, 1989), *cotA* (Sandman *et al.*, 1988) and *sigK* (Kunkel *et al.*, 1988) each contain sequences similar to CATA---TA at about position −10 relative to their transcriptional startsites. Figure 7 also shows that the *cotB* and *cotC* promoters, which were transcribed weakly by σ^K RNA polymerase in the absence of GerE, display some similarity to the CATA---TA sequence. Interestingly, the putative −10 consensus sequence for σ^K-recognized promoters is similar to the sequence CATACA-T, which is conserved in the −10 region of promoters transcribed by RNA polymerase containing the related sporulation sigma factor, σ^E (see Roels *et al.* (1992) and Foulger & Errington (1991) for recent compilations of promoters recognized by σ^E RNA polymerase). Unlike promoters recognized by σ^E RNA polymerase, however, promoters recognized by σ^K RNA polymerase that have been characterized to date display only a limited region of similarity to each other in their −35 regions. The sequence AC is, however, found 17 bp upstream from the −10 region in the four promoters transcribed by σ^K RNA polymerase in the absence of GerE, but only the C is found at the corresponding position in *cotB* and *cotC*. We note that three other promoters that are inferred to be transcribed by σ^K RNA polymerase in the absence of GerE, namely, *spoVJ*P2 (Foulger & Errington, 1991), *cotE*P2 (Zheng & Losick, 1990), and the promoter for the newly discovered coat protein gene *cotF* (Cutting *et al.*, 1991*b*), contain −35 and −10 sequences that strongly conform to the sequences AC and CATA---TA, respectively, at a spacing of 16 to 17 bp. Mutational analyses will be needed to determine whether the AC and CATA---TA sequences are important for promoter recognition by σ^K RNA polymerase.

(c) *Effect of GerE on the transcription of* cotD *and* sigK

GerE stimulated *cotD* transcription *in vitro* by σ^K RNA polymerase two- to threefold (Fig. 6(a)). This result is in qualitative agreement with the finding that *cotD-lacZ* expression is reduced about sevenfold in *gerE* mutant cells (Zheng & Losick, 1990). Inspection of the *cotD* promoter region (Zheng & Losick, 1990) reveals a 12 bp sequence (AAAA-TAGGTCTT) at positions −43 to −54 with ten matches to a sequence (positions −69 to −80) protected by GerE in the *cotB* promoter region (Fig. 1). Within the 12 bp sequence in the *cotD* promoter region is a 5 bp sequence (TAGGT) that conforms to the putative consensus binding sequence for GerE described above. It will be interesting to determine whether GerE binds to this sequence, since it would appear to position GerE appropriately to stimulate RNA polymerase, as discussed above.

GerE completely inhibited *sigK* transcription by partially purified σ^K RNA polymerase (Fig. 6(b)). The partially purified σ^K RNA polymerase used in this experiment contained a small amount of SpoIIID, thus permitting adequate transcription of *sigK*. Inhibition of *sigK* transcription by GerE was unexpected, since expression of a *sigK-lacZ* fusion was about normal in *gerE* mutant cells (Kunkel *et al.*, 1988). Inspection of the *sigK* promoter region (Kunkel *et al.*, 1988) reveals a 15 bp sequence (ACATATAGGCTTTTG) at positions −4 to +11 with 12 matches to a sequence (positions −41 to −55) protected by GerE in the *cotB* promoter region (Fig. 1). Within the 15 bp sequence in the *sigK* promoter region is a 5 bp sequence (TAGGC) that conforms to the putative consensus GerE binding sequence. In addition, this 5 bp sequence is repeated in inverted orientation at positions +11 to +15. Thus, there may be two GerE binding sequences near the start-site of *sigK* transcription and GerE bound at these sites may prevent σ^K RNA polymerase from transcribing *sigK*. If these sites do mediate repression of *sigK* transcription by GerE, it could explain why a *sigK-lacZ* fusion was expressed equally in wild-type or *gerE* mutant cells, since the fusion was created by insertion of a transposon (Tn*917lac*) 4 bp downstream from the *sigK* tran-

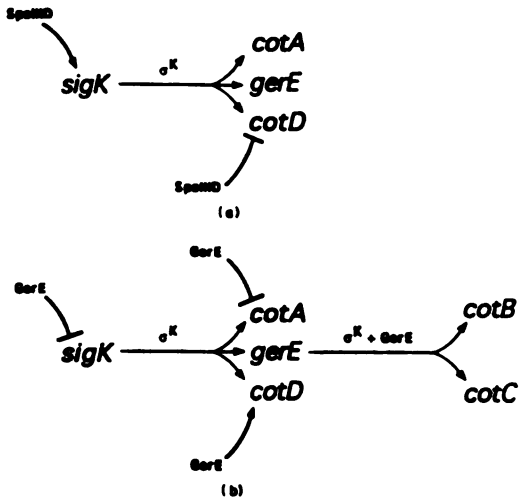

Figure 8. Regulatory effects of SpoIIID and GerE during stages IV and V of sporulation. The effects of SpoIIID and GerE on transcription by σ^K RNA polymerase *in vitro* are illustrated. As noted in the text, studies *in vivo* also support some of the regulatory effects depicted. (a) During stage IV of sporulation, SpoIIID stimulates transcription of *sigK* and inhibits transcription of *cotD*. The *gerE* gene is transcribed by σ^K RNA polymerase. (b) During stage V of sporulation, GerE inhibits transcription of *sigK* and *cotA*, and stimulates transcription of *cotD*, *cotB* and *cotC*.

scriptional startsite (Kunkel *et al.*, 1988), and this would have presumably disrupted the putative GerE binding site.

(d) *Opposite effects of GerE and SpoIIID help to drive the mother-cell regulatory cascade*

GerE and SpoIIID exert opposite effects on σ^K-directed transcription of both *cotD* and *sigK* (Fig. 8). It has been shown that SpoIIID stimulates *sigK* transcription and inhibits *cotD* transcription *in vitro* (Kroos *et al.*, 1989; and Fig. 8(a)). These properties of SpoIIID led to the suggestion that inactivation or sequestering of SpoIIID during sporulation causes a switch from transcription of *sigK* (and perhaps other stage IV sporulation genes) to transcription of *cotD* (and perhaps other stage V genes) (Kroos *et al.*, 1989). Recently, it has been shown that the level of SpoIIID decreases at the appropriate time during sporulation to cause such a switch (Halberg & Kroos, 1992). Furthermore, the decrease in the level of SpoIIID correlates with the appearance of σ^K, suggesting that the appearance of σ^K initiates the switch. We have shown here that σ^K RNA polymerase transcribes *gerE* (Fig. 6(c)). Thus, the appearance of σ^K RNA polymerase beginning at about hour 4 of sporulation (Cutting *et al.*, 1989; Lu *et al.*, 1990) would result not only in a declining level of SpoIIID, but also in a rising level of GerE. We have also shown here that GerE inhibits *sigK* transcription (Fig. 6(b)) and stimulates *cotD* transcription (Fig. 6(a)) by σ^K RNA polymerase *in vitro*

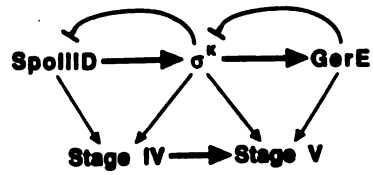

Figure 9. Regulatory interactions controlling the levels of SpoIIID, σ^K and GerE govern the stage IV to V transition in the mother cell. SpoIIID stimulates *sigK* transcription, leading to σ^K production (Kroos *et al.*, 1989). σ^K RNA polymerase transcribes *gerE*, leading to GerE production. The appearance of σ^K causes a decrease in the level of SpoIIID (Halberg & Kroos, 1992). GerE inhibits transcription of *sigK*, down-regulating σ^K production. Thus, σ^K RNA polymerase functions during both stage IV and stage V, but a declining level of SpoIIID and a rising level of GerE switch the pattern of σ^K-directed gene expression from the stage IV pattern to the stage V pattern.

(Fig. 8(b)). Because GerE exerts the opposite effects of SpoIIID on σ^K-directed transcription of *sigK* and *cotD*, the appearance of GerE would reinforce the switch in the pattern of mother-cell gene expression previously postulated to be brought about by inactivation or sequestering of SpoIIID. The reason for the apparent redundancy in the switch is unclear. Perhaps SpoIIID prevents premature expression of *cotD* (and perhaps other stage V genes) during the stage (IV) of spore cortex formation so that the σ^K produced initially directs expression of *sigK* (autogenous regulation), *gerE*, and genes involved in cortex formation (Halberg & Kroos, 1992). Accumulation of GerE would terminate this period by diverting σ^K RNA polymerase away from transcription of *sigK* (and perhaps other stage IV genes) and would initiate the stage (V) of spore coat formation by directing σ^K RNA polymerase to transcribe genes encoding spore coat proteins. According to this model, the regulatory interactions illustrated in Figure 9 co-ordinate the levels of SpoIIID, σ^K and GerE so as to produce a molecular switch governing the transition from the stage IV pattern of mother-cell gene expression to the stage V pattern.

The effects of GerE on transcription of *gerE* and *cotA* by σ^K RNA polymerase *in vitro* are consistent with the effects of a *gerE* mutation on expression of these genes *in vivo*. GerE had no effect on transcription of the *gerE* gene *in vitro* (Fig. 6(c)) and expression of a *gerE-lacZ* fusion is normal in a *gerE* mutant (Cutting *et al.*, 1989). The effect of GerE on *cotA* transcription *in vitro* varied from little effect with a template containing 115 bp of DNA upstream from the transcriptional startsite to a modest (but reproducible), twofold inhibition with a template containing approximately 430 bp of upstream DNA (Fig. 6(d)). If GerE inhibits *cotA* transcription by binding to DNA, this result suggests that it must do so by binding to a site(s) more than 115 bp upstream from the startsite of transcription. In a previous study (Cutting *et al.*,

1989), a *cotA-lacZ* fusion containing as little as 300 bp of DNA upstream from the *cotA* transcriptional startsite was found to be expressed about threefold higher in a *gerE* mutant relative to wild-type cells.

In summary, we have presented biochemical evidence that GerE is a regulatory protein capable of either stimulating or inhibiting transcription of particular genes in the mother-cell line of gene expression. In this respect, GerE appears to be analogous to SpoIIID; however, the ordered appearance of first SpoIIID, then GerE, and the opposite effects of these two proteins on the transcription of genes like *sigK* and *cotD* presumably ensures proper flow of the regulatory cascade (Zheng & Losick, 1990) controlling mother-cell gene expression. In the cases of *cotB* and *cotC*, GerE binds to specific sequences immediately adjacent to the promoter and stimulates transcription by σ^K RNA polymerase. This finding supports the previous proposal (Zheng & Losick, 1990) that GerE directly activates the expression of genes in the terminal temporal class of mother-cell expressed genes.

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CHAPTER III

Regulation of the Transcription of a Cluster of *Bacillus subtilis* Spore Coat Genes

Regulation of the Transcription of a Cluster of *Bacillus subtilis* Spore Coat Genes

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The pattern of transcription has been examined for a cluster of genes encoding polypeptides some or all of which are assembled into a cross-linked component of the *Bacillus subtilis* spore coat. Three promoters, designated P_{VWX}, P_X and P_{YZ}, were indicated by reverse transcriptase mapping. On the basis of Northern hybridization, it appeared that the *cotV*, *W* and *X* genes were transcribed as a polycistronic mRNA from P_{VWX} as well as a monocistronic *cotX* mRNA from P_X. The *cotY* and *cotZ* genes are cotranscribed from the P_{YZ} promoter with a smaller *cotY* mRNA resulting from premature termination or RNA processing. All four transcripts were synthesized late during sporulation and were not produced in mutants lacking sigma K, which directs RNA polymerase to transcribe genes in the mother-cell compartment of sporulating cells. The DNA-binding protein GerE, which affects transcription of many genes in the mother cell during the late stages of sporulation, was also shown to be involved. There was essentially no *cotX* mRNA in a *gerE* mutant and the amounts of *cotVWX*, *cotYZ* and *cotY* mRNAs were somewhat reduced. *In vitro* run-off transcription studies with σ^K RNA polymerase and GerE confirmed the presence of the three promoters, and directly showed that GerE was necessary for transcription from P_X as well as enhanced transcription from the P_{VWX} and P_{YZ} promoters. The DNase I footprints of GerE for all three promoters were immediately upstream of the −35 regions. These GerE binding sites were compared to those in other GerE-responsive promoters and a larger consensus sequence for GerE binding was recognized. This complex transcriptional pattern of the *cotVWXYZ* cluster is probably necessary to ensure that an optimal amount of each protein is made for the assembly of the spore coat.

Keywords: spore coat protein gene cluster and operons; spore promoters; *in vitro* transcription; DNA-binding protein; footprinting

1. Introduction

During sporulation of *Bacillus subtilis*, a number of small polypeptides are synthesized within the mother-cell chamber and deposited on the developing forespore to form a thick, multilayered coat which has a protective function (Aronson & Fitz-James, 1976; Pandey & Aronson, 1979; Goldman & Tipper, 1978; Jenkinson, 1981) and may also have an indirect role in germination (Moir, 1981; Bourne *et al.*, 1991; Zhang *et al.*, 1993). There are at least fifteen soluble spore coat polypeptides and about 30% of the spore coat protein is resistant to a variety of solubilization treatments. This "insoluble fraction" probably consists of cross-linked polypeptides (Pandey & Aronson, 1979; Zhang *et al.*, 1993).

Seven genes encoding soluble coat proteins, *cotA* through *F* and *cotT*, have been cloned and analyzed (Donovan *et al.*, 1987; Zheng *et al.*, 1988; Aronson *et al.*, 1989; Cutting *et al.*, 1991). The transcription units for these genes are monocistronic and scattered widely on the *B. subtilis* chromosome. The expression of these genes is restricted to the mother cell chamber and transcription involves both mother cell-specific σ^E and σ^K RNA polymerases (Sandman *et al.*, 1988; Kroos *et al.*, 1989; Zheng & Losick, 1990; Zheng *et al.*, 1992). In addition, two sporulation-specific DNA-binding proteins, SpoIIID and GerE, function as regulators of *cot* gene expression by acting as transcriptional activators or repressors (Kroos *et al.*, 1989; Kunkel *et al.*, 1989; Zheng *et al.*, 1992; Halberg & Kroos, 1992).

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The spore coat insoluble fraction accounts for a large proportion of the total coat protein. These cross-linked proteins could be responsible for the resilience of the spore coat as well as the hydrophobicity of the spore (Zhang *et al.*, 1993). A cluster of five genes, *cotVWXYZ*, some or all of which encode polypeptides present in the spore coat insoluble fraction of *B. subtilis*, has been cloned and the products characterized (Zhang *et al.*, 1993). The *cotX* gene encodes a glutamine-, lysine- and cysteine-rich protein which appears to be extensively cross-linked and is probably a major component of the coat insoluble fraction. Two other proteins, CotY and CotZ, are similar in sequence with 9·3% and 6·7% cysteine, respectively, some or all of which are disulfide cross-linked. The clustering of these spore coat protein genes and the phenotypic effects of null mutants (Zhang *et al.*, 1993) indicated that these genes may be coordinately controlled in order to produce the appropriate amounts of the proteins necessary for the interactions required to form the coat insoluble fraction. In this report, we present results which show that the genes in the *cotVWXYZ* cluster are organized into two operons as well as two overlapping monocistronic transcription units. Expression of the *cotVWXYZ* genes involves the mother-cell-specific sigma factor, σ^K, the DNA-binding protein GerE, and probably an antitermination mechanism.

2. Materials and Methods

(a) *Strains and plasmids*

The wild-type strain, *B. subtilis* JH642 (*trpC2*, *pheA1*), was obtained from Dr J. Hoch (Scripps Research Institute). Strain 1AA-1 (*gerE36*, *leuB*) was described by Feng & Aronson (1986). Strains 55.3 (*spoIIGB55*, *trpC2*; Errington & Mandelstam, 1986) and BK410 (*spoIIIC94*; Kunkel *et al.*, 1989) and plasmids containing the *cotD* (Donovan *et al.*, 1987) and *cotE* (Zheng *et al.*, 1988) genes were obtained from Dr R. Losick (Harvard University).

Plasmid pJZ6 was previously described (Zhang *et al.*, 1993). Plasmid pJZ22 was derived from pJZ6 by removing a 1175 bp *Bgl*II fragment from the 1771 bp *Hin*dIII insert, and was used for *in vitro* transcription from promoter P_x. Plasmid pJZ23 was constructed by digesting pJZ6 with *Bst*XI (9 bp downstream of the *cotX* initiation codon) and *Sph*I (on the pUC18 vector) to remove a 1378 bp fragment, and then religation with T4 ligase after treatment with Klenow fragment in the presence of 50 μM each of the deoxynucleoside triphosphates. This plasmid was used for the experiment of GerE-footprinting on the P_x promoter. A 2 kb *Hin*dIII fragment containing the entire *cotV* coding region and the first 13 codons of the *cotW* gene (Zhang *et al.*, 1993) was cloned into the *Hin*dIII site of a modified pUC18 lacking the *Eco*RI site. The resulting plasmid was digested with *Eco*RI (a unique site 57 bp downstream of the *cotV* initiation codon) and *Pst*I (in the multicloning site) to remove a 410 bp fragment. Blunt-ends were generated by filling in with Klenow fragment and 50 μM each of the deoxynucleoside triphosphates and the construct was then ligated. This plasmid, pJZ30, was used for *in vitro* transcription and for GerE-footprinting on promoter P_{vwx}.

(b) *Total RNA preparation*

Cells of various *B. subtilis* strains were grown in a nutrient sporulation medium (Shaeffer *et al.*, 1963) at 37°C in a New Brunswick rotary shaker at 250 rpm. Growth was monitored by measuring the A_{560} in a Perkin-Elmer Junior Model 35 spectrophotometer. Cells (30 to 50 ml) were harvested at the end of exponential growth and at hourly intervals (designated t_1, t_2 etc.) thereafter by centrifugation at 5000 *g* for 10 min. Total RNA was extracted as described by Wu *et al.* (1989) and further treated with ribonuclease-free DNase I (Boehringer-Mannheim) at 37°C for 1 h. The phenol/chloroform/isoamyl alcohol (25 : 24 : 1) extraction was repeated 3 to 4 times followed by 2 extractions with chloroform/isoamyl alcohol (24 : 1). The RNAs were precipitated with 2 vol ethanol and one tenth vol of 3 M Na acetate (pH 5). The final precipitates were redissolved in diethylpyrocarbonate-treated water (Maniatis *et al.*, 1982). The concentrations were determined ($A_{260\,nm}$) and 40 units of the RNase inhibitor RNasin (Promega) was added prior to storage at −70°C.

(c) *Northern hybridization analysis*

A procedure modified from that of Miller (1987) for RNA agarose-formaldehyde gel electrophoresis was used. Thirty μg of RNA was denatured by heating at 65°C for 5 min in 15 to 20 μl of a solution containing gel running buffer (0·5 M 3-(*N*-morpholino) propanesulfonic acid (Mops), 0·01 M Na_2 EDTA (pH 7), 3·3 M formaldehyde, 50% formamide) and resolved on a 1% agarose gel containing 2·2 M formaldehyde in running buffer. Two μg of an RNA ladder (0·24 to 9·49 kb; BRL) was loaded on the gel and served as size standards. RNAs were blotted onto a 0·45 μm BA-S85 reinforced nitrocellulose membrane (Schleicher & Schuell) using a VacuGene vacuum blotting system (Pharmacia) in 20 × SSC (1 × SSC contains 0·15 M NaCl, 0·015 M Na citrate) for 1 h. DNA fragments prepared as described below were labeled with [α-^{32}P]dCTP by using a Multiprime DNA labeling kit (Amersham). Hybridization was carried out by the method of Mahmoudi & Lin (1989).

(d) *Preparation of Northern hybridization probes*

Phage mpJZ3(7A) contains a deletion of the 1·7 kb *Hin*dIII fragment (see Figure 1) generated by exonuclease III (Zhang *et al.*, 1993). This deleted fragment containing the C terminus of *cotW* (lacking the first 12 codons, but including 12 bp downstream of the *cotW* stop codon; see Figure 1) was excised by *Hin*dIII-*Eco*RI digestion and used as probe A. Probe B was a 1·3 kb fragment containing the sequence encoding residues 9 to 54 of CotX (see Figure 1) and was isolated from pJZ13 (Zhang *et al.*, 1993) by *Eco*RI-*Pst*I digestion. Probe C includes codons 55 to 146 of the *cotY* coding region and was isolated by digestion of phage mpJZ4(6C) (another deletion of the 1·7 kb *Hin*dIII fragment derived as described above) with *Eco*RI-*Hin*cII. Probe D is a 154 bp *Pvu*II-*Hin*dIII fragment containing 52 bp upstream of the start codon plus the first 34 codons of *cotZ* (see Figure 1), and was isolated from plasmid pJZ8 (which contains the 1771 bp *Hin*dIII fragment (see Figure 1) in pBR322).

A 2·0 kb *Hin*dIII fragment containing the *cotE* gene was isolated from plasmid pLZ100 (Zheng *et al.*, 1988). A 1·8 kb *Hin*dIII fragment containing the *cotD* gene was from plasmid pBD156 (Donovan *et al.*, 1987) and a 1·2 kb *Hin*dIII fragment containing the *cotT* gene was from a

pHP13 clone (Aronson *et al.*, 1989). All of these DNA fragments were labeled with [α-^{32}P]dCTP and Klenow fragment employing a Multiprime labeling kit (Amersham).

(e) *Primer extension assay*

The primer extension method used was modified from that of Ferrari *et al.* (1988). Eighteen or nineteen base oligonucleotide primers were synthesized in the Purdue University Laboratory for Macromolecular Structure and labeled at their 5'-termini by incubation for 2 h at 37°C with T4 polynucleotide kinase and [γ-^{32}P]ATP. Reactions were terminated by heating at 65°C for 20 min. About 30 pmol of labeled primer was mixed with 30 μg of total RNA. NaCl was added to a final concentration of 0·4 M followed immediately by 20 units of RNasin. Annealing was carried out by incubation at 37°C for 2 h followed by precipitation with 2 vol ethanol at −20°C for 1 h. After centrifugation, the precipitates were washed with 70% ethanol, air-dried and redissolved in 50 μl AMV reverse transcriptase buffer (Promega) plus 0·5 mM each of the deoxynucleoside triphosphates and 20 units of RNasin. The reaction was initiated by the addition of 20 units of AMV reverse transcriptase (Promega). After incubation at 42°C for 2 h, the reaction was terminated by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and the nucleic acids were precipitated with 2 vol ethanol and one tenth vol of 3 M Na acetate (pH 5) at −70°C for 1 h. After centrifugation, the precipitates were washed with 70% ethanol, dried and redissolved in 6 μl Sequenase buffer plus 4 μl Sequenase stop solution (U.S. Biochemical). After heating at 90°C for 3 min, 2 to 3 μl of the mixture was fractionated on a 6% sequencing gel. The same primers were used in sequencing reactions from M13 templates with [α-^{32}P]dATP and the Sequenase kit (U.S. Biochemical) or with [α-^{32}P]dCTP and a modified labeling mixture in the Sequenase kit. These reactions were loaded on the same gel and served as size standards.

(f) *Production of GerE in* E. coli

GerE and control protein were prepared as described previously (Zheng *et al.*, 1992) with the following modifications. *E. coli* cultures were grown at 30°C to an A_{595} of 0·5 in LB medium (Maniatis *et al.*, 1982) containing kanamycin sulfate (50 μg/ml) and ampicillin (75 μg/ml). Cells were induced by a temperature shift to 42°C for 25 min. Rifampicin was added to a final concentration of 200 μg/ml, and the cultures incubated at 42°C for 10 min and then at 30°C for 60 min. Cells were collected by centrifugation, resuspended in 0·05 vol of lysis buffer (10 mM Tris·HCl (pH 8·4), 10 mM $MgCl_2$, 1 mM EDTA, 0·3 mg/ml phenylmethanesulfonyl fluoride, 0·5 mg/ml lysozyme, 0·2 mM dithiothreitol, and 0·1 mg/ml DNase I) and incubated for 10 min at 37°C. After addition of 0·5 vol of sample buffer (0·375 M Tris·HCl (pH 6·8), 15% 2-mercaptoethanol, 60 mg/ml SDS, 30% glycerol and 3 mg/ml bromophenol blue) and immersion in boiling water for 3 min, the proteins were separated by SDS/PAGE (18% polyacrylamide gel; Thomas & Kornberg, 1978). GerE was excised from the gel, eluted from the gel slice and renatured as described (Hager & Burgess, 1980). Control protein was prepared by excising the region of the gel corresponding to GerE from a lane containing proteins from *E. coli* cells not expressing GerE, as described previously (Zheng *et al.*, 1992). Control protein was then eluted from the gel slice and renatured as described above.

(g) In vitro *transcription*

σ^K RNA polymerase was partially purified from *gerE* mutant cells as described previously (Kroos *et al.*, 1989). The enzyme was comparable in protein composition and in *cotD*- and *sigK*-transcribing activities to fraction 24 shown in Figure 2 of Kroos *et al.* (1989). σ^K RNA polymerase was reconstituted from *B. subtilis* core RNA polymerase and gel-purified, renatured σ^K as described previously (Kroos *et al.*, 1989). Transcription reactions (45 μl) were performed as described previously (Carter & Moran, 1986) except that RNA polymerase was allowed to bind to the DNA template for 10 min at 37°C before the addition of nucleotides (the labeled nucleotide was [α-^{32}P]CTP). Six μg of heparin was added 2 min after the addition of the nucleotides to prevent reinitiation. After the reactions were stopped, 10 μl of the reaction mixtures was subjected to electrophoresis and transcripts were detected by autoradiography. The signal intensities were quantified using a Visage 110 Image Analyzer (BioImage).

(h) *DNase I footprinting*

DNase I footprinting experiments were performed according to method (2) as described by Zheng *et al.* (1992), except 0·05 pmol of probe (prepared as described below) was used and a five-fold (w/w) excess of poly-(dI-dC) as compared to probe was added as competitor. In order to generate markers, the probes were subjected to the chemical cleavage reactions of Maxam & Gilbert (1980) as described previously (Maniatis *et al.*, 1982). DNA probes labeled at only one end were prepared as follows; for analysis of the *cotX* promoter region, pJZ23 was digested with *Xba*I, which cleaves 54 bp downstream of the transcriptional start site of the P_X promoter, and labeled either in the nontranscribed strand using the fill-in reaction of the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP, or labeled in the transcribed strand by treatment with alkaline phosphatase followed by phage T4 polynucleotide kinase and [γ-^{32}P]ATP. In both cases, the labeled DNA was digested with *Hin*dIII and the 409 bp *Hin*dIII-*Xba*I fragment was purified after electrophoresis in a nondenaturing polyacrylamide gel using the crush and soak method (Sambrook *et al.*, 1989). For analysis of the *cotVWX* promoter region, pJZ30 was digested with *Sal*I which cleaved 93 bp downstream of the transcriptional start site of the P_{VWX} promoter, end-labeled as described above, digested with *Hin*dIII, and the 1·6 kb *Hin*dIII-*Sal*I singly, end-labeled fragments were purified as described above. For analysis of the *cotYZ* promoter region, pJZ6 was digested with *Nco*I which cleaves 60 bp downstream of the transcriptional start site of the P_{YZ} promoter, end-labeled as described above, digested with *Eco*RV, and the 240 bp *Eco*RV-*Nco*I singly, end-labeled fragments were purified as described above.

3. Results

(a) *Organization and expression of transcripts from the* cotVWXYZ *gene cluster*

Total RNA prepared from samples of *B. subtilis* JH642 cultures, collected at one hour intervals after the cells had entered the stationary phase, was fractionated in a 1% agarose gel and blotted onto nitrocellulose (see Materials and Methods). The

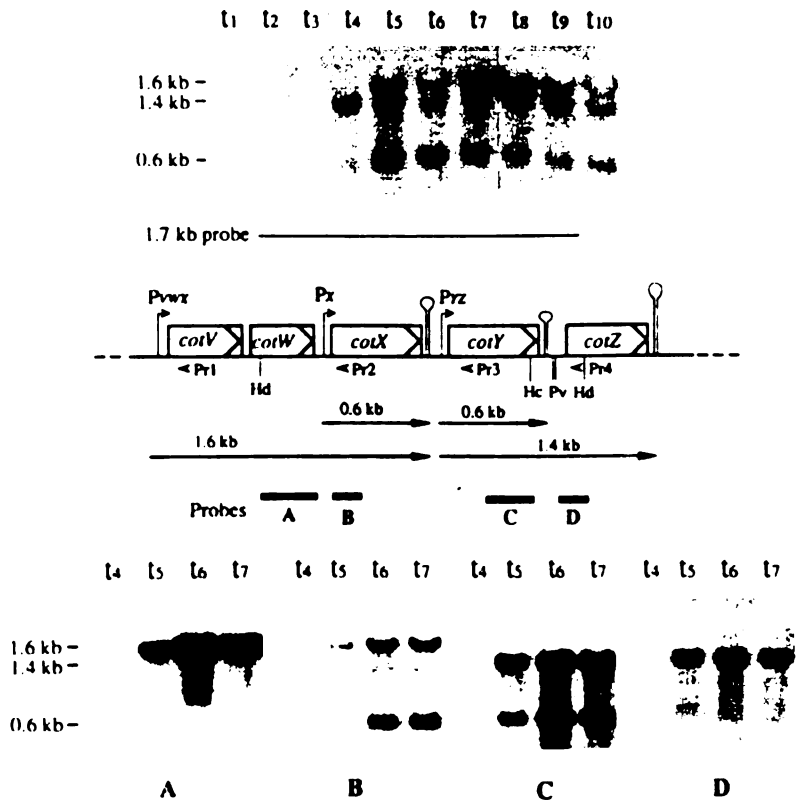

Figure 1. Northern hybridization analysis of the *cotVWXYZ* gene cluster. Total RNA was prepared from *B. subtilis* JH642 cells at hourly intervals after cells had entered the stationary phase (designated t_1 to t_{10}). Thirty μg of RNA was fractionated in 1% formaldehyde-agarose gels and blotted to nitrocellulose (Materials and Methods). Top: hybridization with a 1·7 kb *Hin*dIII fragment. Bottom: hybridization with probes A, B, C and D prepared as described in Materials and Methods. The sizes and relative locations within the gene of each probe are shown with thick bars. The diagram of the organization of the genes is based on the results reported here, primer extension mapping and *in vitro* transcription experiments (Figures 2 and 4). The 5 open reading frames are shown as boxes. Three promoters, P_{VWX}, P_X and P_{YZ}, and the orientation of transcription are marked with bent arrows. Three hairpin loops indicate potential transcription terminators (Zhang *et al.*, 1993). The 4 arrows immediately below the diagram show the orientation and sizes of transcripts produced from this gene cluster. The sizes of hybridizing RNA bands are indicated to the left of the gels. RNA size standards of 0·24 to 9·49 kb (BRL) were used. Orientations and relative binding sites of oligonucleotides (marked Pr1 to Pr4) used for primer extension experiments are indicated with arrow heads. Abbreviations: Hd, *Hin*dIII; Hc, *Hin*cII; Pv, *Pvu*II.

1·7 kb *Hin*dIII fragment containing the C terminus (149 codons) of *cotW*, all of *cotX* and *cotY*, and the N terminus (34 codons) of *cotZ* hybridized to at least three mRNAs of 1·6 kb, 1·4 kb and 0·6 kb, which were first detected at t_4 to t_5 (Figure 1, top). The *cotE* mRNA, which is expressed starting at about stage II of sporulation (Zheng & Losick, 1990), was first detected at t_1, whereas transcripts of the *cotD* and *cotT* genes appeared at about the same time as those of the *cotVWXYZ* cluster (data not shown).

In order to delineate the transcription pattern in detail, four subfragments were prepared from the 1·7 kb probe and each was labeled with [α-^{32}P]dCTP and Klenow fragment and used as a Northern hybridization probe (see Materials and Methods). As depicted in Figure 1, probe B which contains the N terminus of the *cotX* gene hybridized to mRNAs of 1·6 kb and 0·6 kb. Probe A, from the C terminus of *cotW*, hybridized only to a 1·6 kb mRNA. Transcription initiating from a promoter located immediately upstream of the *cotX* coding region could produce the 0·6 kb mRNA if there was termination at a potential Rho-independent terminator immediately downstream of the *cotX* coding region (Figure 1; Zhang *et al.*, 1993). There are no potential stem-loop structures between the *cotV* and *cotW* genes nor between *cotW* and *cotX* (Zhang *et al.*, 1993). Assuming that the only site of termination is after *cotX*, the *cotV*, *cotW* and *cotX* genes are probably cotranscribed, therefore, from another promoter upstream of *cotV* to produce the 1·6 kb mRNA.

The existence of two promoters, designated P_X and P_{VWX}, was suggested by primer extension mapping (Figure 2). The 5′ end of the 1·6 kb mRNA was mapped to 24 bp upstream of the first codon of *cotV* (P_{VWZ} in Figure 1) with primer Pr1 (which corresponds to the region between codons 37 and 42

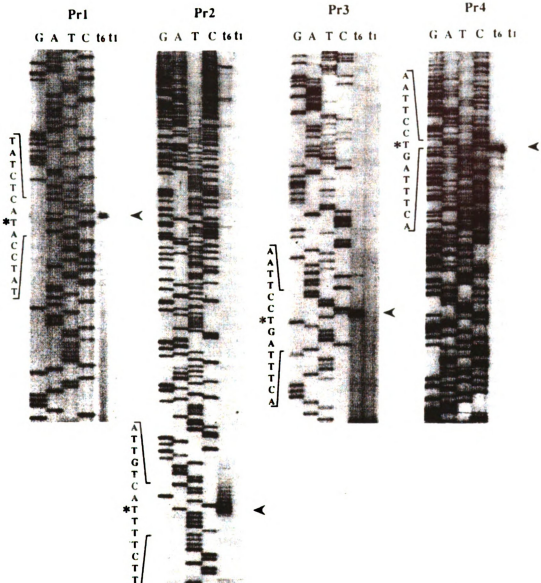

Figure 2. Reverse transcriptase mapping of the 5′ termini of the 1·6 kb *cotVWX* mRNA (Pr1), the 0·6 kb *cotX* mRNA (Pr2), the 0·6 kb *cotY* mRNA (Pr3) and the 1·4 kb *cotYZ* mRNA (Pr4). RNA was prepared from *B. subtilis* JH642 1 and 6 h after the onset of the stationary phase (t_1 and t_6; Materials and Methods). The products of primer extension were fractionated in a 6% sequencing gel. Sequencing reaction mixtures with the same primers were loaded in adjacent lanes and served as size markers. Pr1 corresponds to the region between codons 37 and 42 of *cotV*, Pr2 to the region between codons 15 and 20 of *cotX*, Pr3 corresponds to the region between codons 9 and 14 of *cotY*; and Pr4 to the region between codons 20 and 25 of *cotZ* (Figure 1; Zhang *et al.*, 1993). Arrow-heads indicate the positions and sizes of major extension products. * identifies the 5′ termini of the mRNAs.

of *cotV*). The 5′ end of the 0·6 kb *cotX* mRNA was mapped with primer Pr2 (which corresponds to codons 15 to 20 of *cotX*) and several major primer extension products of 80 to 87 bases in length with single base differences among them were generated. This cluster could correspond to a 5′ terminus of the 0·6 kb *cotX* mRNA located about 21 bp upstream of the first codon of *cotX* (Figures 1 and 2). In addition, larger products were detected, some of which could be prematurely terminated reverse transcriptase extensions of the 1·6 kb *cotVWX* mRNA. The longest could correspond to the 5′ terminus of this mRNA which was not well resolved in this gel. The *in vitro* transcription studies presented below indicate that the synthesis of *cotX* mRNA resulted from transcription from an internal promoter, P_X, rather than from processing of the 1·6 kb *cotVWX* mRNA.

A DNA fragment from the middle portion of the

cotY coding region. C, hybridized to 1·4 kb and 0·6 kb mRNAs, while probe D, from the N-terminal fragment of *cotZ*, hybridized only to a 1·4 kb mRNA (Figure 1). There is a potential Rho-independent terminator (a stem-loop followed by an A-T rich sequence) immediately downstream of *cotZ* coding region (Figure 1; Zhang *et al.*, 1993). Another potential stem-loop structure could be formed in the region between *cotY* and *cotZ* coding regions and may also function as a transcriptional terminator or a site for RNA processing (Figure 1; Zhang *et al.*, 1993). Primer extension with Pr3 (which corresponds to codons 9 to 14 of *cotY*) mapped a 5′ terminus 39 bp upstream of the first codon of *cotY* (Figure 2). In addition, primer Pr4 (which corresponds to codons 20 to 25 of *cotZ*) produced a 731 bp extension product, indicating that the 5′ terminus of the 1·4 kb mRNA is the same as that of the 0·6 kb *cotY* mRNA. No potential internal promoters were found in this region. It appears that both the 1·4 kb and 0·6 kb mRNAs are transcribed from the same promoter, P_{YZ}, immediately upstream of the *cotY* coding region. Termination at the downstream stem-loop structure could produce the 1·4 kb mRNA and termination or processing at the internal stem-loop structure could generate the 0·6 kb mRNA (Figure 1). Total RNA from t_1 cells was used as a control in each of the primer extension experiments and in no case were any of these extension products found (Figure 2).

(b) *Regulation of transcription of the* cotVWXYZ *cluster*

None of the four *cotVWXYZ* mRNAs was detected in RNA preparations from *B. subtilis* strain 55·3 which has a mutation in the *sigE* gene (*spoIIGB*) or from strain BK410, which contains a deletion (*spoIIIC94*; Errington *et al.*, 1988) encompassing the C-terminal half of the *sigK* gene (data not shown). The absence of detectable transcripts in the *sigK* mutant, which still produces σ^K RNA polymerase (Trempy *et al.*, 1985), suggests that σ^K RNA polymerase, and not σ^E RNA polymerase, transcribes the *cotVWXYZ* gene cluster. The lack of transcription in the *sigE* mutant presumably results from the failure to produce σ^K (Kunkel *et al.*, 1988; Stragier *et al.*, 1989; Lu *et al.*, 1990). In a *gerE* mutant strain (1AA-1), the 1·6 kb *cotVWX*, the 1·4 kb *cotYZ* and the 0·6 kb *cotY* mRNAs were present, albeit in slightly reduced amounts relative to the wild-type, but the 0·6 kb *cotX* mRNA was essentially absent (Figure 3). These results suggest that GerE is required for transcription from the P_X promoter and may enhance transcription from the P_{VWX} and P_{YZ} promoters. The RNA hybridizing to probe B was smeared, probably due to the instability of the 1·6 kb *cotVWX* mRNA. Such smearing was not observed when the same RNA was hybridized to probe C.

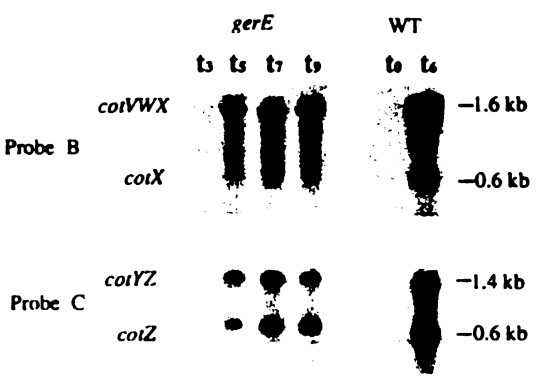

Figure 3. *CotVWXYZ* gene transcription in *B. subtilis* wild-type strain JH642 (WT) and *gerE* mutant 1AA-1 (*gerE*). Preparation of total RNA, fractionation and hybridization to probe B (from the N terminus of the *cotX* gene) and probe C (from the middle of the *cotY* gene) were as described in the legend to Figure 1.

(c) In vitro *transcription of genes in the* cotVWXYZ *cluster*

In order to determine directly the effects of σ^K RNA polymerase and GerE on transcription from promoters in the *cotVWXYZ* cluster, linearized DNA templates were transcribed with σ^K RNA polymerase partially purified from a *gerE* mutant in the presence or absence of gel-purified GerE (see Materials and Methods; Figure 4). Use of σ^K RNA polymerase resulted in run-off transcripts of the expected sizes from P_{VWX} and there was a two- to threefold stimulation of transcription when GerE was present (Figure 4A). This level of stimulation by GerE was reproducible in three separate experiments. The larger of the two transcripts in lane 4 of Figure 4A is probably an artifact resulting from run-off transcription of DNA templates cleaved with that particular restriction enzyme (*Sac*I) since only transcripts of the expected sizes were observed in the other lanes. Run-off transcripts of the expected sizes from P_X were produced only when σ^K RNA polymerase was supplemented with GerE (Figure 4B), consistent with the finding that the 0·6 kb *cotX* transcript was not detected in a *gerE* mutant (Figure 3). σ^K RNA polymerase produced run-off transcripts of the expected sizes from P_{YZ} and there was a twofold stimulation of transcription when GerE was present (Figure 4C). σ^K RNA polymerase reconstituted from *B. subtilis* core RNA polymerase and gel-purified σ^K also produced run-off transcripts of the expected sizes from P_{VWX}, P_X, and P_{YZ} in the presence of GerE (data not shown). These results support the existence of three promoters in the *cotVWXYZ* cluster and each is transcribed *in vitro* by σ^K RNA polymerase with different levels of dependence upon GerE.

(d) *GerE binding sites in the P_{VWX}, P_X and P_{YZ} promoter regions*

GerE was shown previously to bind to DNA at particular sites upstream of the transcriptional start

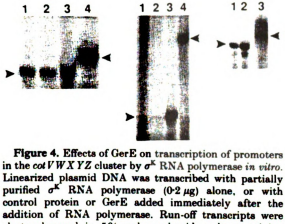

Figure 4. Effects of GerE on transcription of promoters in the *cotVWXYZ* cluster by σ^K RNA polymerase *in vitro*. Linearized plasmid DNA was transcribed with partially purified σ^K RNA polymerase (0·2 μg) alone, or with control protein or GerE added immediately after the addition of RNA polymerase. Run-off transcripts were electrophoresed in 5% polyacrylamide gels containing 8 M urea and were detected by autoradiography. Arrowheads denote the positions of run-off transcripts of the expected sizes, as judged from the migration of end-labeled DNA fragments of *Mspi*-digested pBR322. A, Transcription of P_{VWX} from pJZ30 (0·05 pmol) digested with *Sma*I (lanes 1 to 3, 110-base transcript) or *Sac*I (lane 4, 118-base transcript) with σ^K RNA polymerase alone (lane 1), or with control protein (lane 2) or 0·75 μg of GerE (lanes 3 and 4) added. B, Transcription of P_X from pJZ22 (0·05 pmol) digested with *Bgl*II (lanes 1 to 3, 188-base transcript) or *Eco*RI (lane 4, 288-base transcript) with σ^K RNA polymerase alone (lane 1), or with control protein (lane 2) or 0·75 μg of GerE (lanes 3 and 4) added. C, Transcription of P_{YZ} from pJZ6 (0·05 pmol) digested with *Nco*I (lanes 1 and 2, 62-base transcript) or *Hinc*II (lanes 3, 481-base transcript) with σ^K RNA polymerase alone (lane 1) or with 1·0 μg of GerE added (lanes 2 and 3).

sites of genes such as *cotB* and *cotC* which are transcriptionally stimulated by this protein (Zheng *et al.*, 1992). Since GerE enhanced transcription from all three promoters in the *cotVWXYZ* cluster, GerE binding in these promoter regions was examined by DNase I footprinting experiments carried out with DNA probes end-labeled on each of the strands (Materials and Methods: Figure 5). GerE protected an approximately 24 bp stretch of DNA extending from position −27 to position −50 upstream of the P_{VWX} transcriptional start site on the nontranscribed strand (Figure 5A). The protection may extend to position −54 but the absence of DNase I digestion products from position −50 to position −54 makes the boundary on this strand uncertain (see broken line in Figure 6A). GerE also protected an approximately 19 bp stretch extending from position −32 to position −50 on the transcribed strand in the P_{VWX} region (Figure 5B), with uncertainty in the upstream boundary of protection between −50 and −56 (Figure 6A). GerE protected a large stretch of approximately 35 bp extending from position −28 to position −62 upstream of the P_X transcriptional start site on the nontranscribed strand (Figure 5C), and this protection may extend to position −69 (Figure 6A). An approximately

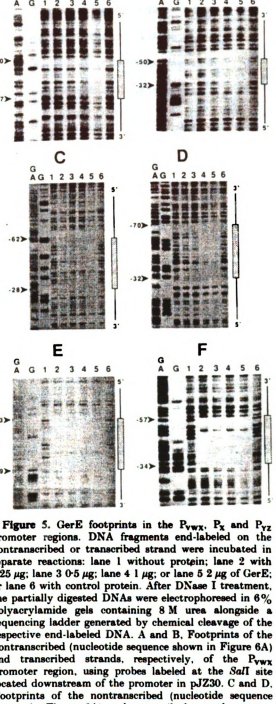

Figure 5. GerE footprints in the P_{VWX}, P_X and P_{YZ} promoter regions. DNA fragments end-labeled on the nontranscribed or transcribed strand were incubated in separate reactions: lane 1 without protein; lane 2 with 0·25 μg; lane 3 0·5 μg; lane 4 1 μg; or lane 5 2 μg of GerE; or lane 6 with control protein. After DNase I treatment, the partially digested DNAs were electrophoresed in 6% polyacrylamide gels containing 8 M urea alongside a sequencing ladder generated by chemical cleavage of the respective end-labeled DNA. A and B, Footprints of the nontranscribed (nucleotide sequence shown in Figure 6A) and transcribed strands, respectively, of the P_{VWX} promoter region, using probes labeled at the *Sal*I site located downstream of the promoter in pJZ30. C and D, Footprints of the nontranscribed (nucleotide sequence shown in Figure 6A) and transcribed strands, respectively, of the P_X promoter region, using probes labeled at the *Xba*I site located downstream of the promoter in pJZ23. E and F, Footprints of the nontranscribed strand (nucleotide sequence shown in Figure 6A) and transcribed strands, respectively, of the P_{YZ} promoter region, using probes labeled at the *Nco*I site located downstream of the promoter in pJZ6. Arrowheads denote the boundaries of protection by GerE and numbers refer to positions relative to the transcriptional start site.

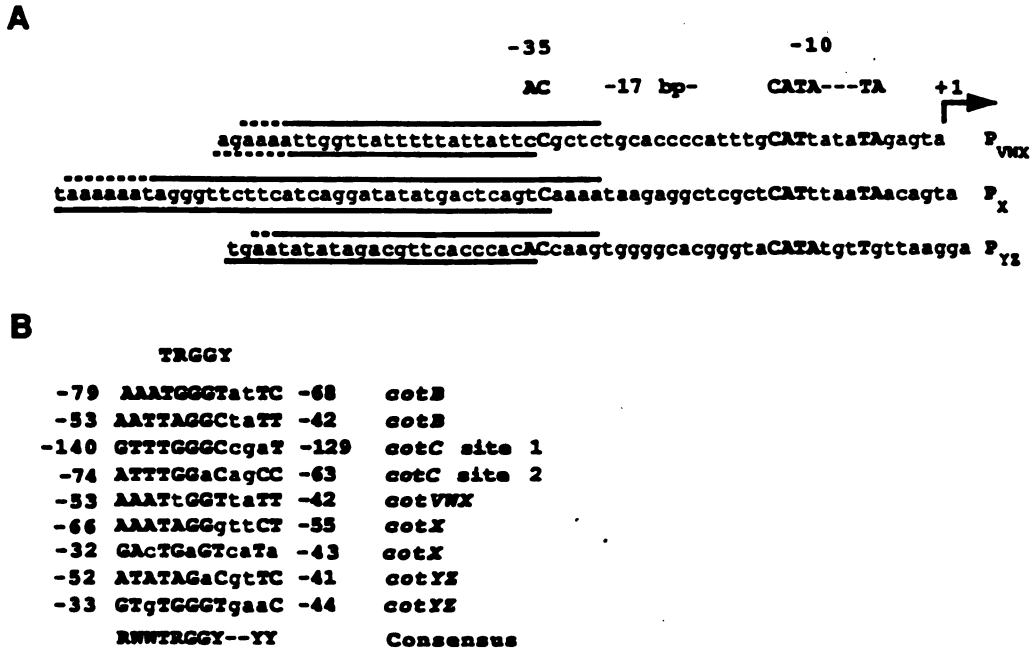

Figure 6. Alignment of promoters in the *cotVWXYZ* cluster with the consensus sequence for σ^K-transcribed promoters (A) and alignment of GerE binding sites (B). A, Nucleotide sequences (Zhang *et al.*, 1993) upstream of the transcriptional start sites of the P_{VWX}, P_X and P_{YZ} promoters (Figure 2) are aligned with respect to conserved nucleotides (boldface, capital letters) found in the −10 and −35 regions of promoters transcribed by σ^K RNA polymerase (Foulger and Errington 1991; Zheng *et al.*, 1992), shown at the top. Overlining and underlining indicate regions on the nontranscribed and transcribed strands, respectively, protected by GerE from digestion with DNase I (Figure 5). The broken portions of lines indicate regions of uncertain protection due to a lack of DNase I digestion in these regions. B, Nucleotide sequences protected by GerE from digestion with DNase I are aligned with respect to the consensus proposed previously (Zheng *et al.*, 1992), shown at the top. GerE binding sites in the *cotB* and *cotC* promoter regions are from Zheng *et al.* (1992) and in the P_{VWX}, P_X and P_{YZ} promoter regions are from Figure 5. Numbers refer to positions relative to the transcriptional start site. Note that the sequences shown for the binding sites between −32 and −43 of the P_X and between −33 and −44 of the P_{YZ} promoter are from the opposite DNA strands as shown in part A of this Figure. The bottom line shows an enlarged consensus sequence for GerE binding based on the sequences shown. R means purine, W means A or T, and Y means pyrimidine. Nucleotides that match the consensus (at least 7 out of 9 at each position) are shown as boldface, capital letters.

39 bp stretch extending from position −32 to position −70 on the transcribed strand in the P_X region was also protected (Figure 5D). The strongest protection by GerE was observed in the P_{YZ} region, where an approximately 25 bp stretch of DNA from position −29 to position −53 (or possibly position −55) was protected on the nontranscribed strand (Figure 5E) and an approximately 24 bp stretch extending from position −34 to position −57 was protected on the transcribed strand (Figure 5F). These results show that GerE binds immediately upstream and partially overlaps the −35 region of all three promoters in the *cotVWXYZ* cluster.

4. Discussion

The *cotVWXYZ* gene cluster is organized into two multicistronic operons plus two overlapping monocistronic transcription units (Figure 1). The *cotV*, *W* and *X* genes are cotranscribed as a 1·6 kb mRNA from the most upstream promoter, P_{VWX}. In addition, a 0·6 kb *cotX* mRNA is synthesized from an internal promoter, P_X, located between the *cotW* and *cotX* coding regions. A 1·4 kb mRNA is the product of cotranscription of the *cotY* and *cotZ* genes, while the 0·6 kb *cotY* mRNA appears to result from transcription from the same promoter (P_{YZ}) and termination or processing at a potential stem-loop structure located between the *cotY* and *cotZ* coding regions (Zhang *et al.*, 1993). The potential stem-loop structure between the *cotY* and *cotZ* genes is not a typical Rho-independent terminator since there is not a stretch of T nucleotides on the nontranscribed strand following the stem-loop structure. Thus, there is likely to be some *trans*-acting factor involved in the control of termination or processing at this internal stem-loop structure resulting in the accumulation of about equal amounts of *cotYZ* and *cotY* mRNA. The *cotVWXYZ* cluster comprises the first example of genes involved in spore coat synthesis or assembly which are organized into operons as well as overlapping single transcriptional units. Seven other genes encoding spore coat proteins, *cotA* through *F* and *cotT*, are expressed from monocistronic transcription units and are scattered widely on the chromo-

some (Donovan *et al.*, 1987; Zheng *et al.*, 1988; Aronson *et al.*, 1989; Cutting *et al.*, 1991).

The clustering of the *cotVWXYZ* genes probably reflects related functions and interactions among their protein products in the formation of the spore coat insoluble fraction. The CotX protein is a major component of the spore coat insoluble fraction and is probably extensively cross-linked (Zhang *et al.*, 1993). The CotY and Z proteins are similar to each other in sequence and are present as disulfide cross-linked multimers in spore coat extracts. There is also evidence of interactions among CotX, Y and Z in the spore coat since deletion of the *cotX* gene resulted in an increase in the amount of soluble CotY and CotZ proteins (Zhang *et al.*, 1993).

As with several other spore coat protein genes, transcription of the *cotVWXYZ* gene cluster is dependent upon σ^K RNA polymerase and to different extents upon the GerE protein. Transcripts were first detected at about stage IV-V of sporulation which coincides with the onset of transcription of two other spore coat genes, *cotD* and *cotT* (data not shown). The *cotD* gene is known to be transcribed by σ^K RNA polymerase in the mother cell (Kroos *et al.*, 1989; Zheng & Losick, 1990; Driks & Losick, 1991) as are the *cotVWXYZ* genes. The *cotVWXYZ* transcripts were not present in a *sigK* mutant (data not shown) and partially purified σ^K RNA polymerase generated transcripts *in vitro* from the P_{VWX} and P_{YZ} promoters, as well as from the P_X promoter when the GerE protein was present (Figure 4). In general, therefore, the transcription of the *cotVWXYZ* cluster fits the pattern of several other spore coat genes. The absolute dependence of the P_X promoter on GerE for transcription *in vitro* was consistent with the observation that there was no 0·6 kb *cotX* mRNA in a *gerE* mutant (Figure 3). The *cotC* promoter also exhibits almost absolute dependence on GerE for transcription by σ^K RNA polymerase *in vitro* (Zheng *et al.*, 1992) and a *cotC-lacZ* fusion was not expressed in a *gerE* mutant (Zheng & Losick, 1990).

In vitro, transcription from the P_{VWX} and P_{YZ} promoters was similar to that from the *cotD* promoter in that there was considerable transcription in the absence of GerE and this basal level of transcription was stimulated two- to threefold by the addition of GerE (Figure 4; Zheng *et al.*, 1992). *In vivo*, the amounts of 1·6 kb *cotVWX* mRNA, 1·4 kb *cotYZ* mRNA and 0·6 kb *cotY* mRNA in a *gerE* mutant were less than in the wild-type (Figure 3) but the effect of GerE on accumulation of these mRNAs appeared to be rather small compared to the sevenfold enhancement of *cotD-lacZ* expression provided by GerE *in vivo* (Zheng & Losick, 1990).

Examination of the P_{VWX}, P_X and P_{YZ} promoter regions reveals nucleotide sequence similarities to other promoters recognized by σ^K RNA polymerase. The three promoter regions are aligned with a proposed σ^K RNA polymerase recognition sequence (Foulger & Errington 1991; Zheng *et al.*, 1992) in Figure 6A. All three promoters have five out of six matches to the consensus in their −10 regions. Only the P_{YZ} promoter, which shows the least dependence upon GerE *in vitro*, has the conserved AC in its −35 region. The P_{VWX} promoter has CC, rather than AC, in its −35 region, indicating that CC can be tolerated since P_{VWX} was transcribed appreciably by σ^K RNA polymerase *in vitro* in the absence of GerE (Figure 4A). The P_X promoter has TC in its −35 region, as does the *cotB* promoter, while the *cotC* promoter has AG at these positions (Zheng *et al.*, 1992). These three promoters exhibit little or no transcription by σ^K RNA polymerase *in vitro* in the absence of GerE, with strong stimulation upon the addition of GerE (Figure 4B; Zheng *et al.*, 1992).

One function of GerE bound to these promoter regions may be to relieve partially or completely a requirement for interactions between σ^K RNA polymerase and nucleotides in the −35 region. For example, the cII protein of bacteriophage λ is a transcriptional activator that apparently modifies σ^{70} RNA polymerase recognition of the −35 region of the λP_{RE} promoter, and it does so by binding to the −35 region on the opposite face of the DNA helix from σ^{70} RNA polymerase (Ho *et al.*, 1983; Shih & Gussin, 1983). Other examples include class II transcription factors of *E. coli* which bind to the −35 region of a promoter and activate transcription apparently by contacting conserved region 4·2 (which is normally involved in recognition of the −35 region of promoters) of σ^{70} (reviewed in Ishihama, 1993; Gardella *et al.*, 1989; Siegele *et al.*, 1989). Despite an extended region of similarity between GerE and region 4 of sigma factors (Kahn & Ditta, 1991), GerE does not appear to recognize the same sequence as σ^K RNA polymerase in the −35 region of promoters (see below). Rather, GerE binds upstream of (and in some cases partially overlapping) the −35 region in the promoters examined to date (Figure 6A; Zheng *et al.*, 1992). This position of binding is typical for class I transcription factors of *E. coli* which contact the C-terminal domain of the α subunit of RNA polymerase when they stimulate transcription (Ishihama, 1993), although the cI protein of λ binds in this position and activates the λP_{RM} promoter apparently by contacting σ^{70} (Li *et al.*, 1994).

Inspection of the sequences protected from DNase I digestion by GerE in the P_{VWX}, P_X and P_{YZ} promoter regions reveals similarities to the consensus sequence for GerE binding, TPuGGPy, proposed previously (Zheng *et al.*, 1992). The protected region in the P_{VWX} promoter region was similar to binding site 2 in the *cotC* promoter region (Zheng *et al.*, 1992) in that GerE binding was relatively weak and 19 to 24 bp were protected. In both cases a sequence with four out of five matches to the previously proposed consensus (TtGGT at position −50 to position −46 for the P_{VWX} region) is present in the protected region (Figure 6A; Zheng *et al.*, 1992). The analogy extends further in that a second GerE binding site is located about 150 bp upstream (and hence beyond the known sequence; Zhang *et al.*, 1993) of the P_{VWX} transcriptional start site (H. Ichikawa & L. Kroos, unpublished results), whereas binding site 1 in the

cotC promoter region is centered about 135 bp upstream of the transcriptional start site (Zheng *et al.*, 1992). Both GerE binding sites were present in the P_{VWX}-bearing template used for *in vitro* transcription (Figure 4A) and we have not yet investigated whether the upstream site is necessary for stimulation of transcription by GerE. The upstream site in the *cotC* promoter region was not necessary for stimulation of transcription by GerE *in vitro* (R. Halberg & L. Kroos, unpublished results cited in Zheng *et al.*, 1992).

The protected region in the P_X promoter (31 to 44 bp) was approximately twice the length of each of two separate protected regions in the *cotC* promoter region (Zheng *et al.*, 1992) and is similar in length to the protected region in the *cotB* promoter which was proposed to encompass two GerE binding sites (Zheng *et al.*, 1992). Within this region of the *cotX* promoter, two sequences separated by 19 bp with four out of five matches to the proposed consensus are found in inverted orientation with respect to each other. The sequences are TAGGg (position −63 to position −59 shown in Figure 6A) and TGaGT (position −35 to position −39 on the DNA strand opposite that shown in Figure 6A). Thus, the P_X promoter region may contain two adjacent GerE binding sites in inverted orientation. In the *cotB* promoter region, the two sequences matching the proposed GerE binding site consensus are in the same orientation and are separated by 21 bp (Zheng *et al.*, 1992).

Relatively strong GerE binding was observed in the P_{YZ} promoter region (Figure 5) and the length of the protected region (20 to 29 bp) is suggestive of a single site. However, within the protected region one sequence with four out of five matches to the previously proposed consensus (TAGaC at positions −49 to −45; Figure 6A) and another sequence which matches the consensus perfectly (TGGGT at positions −36 to −40 on the DNA strand opposite that shown in Figure 6A) are found in inverted orientation with respect to each other and are separated by 4 bp. An analogous arrangement of two 5 bp sequences (both TAGGC) in inverted orientation and separated by 4 bp was noted in the *sigK* promoter region (Zheng *et al.*, 1992) and GerE binds strongly to this region (R. Halberg, H. Ichikawa & L. Kroos, unpublished results). An alignment of sequences bound by GerE is shown in Figure 6B. The additional binding sites in the P_{VWX}, P_X and P_{YZ} promoter regions permit definition of a larger consensus sequence for GerE binding, RWWTRGGY--YY (R means purine, W means A or T and Y means pyrimidine).

The complex transcriptional pattern of the *cotVWXYZ* cluster, including overlapping operons with monocistronic transcriptional units and differential regulation by GerE, is apparently important for ensuring that an optimal amount of each protein is made at the appropriate times for assembly into the spore coat insoluble fraction. The actual protein ratios are difficult to establish because of the insolubility but are probably reflected in the steady state amounts of the various mRNAs. If that were the case, there would be relatively more of the CotX and CotY proteins, each of which is pivotal for the cross-linking and assembly of the coat insoluble fraction.

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CHAPTER IV

Negative Regulation by the *Bacillus subtilis* GerE Protein

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Negative Regulation by the *Bacillus subtilis* GerE Protein*

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GerE is a transcription factor produced in the mother cell compartment of sporulating *Bacillus subtilis*. It is a critical regulator of *cot* genes encoding proteins that form the spore coat late in development. Most *cot* genes, and the *gerE* gene, are transcribed by σ^K RNA polymerase. Previously, it was shown that the GerE protein inhibits transcription *in vitro* of the *sigK* gene encoding σ^K. Here, we show that GerE binds near the *sigK* transcriptional start site, to act as a repressor. A *sigK-lacZ* fusion containing the GerE-binding site in the promoter region was expressed at a 2-fold lower level during sporulation of wild-type cells than *gerE* mutant cells. Likewise, the level of SigK protein (*i.e.* pro-σ^K and σ^K) was lower in sporulating wild-type cells than in a *gerE* mutant. These results demonstrate that σ^K-dependent transcription of *gerE* initiates a negative feedback loop in which GerE acts as a repressor to limit production of σ^K. In addition, GerE directly represses transcription of particular *cot* genes. We show that GerE binds to two sites that span the −35 region of the *cotD* promoter. A low level of GerE activated transcription of *cotD* by σ^K RNA polymerase *in vitro*, but a higher level of GerE repressed *cotD* transcription. The upstream GerE-binding site was required for activation but not for repression. These results suggest that a rising level of GerE in sporulating cells may first activate *cotD* transcription from the upstream site then repress transcription as the downstream site becomes occupied. Negative regulation by GerE, in addition to its positive effects on transcription, presumably ensures that σ^K and spore coat proteins are synthesized at optimal levels to produce a germination-competent spore.

Starvation induces the Gram-positive bacterium *Bacillus subtilis* to initiate a series of morphological changes that result in the formation of a dormant spore (1). Early in the sporulation process a septum forms that divides the cell into a larger mother cell compartment and a smaller forespore compartment. Each compartment contains a copy of the genome, and different genes are expressed in each compartment. Gene expression drives further morphogenesis, including migration of the septum to engulf the forespore in a double membrane, deposition of cell wall-like material called cortex between the membranes, and synthesis in the mother cell of proteins that assemble on the surface of the forespore to produce a tough shell known as the coat. The developmental process culminates with lysis of the mother cell to release a mature spore. When nutrients become available again, the spore germinates, producing a cell that resumes growth and division.

The program regulating transcription of sporulation genes is exceptionally well understood (2). It involves the synthesis and activation of four compartment-specific σ subunits of RNA polymerase (RNAP),[1] each of which directs the enzyme to transcribe a particular set of genes. σ^F and σ^G control forespore-specific gene expression. In the mother cell, activation of σ^E is followed by the synthesis and activation of σ^K. In addition, two small, DNA-binding proteins, SpoIIID and GerE, activate or repress transcription of many mother cell-specific genes (3–6). The mother cell transcription factors form a hierarchical regulatory cascade in which the synthesis of each factor depends upon the activity of the prior factor, in the order σ^E, SpoIIID, σ^K, and finally GerE (7).

In addition to positive regulation between one transcription factor and the next in the mother cell cascade, there is evidence of negative regulation as well. σ^K RNAP initiates a feedback loop that inhibits transcription of the *sigE* gene encoding σ^E (8). Since σ^E RNAP transcribes the *spoIIID* gene (9–11), production of SpoIIID is also negatively regulated (8, 12). This facilitates the switch from the early σ^E- and SpoIIID-directed pattern of gene expression to the late σ^K- and GerE-directed pattern. GerE is not a component of this feedback loop (8); however, there was reason to believe that GerE might initiate a second feedback loop. GerE was shown previously to inhibit transcription *in vitro* of the *sigK* gene encoding σ^K (4). Whether GerE inhibits *sigK* transcription *in vivo* was in doubt, though, because a *sigK-lacZ* fusion was not overexpressed in *gerE* mutant cells (13). We have resolved this paradox by mapping a GerE-binding site in the *sigK* promoter region. This showed that the *sigK-lacZ* fusion examined previously did not contain the entire GerE-binding site. Here, we demonstrate that GerE represses *sigK* expression about 2-fold *in vivo*, and we discuss the implications of this negative feedback.

Overexpression of *sigK* is not the only defect in *gerE* mutant cells. Expression of some *cot* genes encoding spore coat proteins is reduced or absent, whereas expression of other genes is increased. The resulting spores have defective coats and germinate inefficiently (14). Previously, it was shown that purified GerE binds to DNA sequences matching the consensus RWWTRGGY-YY (where R is purine; W is A or T; and Y is pyrimidine) and activates transcription *in vitro* from the *cotB* and *cotC* promoters (4) and from three promoters in the *cotVWXYZ* cluster (3). The position of binding ranges in the different promoters from well upstream of the −35 region to partially overlapping it. Here, we show that GerE binds to the *cotD* promoter region at a position typical for activation of transcription, and to a position downstream, which may cause repression of transcription. Our mapping of GerE-binding sites in the

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[1] The abbreviations used are: RNAP, RNA polymerase; TSS, transcriptional start site; PCR, polymerase chain reaction; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; bp, base pair.

cotD and *sigK* promoters provides the first information about how GerE acts as a transcriptional repressor.

EXPERIMENTAL PROCEDURES

DNase I Footprinting—DNA fragments labeled at only one end were prepared as follows. For analysis of the *sigK* promoter region, a *Hin*dIII-*Xba*I fragment of pBK16 (15) was labeled at the 3′ ends by the Klenow enzyme fill-in reaction and [α-^{32}P]dCTP or at the 5′ ends by treatment with alkaline phosphatase followed by T4 polynucleotide kinase and [γ-^{32}P]ATP. In both cases, the labeled DNA was digested with *Pst*I, which cleaved off a small fragment containing the labeled *Hin*dIII end, so it did not interfere with subsequent DNase I footprinting. The labeled *Xba*I end is 164 bp downstream of the *sigK* transcriptional start site (TSS). For analysis of the *cotD* promoter region, pLRK100 (15) was digested with *Eco*RI, which cleaves 227 bp upstream of the *cotD* TSS, and labeled either at the 3′ end by the Klenow enzyme fill-in reaction and [α-^{32}P]dATP or at the 5′ end by treatment with alkaline phosphatase followed by T4 polynucleotide kinase and [γ-^{32}P]ATP. In both cases, the labeled DNA was digested with *Hin*dIII, which cleaves downstream of the *cotD* promoter, to produce a 443-bp fragment that was purified by elution from an 8% polyacrylamide gel (16). Labeled DNA fragments were incubated with different amounts of GerE gel purified from *Escherichia coli* engineered to overproduce the protein as described previously (3) and then mildly digested with DNase I according to method 2 as described previously (4), except 3 pmol of probe was used and a 7-fold (w/w) excess of poly(dI-dC) was added to compete with probe was added as competitor. After DNase I treatment, the partially digested DNAs were electrophoresed in a 7% polyacrylamide gel containing 8 M urea alongside a sequencing ladder generated by subjecting the appropriate end-labeled DNA to the chemical cleavage reactions of Maxam and Gilbert as described previously (16).

Construction of a sigK-lacZ Fusion—DNA between −115 and +28 relative to the *sigK* TSS was amplified by the polymerase chain reaction (PCR) using pBK16 (15) as the template. The upstream primer was 5′ GGGAATTCGATGAAGAATATTTTTAAC 3′ and the downstream primer was 5′ GCGAAGCTTCCACAAAAGTATGTA 3′. *Eco*RI and *Hin*dIII restriction sites (underlined) were designed into the 5′ ends of the upstream and downstream primers, respectively, to allow directional subcloning of the PCR product into *Eco*RI-*Hin*dIII-digested pTK*lac* (17). The resulting plasmid pHI16 was linearized by digestion with *Bsa*I and transformed into *B. subtilis* ZB307, in which marker replacement-type recombination created an SPβ::*sigK-lacZ* specialized transducing phage as described previously (18). A phage lysate was prepared by heat induction and used to transduce *B. subtilis* SG38 (*spo*$^+$ *trpC2*) and 522.2 (*gerE36 trpC2*) (19) with selection for resistance to chloramphenicol (5 μg/ml) on LB agar as described previously (20).

Measurement of β-Galactosidase Activity—Sporulation was induced by nutrient exhaustion in DSM at 37 °C as described previously (20). Samples (1 ml) were collected at hourly intervals during sporulation, cells were pelleted, and pellets were stored at −70 °C prior to the assay. The specific activity of β-galactosidase was determined by the method of Miller (21), using *o*-nitrophenol-β-D-galactopyranoside as the substrate. One unit of enzyme hydrolyzes 1 μmol of substrate per min per unit of initial cell absorbance at 595 nm.

Western Blot Analysis—Cells were induced to sporulate by nutrient exhaustion in DSM at 37 °C as described previously (20). Samples (1 ml) were collected at hourly intervals during sporulation, and whole-cell extracts were prepared as described previously (22). Protein concentrations were determined by the method of Bradford (23). Proteins (5 μg) were separated by sodium dodecyl sulfate (SDS)-14% Prosieve (FMC BioProducts) polyacrylamide gel electrophoresis with Tris/Tricine electrode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS) and electroblotted onto Immobilon-P membranes (Millipore). Blots were incubated as described previously with polyclonal anti-pro-σ^K antibodies that detect both pro-σ^K and σ^K (22). The secondary antibody was horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Bio-Rad). Chemiluminescence detection (ECL) was performed according to the manufacturer's instructions (Amersham Pharmacia Biotech). The signal intensities were quantified using a GS 505 Molecular Imager System (Bio-Rad).

In Vitro Transcription—σ^K RNAP was partially purified from *gerE* mutant cells as described previously (15). The enzyme was comparable in protein composition and in *cotD*- and *sigK*-transcribing activities to fraction 24 shown in Fig. 2 of Kroos *et al.* (15). Transcription reactions (45 μl) were performed as described previously (24), except that RNAP was allowed to bind to the DNA template for 10 min at 37 °C before the addition of nucleotides (the labeled nucleotide was [α-^{32}P]CTP). Heparin (6 μg) was added 2 min after the addition of nucleotides to prevent reinitiation. After the reactions were stopped, 20 μl of each reaction mixture was subjected to electrophoresis in a 5% polyacrylamide gel containing 8 M urea, and transcripts were detected by autoradiography. The signal intensities were quantified using a Storm 820 PhosphorImager (Molecular Dynamics). To prepare a DNA template containing the *cotD* promoter and lacking the upstream GerE-binding site, DNA between −44 and +227 relative to the TSS was amplified by the PCR using pLRK100 (15) as the template. The upstream primer was 5′ CGGTTTTGCATCAGAACATGT 3′ (the underlined portion corresponds to the nontranscribed strand of the *cotD* promoter region), and the downstream primer was 5′ GGAAGCTTGCATGCCTGCA 3′ (the *underlined portion* corresponds to a *Hin*dIII site downstream of the *cotD* promoter in pLRK100).

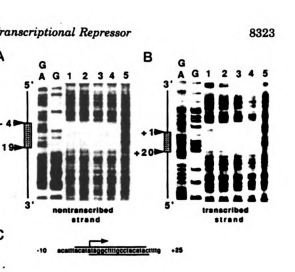

FIG. 1. **GerE footprints in the *sigK* promoter region.** Radioactive DNA fragments separately end-labeled on the nontranscribed (*A*) or transcribed (*B*) strand were incubated in separate reactions with a carrier protein (bovine serum albumin, 310 pmol) only (*lane 5*) or with 6 pmol (*lane 1*), 12 pmol (*lane 2*), 60 pmol (*lane 3*), or 120 pmol (*lane 4*) of gel-purified GerE in addition to the carrier protein and then subjected to DNase I footprinting in a total volume of 45 μl. *Hatched bases* indicate the region protected from DNase I digestion by GerE. *Arrowheads* denote the boundaries of protection, and numbers to the *left* refer to positions relative to the TSS, as deduced from sequencing ladders generated by chemical cleavage of the respective end-labeled DNA at purines (*lane G over A*) or guanines (*lane G*). *C*, position of the GerE-binding site in the *sigK* promoter region. The nucleotide sequence of the nontranscribed strand of the *sigK* promoter region is shown (13). *Overlining* and *underlining* indicate regions on the nontranscribed and transcribed strands, respectively, protected by GerE from DNase I digestion. The TSS is shown by an *arrow*. *D*, nucleotide sequences within the GerE-protected region of the *sigK* promoter are aligned with the consensus sequence for GerE binding (3). Matches to the consensus sequence are shown as *capital letters*, and *numbers* refer to positions relative to the TSS. Note that the sequence shown for the binding site between +18 and +7 is from the strand opposite that shown in *C*.

RESULTS

Location of the GerE-binding Site in the sigK Promoter Region—Purified GerE was shown previously to strongly inhibit transcription *in vitro* of the *sigK* gene by σ^K RNAP (4). However, expression of a *sigK-lacZ* fusion *in vivo* was unaffected by a *gerE* mutation (13). The discrepancy between the two results could be explained if the *sigK-lacZ* fusion did not contain a binding site for GerE that mediates repression. To see if GerE binds specifically in the *sigK* promoter region and, if so, to determine the position of binding, we performed DNase I footprinting experiments.

Fig. 1 shows that GerE protected a stretch of DNA from DNase I digestion that included the TSS of *sigK* and extended

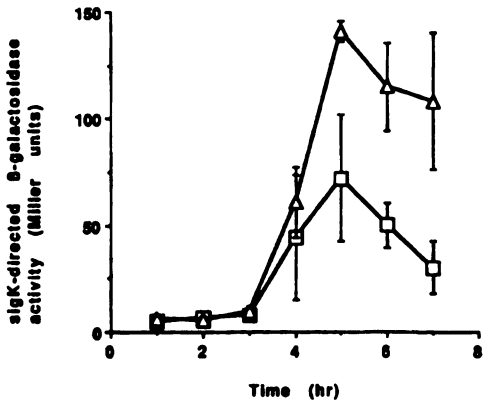

FIG. 2. *sigK-lacZ* expression in wild-type and *gerE* mutant cells. *sigK*-directed β-galactosidase activity was measured at the indicated times during sporulation of congenic wild-type (SG38, □) and *gerE* mutant (522.2, △) strains. *Points* on the graph are averages for isolates of each type, and *error bars* show 1 S.D. of the data.

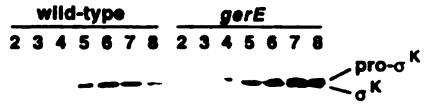

FIG. 3. Levels of pro-σ^K and σ^K during sporulation of wild-type and *gerE* mutant cells. Whole-cell extracts were prepared from wild-type (SG38) and *gerE* mutant (522.2) cells collected at the indicated number of hours after the onset of sporulation in DSM. Proteins were fractionated by SDS-PAGE and subjected to Western blot analysis with anti-pro-σ^K antibodies, which detect both pro-σ^K and σ^K.

downstream. The protection spanned positions −4 to +19 on the nontranscribed strand (Fig. 1*A*) and positions +1 to +20 on the transcribed strand (Fig. 1*B*). Complete protection from DNase I digestion was observed at the lowest concentration of GerE tested, indicating that GerE binds with relatively high affinity to this site as compared with other GerE-binding sites mapped previously (3, 4). Fig. 1*C* shows the sequence of the *sigK* promoter in the region protected by GerE. Within the protected region are two sequences in inverted orientation that overlap slightly and match the consensus sequence for GerE binding (Fig. 1*D*).

We conclude that GerE binds to a site in the *sigK* promoter region that overlaps the TSS and extends downstream. Presumably, GerE binding to this site represses *sigK* transcription *in vitro* (4) by interfering with RNA polymerase binding and/or a subsequent step in initiation. The location of the GerE-binding site provides a plausible explanation for the lack of an effect of a *gerE* mutation on *sigK-lacZ* expression reported previously (13). The *sigK* portion of the fusion extended only to +4, so it did not contain the entire GerE-binding site.

GerE Inhibits sigK Expression in Vivo—To determine whether GerE affects *sigK* expression *in vivo*, we constructed a new *sigK-lacZ* transcriptional fusion that includes the GerE-binding site. DNA between −115 and +28 relative to the *sigK* TSS was fused to *lacZ*, and the fusion was recombined into phage SPβ. The resulting SPβ::*sigK-lacZ* phage was transduced into wild-type and *gerE* mutant *B. subtilis*, creating lysogens. Fig. 2 shows the average β-galactosidase activity during sporulation of three isolates of each type. The average maximum activity was 2-fold higher in *gerE* mutant cells than in wild-type cells, demonstrating that GerE inhibits *sigK* expression *in vivo*.

We also compared the level of *sigK* gene products in developing wild-type and *gerE* mutant cells. The primary translation product of *sigK* is pro-σ^K, an inactive precursor that is proteolytically processed to active σ^K (22). We used anti-pro-σ^K antibodies to detect both pro-σ^K and σ^K in extracts of cells subjected to Western blot analysis. Fig. 3 shows that the levels of pro-σ^K and σ^K are higher in *gerE* mutant cells than in wild-type cells late in sporulation. Quantitation of the combined pro-σ^K plus σ^K signal for the experiment shown in Fig. 3 and three additional experiments showed that the maximum level of SigK gene products in wild-type cells during sporulation, on average, reached 57% (±6%, 1 S.D.) of the level in *gerE* mutant cells. In all four experiments, the level of both pro-σ^K and σ^K was elevated in T_7 and T_8 samples from the *gerE* mutant compared with wild type. These results show that GerE normally inhibits the accumulation of pro-σ^K and σ^K during the late stages of sporulation. It is likely that GerE represses transcription of the *sigK* gene, reducing the synthesis of pro-σ^K and σ^K. This would explain the similar 2-fold decrease of *sigK*-directed β-galactosidase activity (Fig. 2) and pro-σ^K plus σ^K (Fig. 3) in wild-type cells compared with *gerE* mutant cells. The alternative explanation that GerE in wild-type cells causes increased turnover of pro-σ^K and σ^K and a similar increase in turnover of β-galactosidase is unlikely because β-galactosidase activity from a *sigK-lacZ* fusion lacking the GerE-binding site identified in Fig. 1 is similar during sporulation of wild-type and *gerE* mutant cells (13), as is β-galactosidase activity from *lacZ* fusions to many other genes.

GerE Inhibits cotD Transcription in Vitro—In addition to possible inhibitory effects of GerE on transcription of genes in the σ^K regulon (due to the inhibition of σ^K accumulation by GerE), GerE stimulates expression of certain genes in the σ^K regulon (3, 4, 7). Expression of a *cotD-lacZ* transcriptional fusion was 7-fold higher in developing wild-type cells than in *gerE* mutant cells (7). It was shown previously that purified GerE stimulates *cotD* transcription by σ^K RNAP 2–3-fold (4). We discovered that at a higher concentration GerE inhibits *cotD* transcription *in vitro*. Fig. 4 shows the result of an *in vitro* transcription experiment with a mixture of DNA templates containing the *cotD* or *cotC* promoter. The *cotC* promoter was included as an internal control because GerE has been shown to bind to a site centered at −68.5 relative to the TSS and to activate transcription by σ^K RNA polymerase (4). Lower levels of GerE stimulated *cotD* transcription about 2-fold (based on quantitation of signals in the experiment shown and one additional experiment), as reported previously (4), but higher concentrations of GerE inhibited *cotD* transcription. The inhibition was specific to *cotD*, since *cotC* transcription was activated at the highest concentration of GerE tested.

Location of the GerE-binding Site in the cotD Promoter Region—To understand how GerE both positively and negatively affects *cotD* transcription, we tested whether GerE binds in the promoter region by performing DNase I footprinting experiments. Fig. 5 shows that GerE protected a stretch of DNA from DNase I digestion that included the −35 region of the *cotD* promoter and extended upstream. On the nontranscribed strand, the protection spanned positions −39 to −25 relative to the TSS (Fig. 5*A*), whereas on the transcribed strand positions −60 to −23 were protected. We could not be certain whether protection extends farther upstream on the nontranscribed strand due to the absence of DNase I cleavage in this region even in the absence of GerE. GerE appears to bind to the *cotD* promoter region with lower affinity than to the *sigK* promoter region, since a higher concentration of GerE was required to observe protection from DNase I digestion (compare Figs. 1 and 5). Fig. 5*C* shows the sequence of the *cotD* promoter in the region protected by GerE. Typically, GerE protects a stretch of

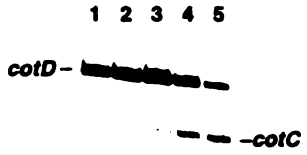

FIG. 4. **Effect of GerE on *cotD* transcription *in vitro*.** DNA templates (0.2 pmol each template) were transcribed with partially purified σ^K RNAP (0.2 μg) alone (*lane 1*) or with 50 (*lane 2*), 100 (*lane 3*), 200 (*lane 4*), or 400 pmol (*lane 5*) of gel-purified GerE added immediately after the σ^K RNAP. DNA templates were pLRK100 (15) linearized with *Hin*dIII (225-base *cotD* transcript) and a *Hae*III-*Eco*RI restriction fragment from pHI1(4) (196-base *cotC* transcript). The positions of run-off transcripts of the expected sizes, as judged from the migration of end-labeled DNA fragments of *Msp*I-digested pBR322, are indicated.

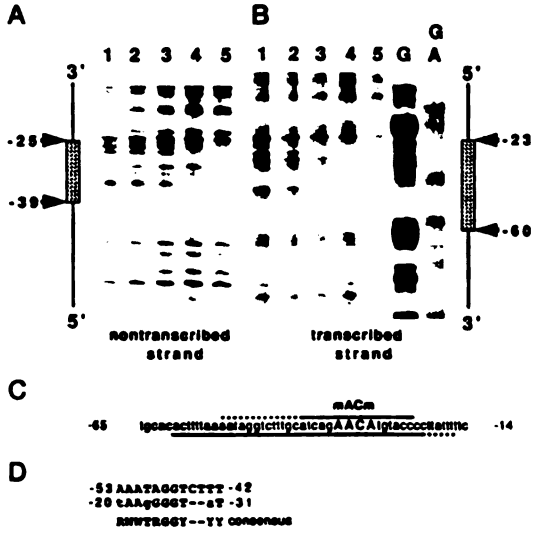

FIG. 5. **GerE footprints in the *cotD* promoter region.** Radioactive DNA fragments separately end-labeled on the nontranscribed (*A*) or transcribed (*B*) strand were incubated in separate reactions with a carrier protein (bovine serum albumin, 310 pmol) only (*lane 1*) or with 12 (*lane 2*), 25 (*lane 3*), 50 (*lane 4*), or 100 pmol (*lane 5*) of gel-purified GerE in addition to the carrier protein and then subjected to DNase I footprinting in a total volume of 45 μl. See Fig. 1 legend for explanation of *boxes*, *arrowheads*, and *numbers*. *C*, position of the GerE-binding site in the *cotD* promoter region. The nucleotide sequence of the nontranscribed strand of the *cotD* promoter region (7) is aligned with respect to conserved nucleotides found in the −35 regions of promoters transcribed by σ^K RNAP (34), shown *above* the sequence. Nucleotides in the *cotD* −35 region that match the consensus are shown as *boldface capital letters*. *Overlining* and *underlining* indicate regions on the nontranscribed and transcribed strands, respectively, protected by GerE from DNase I digestion. The *dashed lines* indicate regions of uncertain protection due to a lack of DNase I digestion in these regions. *Numbers* refer to positions relative to the TSS. M indicates A or C. *D*, nucleotide sequences within the GerE-protected region of the *cotD* promoter are aligned with the consensus sequence for GerE binding (3). Matches to the consensus sequence are shown as *capital letters*, and *numbers* refer to positions relative to the TSS. Note that the sequence shown for the binding site between −20 and −31 is from the strand opposite that shown in *C*.

about 20 bp from DNase I digestion (3, 4). The long region of protection (nearly 40 bp) observed on the transcribed strand in the *cotD* promoter region suggests that GerE binds to two sites. Within this region is a perfect match (positions −53 to −42) to the consensus sequence for GerE binding (Fig. 5*D*). A second match (7 out of 10) to the consensus sequence is present at positions −20 to −31, in inverted orientation with respect to

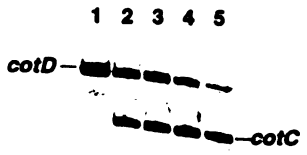

FIG. 6. **Effect of GerE on transcription *in vitro* of a *cotD* template lacking the upstream GerE-binding site.** The amounts of DNA templates, σ^K RNAP, and GerE were the same as in the Fig. 4 legend. The *cotD* template was a 275-bp PCR fragment containing 44 bp upstream of the TSS (228-base transcript) prepared as described under "Experimental Procedures." The *cotC* template was described in the Fig. 4 legend. The positions of run-off transcripts of the expected sizes, as judged from the migration of end-labeled DNA fragments of *Msp*I-digested pBR322, are indicated.

the first match (Fig. 5*D*), which probably accounts for GerE binding in this region.

The Upstream GerE-binding Site in the cotD Promoter Is Necessary for Activation of Transcription in Vitro but Not for Repression—We hypothesized that GerE bound at the upstream site in the *cotD* promoter (*i.e.* recognizing the perfect match to the consensus centered at −47.5) activates transcription, because GerE binds at a similar position in the *cotB* (4) and *cotVWX* (3) promoters and activates transcription. GerE bound at the *cotD* promoter downstream site (centered at −25.5) might repress transcription, explaining the inhibition of *cotD* transcription we observed (Fig. 4). To test these ideas, we repeated the *in vitro* transcription experiments with a *cotD* DNA template lacking the upstream GerE-binding site. As shown in Fig. 6, GerE failed to activate transcription of this template. However, GerE still repressed transcription of this template, under conditions that permitted GerE to activate *cotC* transcription. We conclude that GerE binding to the upstream site in the *cotD* promoter is required to activate transcription. GerE binding to the downstream site probably represses transcription, although we cannot rule out the possibility that GerE binding farther downstream is also necessary and was not detected by DNase I footprinting.

DISCUSSION

Our results strongly support the model that GerE is a repressor of *sigK* transcription which lowers the level of pro-σ^K and σ^K in cells during the late stages of sporulation. The previous finding that GerE inhibits *sigK* transcription *in vitro* (4) can be explained by GerE binding near the TSS (Fig. 1) and acting as a repressor. The previous observation that expression of a *sigK-lacZ* fusion lacking the GerE-binding site in the promoter region is unaffected by a *gerE* mutation (13), together with our finding that expression of a *sigK-lacZ* fusion containing the GerE-binding site is 2-fold lower in wild-type cells than in *gerE* mutant cells (Fig. 2), demonstrates the importance of the GerE-binding site for *sigK* regulation *in vivo*. The lowering of *sigK* expression by GerE in wild-type cells appears to result in a comparable decrease in the level of SigK gene products (Fig. 3). The simplest interpretation of these results is that GerE represses *sigK* transcription, limiting the synthesis of pro-σ^K and σ^K during sporulation.

The negative effect of GerE on the σ^K level during sporulation would lower expression of genes for which σ^K is the limiting factor for transcription. Four genes in the σ^K regulon show a lower level of expression in wild-type cells than in *gerE* mutant cells. These are *cotA* (25), *spoVF* (26), *csk22* (27), and *cotM* (28). In the case of *cotA*, there is evidence of a direct effect of GerE on transcription. Purified GerE inhibits *cotA* transcription *in vitro* (4). It is unknown how much of the increased *cotA* expression observed in a *gerE* mutant (25) results from loss of

direct inhibition by GerE and how much results from an elevated level of σ^K. Also unknown is whether GerE represses *spoVF*, *csk22*, or *cotM* directly, or whether these genes are overexpressed in a *gerE* mutant because GerE fails to repress *sigK*. It should be possible to answer some of these questions and, more generally, to assess the importance of GerE repression of *sigK* during sporulation and germination, by deleting the GerE-binding site in the *sigK* promoter.

The *cotD* gene, like many other genes in the σ^K regulon, is positively regulated by GerE. For several genes, GerE has been shown to bind upstream of the promoter −35 region and stimulate transcription by σ^K RNAP *in vitro* (3, 4). GerE can increase *cotD* transcription *in vitro* as shown previously (4), but we discovered that a higher concentration of GerE causes repression (Fig. 4). DNase I footprinting revealed that GerE protects not only DNA upstream of the −35 region in the *cotD* promoter, but the protection extends downstream through the −35 region (Fig. 5). Binding that extends through the −35 region has not been observed in other promoters activated by GerE (3, 4). We reasoned that GerE binding in the *cotD* −35 region might repress transcription, and we showed that truncation of *cotD* promoter DNA at −44 prevented activation, but allowed repression, by GerE (Fig. 6). These *in vitro* results suggest the model that a rising level of GerE in sporulating cells first activates *cotD* transcription by binding upstream of the −35 region and then represses transcription by binding to a second site just downstream. It should be straightforward to test the requirement for the upstream GerE-binding site for activation *in vivo*, by making the appropriate 5′ deletion (*e.g.* to −44) and measuring expression of a fusion to a reporter gene. However, testing the role of the downstream GerE-binding site in repression *in vivo* will be more difficult. As noted above, one must be careful to distinguish between direct repressive effects of GerE and indirect effects due to GerE repression of *sigK*.

Fig. 7 illustrates the regulatory interactions between the four mother cell-specific transcription factors (*circled*) and our model for regulation of *cotD* transcription at different times during sporulation. Early in sporulation, σ^E and SpoIIID are active in the mother cell (Fig. 7*A*). At this time, *sigK* is transcribed by σ^E RNAP, with SpoIIID serving as an essential activator (6, 13). As we have shown for GerE (Figs. 5 and 6), it was shown previously that SpoIIID binds in the −35 region of the *cotD* promoter and represses transcription *in vitro* (6). Fig. 7*B* shows the regulatory interactions in the mother cell slightly later, at about 4 h into the sporulation process, when the primary product of the *sigK* gene, pro-σ^K, is processed to active σ^K in response to a signal from the forespore (22, 29). σ^K RNAP initiates a negative feedback loop that inhibits transcription of the *sigE* gene encoding σ^E, which in turn lowers expression of *spoIIID* (8, 12). As the levels of σ^E RNAP and SpoIIID fall, *cotD* transcription would no longer be repressed by SpoIIID. At the same time, σ^K RNAP transcribes *gerE* (4, 30), and, according to our model, the GerE produced initially activates *cotD* transcription and represses *sigK* transcription. For a period, all four mother cell-specific transcription factors probably affect transcription of *sigK*, because SpoIIID activates both σ^E RNAP and σ^K RNAP to transcribe *sigK* (6, 13), and GerE represses *sigK* transcription (4) (Figs. 1–3). The positive autoregulatory loop created by σ^K RNAP transcription of *sigK* is kept in check by two negative feedback loops (Fig. 7*B*). One inhibits transcription of *sigE* and therefore inhibits production of σ^E and SpoIIID (8). The other leads to synthesis of GerE, which represses *sigK* transcription directly (4) (Figs. 1–3). As the level of GerE rises later in sporulation, GerE may also repress *cotD* (Fig. 7*C*). Presumably, the complex regulatory interactions depicted in Fig. 7 ensure that the four transcription factors, as well as

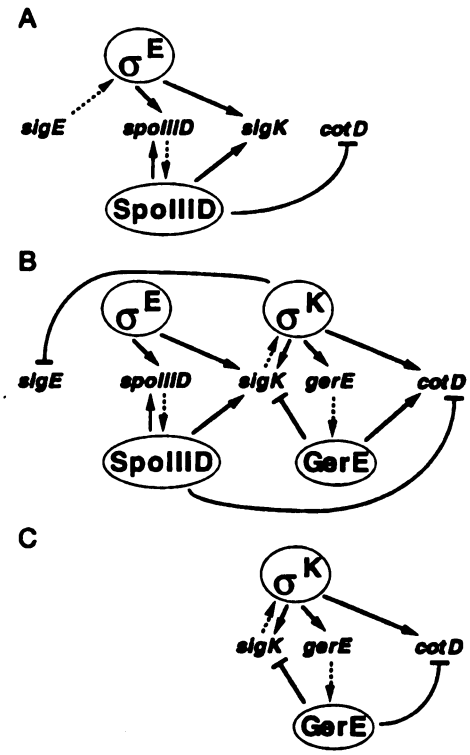

FIG. 7. **Regulatory interactions between mother cell-specific transcription factors and a model for regulation of *cotD* transcription at different times during sporulation.** *Dashed arrows* show gene (*italicized*) to product (proteins are *circled*) relationships. *Solid arrows* represent positive regulation of transcription. *Lines* with a *barred end* represent negative regulation of transcription. *A*, *B*, and *C* represent early, intermediate, and late stages of sporulation, respectively, as explained in the text.

structural proteins like CotD under their control, are made at optimal levels for formation of the spore cortex and coat.

Our mapping of GerE-binding sites in the *sigK* and *cotD* promoters and our *in vitro* transcription experiments with *cotD* promoter fragments differing at the 5′ end provide the first insight into how GerE acts as a repressor. In both the *sigK* and *cotD* promoters, GerE binds within the region likely to be bound by σ^K RNAP, although the footprints of σ^K RNAP on these promoters have not yet been determined. The positions of GerE binding in these promoters suggest that GerE may repress transcription by interfering with the initial binding of σ^K RNAP or a subsequent step in transcription initiation. It may be possible to distinguish between these possibilities by measuring the ability of σ^K RNAP to bind to these promoters in the presence or absence of GerE. In the case of *cotD*, it is unlikely that GerE inhibits elongation of transcripts or inhibits transcription by any other mechanism that does not depend on sequence-specific DNA binding, because GerE stimulated *cotC* transcription in the same reaction mixtures in which it inhibited *cotD* transcription (Figs. 4 and 6). These same *in vitro* transcription experiments, with *cotD* promoter fragments differing at the 5′ ends, suggest that at a low GerE concentration, binding to a site upstream of the −35 region activates transcription, and at a higher GerE concentration, binding to a second site just downstream represses transcription. In the DNase I footprint experiment shown in Fig. 5, there is no

indication that GerE binds with higher affinity to the upstream site (a perfect match to the GerE binding consensus sequence) than the downstream site (a 7 out of 10 match to the consensus). Although we cannot rule out a slight difference in affinities, another possible explanation is that σ^K RNAP competes with GerE for binding to the downstream site but not the upstream site.

GerE appeared to bind with higher affinity to the *sigK* promoter region than to the *cotD* promoter region (compare Figs. 1 and 5) or many other GerE-binding sites mapped previously (3, 4). Within the region of the *sigK* promoter protected by GerE from DNase I digestion are two sequences in inverted orientation that overlap by 4 bases and match the consensus sequence for GerE binding perfectly or in 7 out of 10 positions. An identical arrangement of sequences matching the GerE consensus was observed previously just upstream of the −35 region in the *cotYZ* promoter, where GerE appeared to bind with high affinity and activated transcription (3). It is unknown whether more than one molecule of GerE at a time binds the *sigK* and *cotYZ* sites. Also unknown is whether GerE is monomeric in solution. GerE is predicted to possess a helix-turn-helix DNA-binding motif similar to that in several proteins whose three-dimensional structure has been determined (31, 32). Some of these well characterized proteins are dimeric and recognize inverted repeats in the DNA-binding sites (33). The consensus GerE-binding sequence is not palindromic, so perhaps GerE can bind as a monomer. This does not exclude the possibility of interaction between monomers at sites that exhibit palindromic character, such as the *sigK* and *cotYZ* sites.

GerE appears to be similar to SpoIIID in terms of its DNA sequence recognition characteristics. The SpoIIID-binding site consensus sequence (WWRRACAR-Y) is of similar length and degeneracy as that recognized by GerE (RWWTRGGY-YY) and also is not palindromic (5, 6). Although SpoIIID appears to be monomeric in solution,[2] many strong SpoIIID-binding sites exhibit a second good match to the consensus in inverted orientation relative to the best match (6). Mutational analysis will be required to assess the contribution of each consensus match to binding of GerE and SpoIIID at sites with palindromic character and to determine whether two monomers interact (*e.g.* bind cooperatively) at these sites.

In summary, we have investigated the biochemical basis for negative regulation by the GerE protein. It appears to act like a classical repressor, binding in promoter regions at sites that overlap the position of RNAP binding. GerE repression at the *sigK* promoter lowers σ^K production during sporulation about 2-fold, potentially regulating the expression of many genes in the σ^K regulon. In addition, GerE binds in the promoter regions of certain genes in the σ^K regulon and directly represses or activates transcription by σ^K RNAP. In the case of *cotD*, it is likely that GerE binds to a site upstream of the promoter −35 region and first activates transcription, then, as its level rises in sporulating cells, GerE binds to a site slightly farther downstream and represses transcription.

[2] B. Zhang and L. Kroos, unpublished work.

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CHAPTER V

Combined Action of Two Transcription Factors Regulates Genes Encoding Spore Coat Proteins of *Bacillus subtilis*

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Combined Action of Two Transcription Factors Regulates Genes Encoding Spore Coat Proteins of *Bacillus subtilis**

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During sporulation of *Bacillus subtilis*, spore coat proteins encoded by *cot* genes are expressed in the mother cell and deposited on the forespore. Transcription of the *cotB*, *cotC*, and *cotX* genes by σ^K RNA polymerase is activated by a small, DNA-binding protein called GerE. The promoter region of each of these genes has two GerE binding sites. 5′ deletions that eliminated the more upstream GerE site decreased expression of *lacZ* fused to *cotB* and *cotX* by approximately 80% and 60%, respectively but had no effect on *cotC-lacZ* expression. The *cotC-lacZ* fusion was expressed later during sporulation than the other two fusions. Primer extension analysis confirmed that *cotB* mRNA increases first during sporulation, followed by *cotX* and *cotC* mRNAs over a 2-h period. *In vitro* transcription experiments suggest that the differential pattern of *cot* gene expression results from the combined action of GerE and another transcription factor, SpoIIID. A low concentration of GerE activated *cotB* transcription by σ^K RNA polymerase, whereas a higher concentration was needed to activate transcription of *cotX* or *cotC*. SpoIIID at low concentration repressed *cotC* transcription, whereas a higher concentration only partially repressed *cotX* transcription and had little effect on *cotB* transcription. DNase I footprinting showed that SpoIIID binds strongly to two sites in the *cotC* promoter region, binds weakly to one site in the *cotX* promoter, and does not bind specifically to *cotB*. We propose that late in sporulation the rising level of GerE and the falling level of SpoIIID, together with the position and affinity of binding sites for these transcription factors in *cot* gene promoters, dictates the timing and level of spore coat protein synthesis, ensuring optimal assembly of the protein shell on the forespore surface.

Upon starvation, the Gram-positive bacterium *Bacillus subtilis* initiates a sporulation process involving a series of morphological changes (1). The rod-shaped cell undergoes asymmetrical division into two compartments, a larger mother cell and a smaller forespore. Different sets of genes are expressed from the genome in each compartment. As sporulation proceeds, the forespore is engulfed by the mother cell, forming a free protoplast surrounded by a double membrane inside the mother cell. Cell wall-like material called cortex is then deposited between the forespore membranes. Transcription of *cot* genes, which encode spore coat proteins, occurs in the mother cell. The coat proteins assemble on the surface of the forespore, forming a tough shell that protects the spore from environmental insults after it is released by lysis of the mother cell. When nutrients become available again, the spore germinates, producing a cell that resumes growth and division.

The sporulation process of *B. subtilis* has been studied as a model to understand the relationship between developmental morphogenesis and gene regulation (2). A central feature of sporulation gene regulation is the synthesis and activation of four compartment-specific σ subunits of RNA polymerase (RNAP).[1] σ^F and σ^G direct RNAP to transcribe genes in the forespore. σ^E and σ^K direct transcription in the mother cell. The four σ factors form a regulatory cascade in which the activation of each σ depends upon the activity of the prior σ in the order σ^F, σ^E, σ^G, and finally σ^K (3). Activation of each of the latter three σ factors appears to be coupled to a morphological step in development and involves signaling between the two compartments.

In the mother cell, two accessory transcription factors, SpoIIID and GerE, modulate RNAP activity at specific promoters (2). GerE is an 8.5-kDa protein that binds to DNA sequences resembling RWWTRGGY–YY (R is purine, W is A or T, and Y is pyrimidine) and activates transcription of many *cot* genes by σ^K (4, 5). GerE can also act as a repressor (6). Likewise, SpoIIID is a 10.8-kDa protein that binds to DNA sequences resembling WWRRACAR-Y and activates or represses transcription of many different genes (7, 8).

We have investigated transcriptional regulation of the *cotB*, *cotC*, and *cotX* genes. These genes were known to be transcribed by σ^K RNAP, with activation by GerE (4, 5). Two GerE binding sites had been mapped in the promoter region of each gene (Fig. 1) (4, 5). Here we report that 5′ deletions that eliminated the more upstream GerE site reduced expression of *cotB* and *cotX* but not *cotC*. Interestingly, we found that the three genes are differentially expressed during development, suggesting an additional level of regulation. SpoIIID appears to provide the additional control, based on the results of *in vitro* transcription and DNase I footprinting experiments presented here and based on how the SpoIIID level has been shown to change during sporulation (9, 10). This is the first study to correlate differential transcription of *cot* genes with the combined action of GerE and SpoIIID. The discovery of such complex regulation of *cot* gene expression leads us to speculate that synthesis of coat proteins is finely tuned to ensure optimal assembly of the spore coat.

MATERIALS AND METHODS

Construction of cot-lacZ Fusion Strains—DNA fragments containing the *cotB*, *cotC*, or *cotX* promoter region flanked by *Eco*RI and *Hin*dIII restriction sites at the upstream and downstream ends, respectively,

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[1] The abbreviations used are: RNAP, RNA polymerase; PCR, polymerase chain reaction; R, purine; W, A, or T; Y, pyrimidine.

were synthesized by the polymerase chain reaction (PCR) and directionally subcloned into *Eco*RI-*Hin*dIII-digested pTKlac (11). The templates of the PCR were pBD136 (4), pHI1 (4), and pJZ22 (5), respectively, for *cotB*, *cotC*, and *cotX*. Plasmids containing the *cotB* promoter region from −85 to +37 (pHI3) or from −60 to +37 (pHI4) were constructed using the upstream primer 5′-GGGAATTCGCGTGAAAATGGGTAT-3′ or 5′-GGGAATTCAAGCGACAATTAGGCT-3′ for pHI3 and pHI4, respectively, and the downstream primer 5′-GCGAAGCTTAATTCCTCCTAGTCA-3′ (the restriction site in the primer is underlined). Plasmids containing the *cotC* promoter region from −153 to +13 (pHI6) or from −79 to +13 (pHI7) were constructed using the upstream primer 5′-CGGAATTCTGTAGGATAAATCGTT-3′ or 5′-CGGAATTCTCTATCATTTGGACAG-3′ for pHI6 and pHI7, respectively, and the downstream primer 5′-CGGAAGCTTTTATTTTTACTACG 3′. Plasmids containing the *cotX* promoter region from −69 to +10 (pHI8) or from −54 to +10 (pHI9) were constructed using the upstream primer 5′-CGGAATTCAAAAAATAGGGTTCTT-3′ or 5′-CGGAATTCTCATCAGGATATATGA-3′ for pHI8 and pHI9, respectively, and the downstream primer 5′-CGGAAGCTTTTCTTTTACTGTTAT-3′. These plasmids were linearized by digestion with *Bsa*I and transformed into *B. subtilis* ZB307, in which marker replacement-type recombination created an SPβ-specialized transducing phage containing the *lacZ* fusion as described previously (12). A phage lysate was prepared by heat induction and used to transduce *B. subtilis* SG38 (*spo*⁺ *trpC2*) and 522.2 (*gerE36 trp*C2) (13) with selection of lysogens resistant to chloramphenicol (5 μg/ml) on LB agar as described previously (14).

Measurement of β-Galactosidase Activity—Sporulation was induced by nutrient exhaustion in Difco sporulation medium at 37 °C as described previously (14). Samples (1 ml) were collected at hourly intervals during sporulation, cells were pelleted, and pellets were stored at −20 °C before the assay. The specific activity of β-galactosidase was determined by the method of Miller (15), using *o*-nitrophenol-β-D-galactopyranoside as the substrate. One unit of enzyme hydrolyzes 1 μmol of substrate/min/unit of initial cell absorbance at 595 nm.

Primer Extension Analysis—At hourly intervals from 3 to 7 h after the onset of sporulation, cells were harvested by centrifugation (11,950 × *g* for 10 min), and RNA was prepared as described previously (16) except the RNA was resuspended in 100 μl of water that had been treated with 0.1% (v/v) diethylpyrocarbonate. The RNA was treated with DNase I to remove contaminating chromosomal DNA. Primer extension reactions were performed as described previously (17, 18). The *cotB* and *cotC* primers were those designated as Pr2 previously (19). The *cotX* primer we used was also called Pr2 previously (5). After the reaction, the extension products were subjected to electrophoresis in a 5% polyacrylamide gel containing 8 M urea, and transcripts were detected by autoradiography. The signal intensities were quantified using a Storm 820 PhosphorImager (Molecular Dynamics).

In Vitro Transcription—σ^K RNAP was partially purified from *gerE* mutant cells as described previously (20). The enzyme was comparable in protein composition and in *cotD*- and *sigK*-transcribing activities with fraction 24 shown in Fig. 2 of Kroos *et al.* (20). GerE was gel-purifed from *Escherichia coli* engineered to overproduce the protein as described previously (4). SpoIIID was gel-purified from fractions of partially purified σ^K RNAP as described previously (20). Transcription reactions (45 μl) were performed as described previously (21), except that RNAP was allowed to bind to the DNA template for 10 min at 37 °C before the addition of nucleotides (the labeled nucleotide was [α-^{32}P]CTP). Heparin (6 μg) was added 2 min after the addition of nucleotides to prevent reinitiation. After the reactions were stopped by adding 40 μl of stop buffer (100 mM Tris·HCl, pH 8.0, 50 mM EDTA, 200 μg of yeast tRNA/ml), each reaction mixture was incubated with 250 μl of ethanol at −70 °C for 1 h to allow precipitation of transcripts. Precipitates were pelleted at 12,000 × *g* for 15 min and resuspended in 10 μl of loading dye (80% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol FF and 1 mg/ml bromphenol blue). The resuspension was placed at 100 °C for 3 min, then subjected to electrophoresis in a 5% polyacrylamide gel containing 8 M urea, and transcripts were detected by autoradiography. The signal intensities were quantified using a Storm 820 PhosphorImager (Molecular Dynamics). To prepare a DNA template containing the *cotX* promoter, DNA between −97 and +82 was amplified by the PCR using pJZ22 (5) as the template, upstream primer 5′-CGGAAAAACGATAACAATTAG-3′ and downstream primer 5′-CATCTAACGGATGGTCACAGTCAG-3′.

DNase I Footprinting—DNA fragments labeled at only one end were prepared as follows. For analysis of the *cotC* promoter region, DNA between −143 and +30 was amplified by PCR using pHI1 (4) as the template: upstream primer, 5′-ATCGTTTGGGCCGATGAAAATC-3′; downstream primer, 5′-CCCATATATACTCCTCCTTTATT-3′. To generate a probe for each DNA strand, two separate reactions were performed containing one or the other of the PCR primers labeled at the 5′ end by treatment with T4 polynucleotide kinase and [γ-^{32}P]ATP and purified by passage through a MicroSpin G-25 Column (Amersham Pharmacia Biotech). DNA probes for analysis of the *cotX* promoter region were prepared as described previously (5). Labeled DNA fragments were incubated with different amounts of gel-purified SpoIIID and then mildly digested with DNase I according to method 2 of Zheng *et al.* (4), except 0.4 units of DNase I was used, and a 7-fold (w/w) excess of poly(dA-dT) or poly(dI-dC) as compared with *cotC* or *cotX* probe, respectively, was added as competitor. After DNase I treatment, the partially digested DNAs were electrophoresed in a 7% polyacrylamide gel containing 8 M urea alongside a sequencing ladder generated with T7 Sequenase V 2.0 (Amersham Pharmacia Biotech) and the appropriate primer for *cotC* or by chemical cleavage of the appropriate end-labeled DNA for *cotX*.

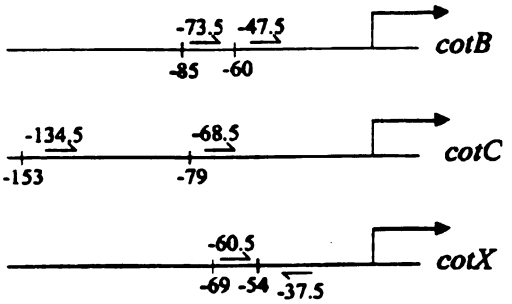

FIG. 1. **GerE binding sites in the *cotB*, *cotC*, and *cotX* promoter regions.** *Large arrows* indicate the position of the transcriptional start site. *Small arrows* indicate the orientation of sequences that match the consensus sequence for GerE binding, and *numbers* above or below these *arrows* indicate the position of the center of the sequence. *Numbers* below *vertical lines* indicate the end points of 5′ deletions used in this study.

RESULTS

Role of Upstream GerE Binding Sites in cot Gene Transcription—The *cotB*, *cotC*, and *cotX* promoter regions each have two GerE binding sites (Fig. 1) (4, 5). To test the importance of the more upstream GerE site in transcription of each gene, we fused promoter DNA fragments containing different amounts of upstream sequence to *lacZ*. These fusions were recombined into the lysogenic phage SPβ, and each phage was transduced into wild-type and *gerE* mutant *B. subtilis*, where the phage integrated into the chromosome at the attachment site. Transductants were induced to sporulate by nutrient exhaustion, and β-galactosidase activities were measured. Fig. 2 shows that deletion of the more upstream GerE site reduced *cotB-lacZ* and *cotX-lacZ* expression by approximately 80% and 60%, respectively. Deletion of the more upstream site centered at −134.5 had no effect on *cotC-lacZ* expression. All the fusions failed to be expressed in the *gerE* mutant (Fig. 2 and data not shown). These results demonstrate that the more upstream GerE site is not important for *cotC* transcription and suggest that the more upstream sites contribute greatly to GerE transcriptional activation of *cotB* and *cotX*.

cot Genes Exhibit Different Patterns of Expression—The data in Fig. 2 suggest that the *cotB*, *cotX*, and *cotC* promoters are regulated differently. Expression of *cotB-lacZ* rose sharply between 4 and 5 h into sporulation and reached a maximum at 6 h. *cotX-lacZ* expression also increased between hours 4 and 5 but continued to rise until hour 7. Expression of *cotC-lacZ* began later, between hours 5 and 6, and rose until hour 7.

To further examine the apparent difference in the pattern of *cot* gene expression, we measured the appearance of *cotB*, *cotX*, and *cotC* mRNAs during sporualtion of wild-type cells using primer extension analysis. Fig. 3*A* shows a representative re-

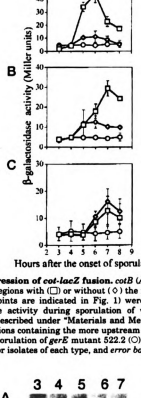

FIG. 2. **Expression of *cot-lacZ* fusion.** *cotB* (*A*), *cotX* (*B*), and *cotC* (*C*) promoter regions with (□) or without (◇) the more upstream GerE site (5′ end points are indicated in Fig. 1) were fused to *lacZ*, and β-galactosidase activity during sporulation of wild-type SG38 was measured as described under "Materials and Methods." Likewise, expression of fusions containing the more upstream GerE site was measured during sporulation of *gerE* mutant 522.2 (○). *Points* on the graph are averages for isolates of each type, and *error bars* show 1 S.D. of the data.

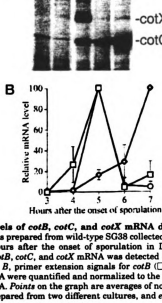

FIG. 3. **Levels of *cotB*, *cotC*, and *cotX* mRNA during sporulation.** RNA was prepared from wild-type SG38 collected at the indicated number of hours after the onset of sporulation in Difco sporulation medium. *A*, *cotB*, *cotC*, and *cotX* mRNA was detected by primer extension analysis. *B*, primer extension signals for *cotB* (□), *cotC* (◇), and *cotX* (○) mRNA were quantified and normalized to the maximum signal for each mRNA. *Points* on the graph are averages of normalized signals from RNA prepared from two different cultures, and *error bars* show 1 S.D. of the data.

sult from an experiment in which primers for all three mRNAs were mixed with RNA, and primer extension was done simultaneously. Similar results were obtained when primer extension was done separately for each gene (data not shown). *cotB* mRNA was detectable at 4 h into sporulation, and its level rose sharply at hour 5. Reproducibly, *cotB* mRNA was undetectable at hour 6, then reappeared at hour 7, indicating that synthesis and/or stability of this mRNA is regulated by an unknown mechanism late in development. *cotX* mRNA was barely detectable at hour 4, rose to its maximum level at hour 5, and fell to a barely detectable level at hour 6. *cotC* mRNA was present at a low level at hour 3. The enzyme responsible for this low level of *cotC* transcription is unknown, but it could be σ^E RNAP because σ^E and σ^K recognize similar sequences in cognate promoters (22, 23). The *cotC* mRNA level increased at hour 5 and continued to rise until hour 7. Fig. 3*B* shows quantification of the experiment shown in Fig. 3*A* plus one independent experiment. The average level at different times is plotted relative to the maximum level for each mRNA to illustrate the different patterns of mRNA accumulation. These results together with the *lacZ* expression data (Fig. 2) suggest that *cotB* transcription is induced slightly earlier than that of *cotX*, whereas full induction of *cotC* transcription lags behind that of *cotX*.

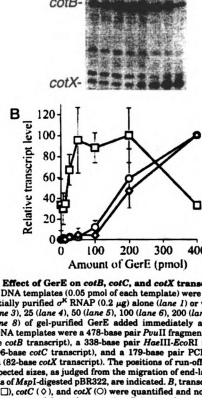

FIG. 4. **Effect of GerE on *cotB*, *cotC*, and *cotX* transcription *in vitro*.** *A*, DNA templates (0.05 pmol of each template) were transcribed with partially purified σ^K RNAP (0.2 μg) alone (*lane 1*) or with 6 (*lane 2*), 13 (*lane 3*), 25 (*lane 4*), 50 (*lane 5*), 100 (*lane 6*), 200 (*lane 7*), or 400 pmol (*lane 8*) of gel-purified GerE added immediately after the σ^K RNAP. DNA templates were a 478-base pair *Pvu*II fragment of pBD136 (170-base *cotB* transcript), a 338-base pair *Hae*III-*Eco*RI fragment of pHI1 (196-base *cotC* transcript), and a 179-base pair PCR-generated fragment (82-base *cotX* transcript). The positions of run-off transcripts of the expected sizes, as judged from the migration of end-labeled DNA fragments of *Msp*I-digested pBR322, are indicated. *B*, transcript signals for *cotB* (□), *cotC* (◇), and *cotX* (○) were quantified and normalized to the maximum signal for each transcript. *Points* on the graph are averages of normalized signals from two experiments, and *error bars* show 1 S.D. of the data.

A Lower Concentration of GerE Activates cotB Transcription than cotX or cotC Transcription—To investigate how different patterns of *cot* gene expression might be established, we performed *in vitro* transcription with σ^K RNAP and different amounts of GerE. Fig. 4*A* shows a representative experiment in which an equimolar mixture of *cotB*, *cotX*, and *cotC* DNA templates was transcribed by σ^K RNAP (partially purified from

gerE mutant cells) in the presence of increasing GerE. In this *in vitro* system, all three genes were transcribed in the absence of GerE, whereas expression of *lacZ* fused to these genes was not observed in *gerE* mutant cells (Fig. 2). The addition of GerE activated transcription of all three genes *in vitro*, as expected (4, 5), but interestingly, a lower concentration of GerE was sufficient to activate *cotB* transcription, whereas a higher concentration was needed to activate *cotX* or *cotC* transcription (Fig. 4*A*). The experiment was repeated, and transcript signals from both experiments were quantified and normalized to the maximum signal obtained for each template. Fig. 4*B* shows that, on average, *cotB* transcription was activated about 3-fold and 0.5 μM GerE was required for half-maximal activation. The activation profiles for *cotX* and *cotC* were very similar. Both genes were activated more than 10-fold, and half-maximal activation required 4 μM GerE. These results suggest that earlier expression of *cotB* during sporulation (Figs. 2 and 3) may result from a lower threshold for activation by GerE, since the level of GerE is believed to increase as σ^K RNAP becomes active (24). The results do not explain the apparent differential expression of *cotX* and *cotC* (Figs. 2 and 3), suggesting there might be an additional level of control.

SpoIIID Is a Potent Repressor of cotC Transcription—We discovered that extracts of sporulating wild-type cells contain a protein that binds to the *cotC* promoter region (data not shown). The kinetics of appearance of this binding activity and its absence from extracts of *spoIIID* mutant cells suggested that the protein is SpoIIID. The SpoIIID protein had been shown previously to inhibit transcription of the *cotD* gene *in vitro* and to bind in the −35 region of the *cotD* promoter (7, 20). We hypothesized that SpoIIID might contribute to the differential regulation of *cot* gene expression we had observed (Figs. 2 and 3). This hypothesis is difficult to test *in vivo*, because SpoIIID is required for production of σ^K RNAP (7, 25), which transcribes the *cot* genes (4, 5). To test whether SpoIIID affects *cot* gene transcription *in vitro*, we modified the experiment shown in Fig. 4. Different amounts of SpoIIID were incubated with a mixture of DNA templates before the addition of σ^K RNAP and a fixed amount of GerE. In this set of experiments, equimolar *gerE* template was included as a control because we knew that SpoIIID has very little effect on its transcription (26). Fig. 5*A* shows a representative result, and Fig. 5*B* summarizes quantification of two experiments. SpoIIID repressed *cotC* transcription about 10-fold, with 50% repression occurring at 0.2 μM. *cotX* transcription was repressed about 2-fold at the highest SpoIIID concentration tested (approximately 1 μM). SpoIIID had very little effect on transcription of *cotB* or *gerE*. These results provide a plausible explanation for the lag between *cotX* and *cotC* expression (Figs. 2 and 3). The level of SpoIIID decreases as active σ^K RNAP accumulates in the mother cell (9, 10). Our *in vitro* transcription results suggest that *cotX* would be released from SpoIIID repression first, followed by *cotC* when the SpoIIID concentration reaches a much lower level.

SpoIIID Binds to Specific Sites in the cotC and cotX Promoter Regions—The inhibitory effect of SpoIIID on *cotC* and *cotX* transcription suggested that SpoIIID might bind to specific DNA sequences in the promoter regions of these genes. To examine specific binding by SpoIIID, we performed DNase I footprinting experiments. SpoIIID protected two regions of *cotC* promoter DNA from digestion with DNase I. The protection spanned positions −43 to −29 and positions −77 to −56 on the transcribed strand (Fig. 6*A*). On the nontranscribed strand, protection spanned positions −40 to −22 and positions −75 to −63 (Fig. 6*B*). Protection was observed at the lowest concentration of SpoIIID tested, indicating that SpoIIID binds with relatively high affinity to these sites as compared with other SpoIIID binding sites mapped previously (7, 8). Fig. 6*C* shows the sequence of the *cotC* promoter in the two regions protected by SpoIIID. Within each protected region is a sequence that matches the consensus sequence for SpoIIID binding (Fig. 6*D*). These results may explain why SpoIIID is a potent repressor of *cotC* transcription (Fig. 5). The upstream SpoIIID binding site centered at position −67.5 (Fig. 6*C*) overlaps the critical GerE site centered at position −68.5 (Fig. 1). The downstream SpoIIID binding site centered at −36.5 overlaps the −35 region of the *cotC* promoter, which may be important for recognition by σ^K RNAP.

SpoIIID also bound specifically to a site in the *cotX* promoter. Protection from DNase I digestion spanned positions −27 to −11 on the transcribed strand (Fig. 7*A*) and at least positions −23 to −15 on the nontranscribed strand (Fig. 7*B*). Fig. 7*C* shows the sequence of the *cotX* promoter in the region protected by SpoIIID. Within this region is a sequence that matches the consensus sequence for SpoIIID binding in 7 of 9 positions (Fig. 7*D*). SpoIIID binds with relatively low affinity to this site in the *cotX* promoter (Fig. 7, *A* and *B*) as compared with the two sites in the *cotC* promoter (Fig. 6, *A* and *B*), which may explain why SpoIIID was a less potent repressor of *cotX* transcription than *cotC* transcription (Fig. 5).

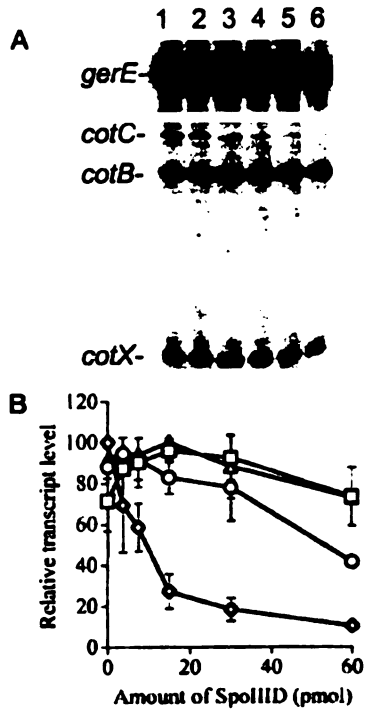

FIG. 5. **Effect of SpoIIID on *cotB*, *cotC*, and *cotX* transcription *in vitro*.** *A*, DNA templates (0.05 pmol each) were incubated without (*lane 1*) or with 4 (*lane 2*), 8 (*lane 3*), 15 (*lane 4*), 30 (*lane 5*), or 60 pmol (*lane 6*) of gel-purified SpoIIID and transcribed with partially purified σ^K RNAP (0.2 μg) and 200 pmol of gel-purified GerE. DNA templates were the same as in Fig. 4 plus a 480-base pair *Eco*RI-*Pvu*II of pSC146 (382-base *gerE* transcript). The positions of run-off transcripts of the expected sizes, as judged from the migration of end-labeled DNA fragments of *Msp*I-digested pBR322, are indicated. *B*, transcript signals for *cotB* (□), *cotC* (◇), *cotX* (○), and *gerE* (△) were quantified and normalized to the maximum signal for each transcript. *Points* on the graph are averages of normalized signals from two experiments, and *error bars* show 1 S.D. of the data.

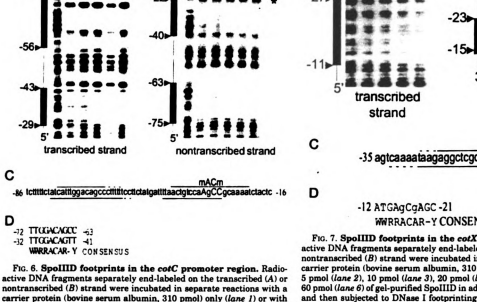

FIG. 6. **SpoIIID footprints in the *cotC* promoter region.** Radioactive DNA fragments separately end-labeled on the transcribed (*A*) or nontranscribed (*B*) strand were incubated in separate reactions with a carrier protein (bovine serum albumin, 310 pmol) only (*lane 1*) or with 4 (*lane 2*), 8 (*lane 3*), 15 (*lane 4*), 30 (*lane 5*), or 60 pmol (*lane 6*) of gel-purified SpoIIID in addition to the carrier protein and then subjected to DNase I footprinting in a total volume of 45 μl. *Filled boxes* indicate the region protected from DNase I digestion by SpoIIID. *Arrowheads* denote the boundaries of protection, and *numbers to the left* refer to positions relative to the transcriptional start site, as deduced from sequencing ladders generated with T7 Sequenase V 2.0 (Amersham Pharmacia Biotech) and the appropriate primer. *Asterisks* indicate the position of sites rendered hypersensitive to DNase I digestion by SpoIIID binding. *C*, positions of SpoIIID footprints in the *cotC* promoter region. The nucleotide sequence of the nontranscribed strand of the *cotC* promoter region is shown (4). Nucleotides in the −35 region that match the consensus for recognition by σ^K RNAP (m indicates A or C) are shown as *boldface capital letters*. *Overlining* and *underlining* indicate regions on the nontranscribed and transcribed strands, respectively, protected by SpoIIID from DNase I digestion. The *dashed lines* indicate regions of uncertain protection due to a lack of DNase I digestion in these regions. *Numbers* refer to positions relative to the transcriptional start site. *D*, nucleotide sequences within the SpoIIID-protected regions of the *cotC* promoter are aligned with the consensus sequence for SpoIIID binding. Matches to the consensus sequence are shown as *capital letters*, and *numbers* refer to positions relative to the transcriptional start site.

DISCUSSION

Our results strongly support the idea that the combined action of GerE and SpoIIID produces differential patterns of *cot* gene expression during *B. subtilis* sporulation. Previously, *cotB* and *cotC* had been thought to be coordinately regulated by the appearance of GerE (4, 19). However, expression of a *cotB-lacZ* fusion begins to increase at least 1 h earlier than expression of a *cotC-lacZ* fusion (Fig. 2), and *cotB* mRNA reaches its maximum level at least 2 h earlier than *cotC* mRNA (Fig. 3). The earlier expression of *cotB* during sporulation may result, in part, from a lower threshold for activation by GerE (Fig. 4), but in addition, SpoIIID was shown to be a potent repressor of *cotC* transcription (Fig. 5). The repressive effect of SpoIIID on *cotC* transcription *in vitro* appears to be due to the presence of two relatively high affinity SpoIIID binding sites in the *cotC* promoter region that overlap binding sites for GerE and σ^K RNAP (Fig. 6). Therefore, we propose that SpoIIID represses *cotC* transcription during sporulation, contributing to the observed lag between *cotB* and *cotC* expression.

FIG. 7. **SpoIIID footprints in the *cotX* promoter region.** Radioactive DNA fragments separately end-labeled on the transcribed (*A*) or nontranscribed (*B*) strand were incubated in separate reactions with a carrier protein (bovine serum albumin, 310 pmol) only (*lane 1*) or with 5 pmol (*lane 2*), 10 pmol (*lane 3*), 20 pmol (*lane 4*), 40 pmol (*lane 5*), or 60 pmol (*lane 6*) of gel-purified SpoIIID in addition to the carrier protein and then subjected to DNase I footprinting in a total volume of 45 μl. See the Fig. 6 legend for explanation of *filled boxes*, *arrowheads*, *numbers to the left*, and *asterisks*. *C*, position of the SpoIIID footprint in the *cotX* promoter region. The nucleotide sequence of the nontranscribed strand of the *cotX* promoter region is shown (39). *Overlining* and *underlining* indicate regions on the nontranscribed and transcribed strands, respectively, protected by SpoIIID from DNase I digestion. The *dashed lines* indicate regions of uncertain protection due to a lack of DNase I digestion in these regions. *Numbers* refer to positions relative to the transcriptional start site. *D*, nucleotide sequences within the SpoIIID-protected region of the *cotX* promoter are aligned with the consensus sequence for SpoIIID binding. Matches to the consensus sequence are shown as *capital letters*, and *numbers* refer to positions relative to the transcriptional start site.

Consideration of our results with the *cotX* promoter provides additional support for the proposal that SpoIIID delays full expression of *cotC* during sporulation. The pattern of *cotX-lacZ* expression and *cotX* mRNA accumulation was more similar to that of *cotB* than *cotC* (Figs. 2 and 3), yet *cotX* and *cotC* transcription *in vitro* exhibited similar dependence on the concentration of GerE (Fig. 4), providing no explanation for the observed differential expression of *cotX* and *cotC* *in vivo*. This difference can be plausibly explained by our finding that SpoIIID is a more potent repressor of cotC transcription *in vitro* than of *cotX* (Fig. 5). SpoIIID appears to be a weak repressor of *cotX* because it binds with relatively low affinity to a site in the promoter that overlaps the binding site for σ^K RNAP (Fig. 7). If SpoIIID does repress transcription from the *cotX* promoter during sporulation, this repression would be expected to be relieved earlier than repression of *cotC*, as the level of SpoIIID decreases in the mother cell (9, 10).

Differential timing of *cotB* and *cotC* expression was overlooked previously due to hybridization of a primer that was thought to be *cotC*-specific with *cgeAB* mRNA (4, 19, 23). Hence, the primer extension analysis reported previously shows that *cotB* and *cgeAB* transcripts appear with similar timing during sporulation (19) and does not conflict with our finding that *cotC* expression lags behind that of *cotB* (Figs. 2 and 3). Expression of a *cotC-lacZ* fusion was shown previously to be induced about 1 h later during sporulation than expression of *cotD-lacZ* (27). Interestingly, the difference in time of

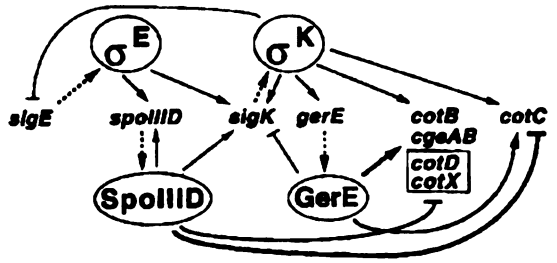

FIG. 8. A model showing how the combined action of SpoIIID and GerE may regulate *cot* genes in the context of interactions between mother cell-specific transcription factors. *Dashed arrows* show gene (*italicized*)-to-product (proteins are *circled*) relationships. *Solid arrows* represent positive regulation of transcription. *Lines* with a *barred end* represent negative regulation of transcription. The *box* distinguishes *cotD* and *cotX*, which are proposed to be weakly repressed by SpoIIID, from *cotB* and *cgeAB*, which are not repressed by SpoIIID, and from *cotC*, which is expressed later because it is strongly repressed by SpoIIID (indicated by the *thick line* with a *barred end*).

induction disappeared when the genes were artificially induced by production of σ^K during growth (27). Under these conditions, SpoIIID would not be present. Therefore, we propose that SpoIIID is responsible for the observed delay during sporulation in *cotC* expression as compared with that of *cotD*. A prediction of this hypothesis is that SpoIIID is a more potent repressor of *cotC* transcription than of *cotD* transcription. SpoIIID was shown previously to bind with relatively high affinity to a site spanning the −35 region of the *cotD* promoter and repress transcription *in vitro* (7); however, the effect of SpoIIID on *cotD* and *cotC* transcription *in vitro* has not been compared directly.

Fig. 8 illustrates how the combined action of SpoIIID and GerE may produce differential regulation of *cot* gene transcription in the context of known regulatory interactions with the two mother cell-specific σ factors, σ^E and σ^K. σ^E RNAP transcribes the *spoIIID* gene (28–32). As SpoIIID accumulates, it activates transcription of *sigK* by σ^E RNAP (7, 25). The primary product of the *sigK* gene, pro-σ^K (not shown in Fig. 8), is processed to σ^K in an activation step coupled to a signal from the forespore (33–35). σ^K RNAP transcribes *gerE* (4, 24). As GerE and σ^K RNAP accumulate, *cotB* (4, 19), *cgeAB* (23), and other genes begin to be transcribed. The other genes include *cotD* (4, 6, 7, 20) and *cotX* (5, 36), but we propose that SpoIIID limits transcription of these genes (*boxed* in Fig. 8) and prevents transcription of *cotC* for about 1 h. Repression by SpoIIID is relieved as its level declines due to degradation of the protein and due to a negative feedback loop initiated by σ^K RNAP that inhibits transcription of *sigE* and other early sporulation genes, thus inhibiting further production of σ^E and SpoIIID (9, 10, 37). The falling levels of σ^E and SpoIIID and the rising levels of σ^K and GerE together with the fact that both SpoIIID and GerE can act as activators or repressors of transcription (4, 6–8) make it possible to regulate the timing and level of individual *cot* gene transcription in a variety of ways.

Our 5′ deletion analysis of *cot* promoters gives further insight into the function of GerE as an activator of transcription. Deletions designed to eliminate the more upstream GerE binding site in the *cotB* and *cotX* promoters greatly reduced expression of *lacZ* fusions (Fig. 2), strongly suggesting that GerE binds to sites centered at −73.5 and −60.5 in the *cotB* and *cotX* promoters, respectively, and contributes to transcriptional activation of these promoters during sporulation. On the other hand, the finding that elimination of the more upstream GerE sites in these promoters did not abolish GerE-dependent expression suggests that the more downstream GerE sites are sufficient for weak transcriptional activation. The more downstream GerE site in the *cotB* promoter has the sequence 5′-AATTAGGCTATT-3′ (4), which matches perfectly the consensus sequence for GerE binding (5). This sequence is centered at −47.5 (4), which seems to be a preferred position for binding in promoters activated by GerE, since the *cotVWX*, *cotYZ*, and *cotD* promoters also have a sequence matching the consensus centered at −47.5 or −46.5, to which GerE appears to bind, activating transcription (5, 6). The more downstream site in the *cotX* promoter has the sequence 5′-GACTGAGTCATA-3′, which matches in 7 of 10 positions in the consensus sequence for GerE binding (5). This sequence is centered at −37.5 and is in the opposite orientation relative to the direction of *cotX* transcription as compared with the site centered at −47.5 in the *cotB* promoter. Assuming that GerE binds in a particular orientation to sequences similar to its nonpalindromic consensus sequence, our results suggest that GerE can activate transcription when bound in opposite orientations to sites centered at −47.5 and −37.5 (Figs. 1 and 2). Our results also suggest that GerE can activate transcription when bound to a site centered as far upstream as −73.5 in the *cotB* promoter or one-half turn of the DNA helix downstream at −68.5 in the *cotC* promoter. Hence, GerE may be less stringent than, for example, the *E. coli* catabolite gene activator protein with respect to the position from which it can activate transcription (38). This idea can be tested further by creating single base pair changes that eliminate GerE binding to individual sites and/or by systematically varying the position of a GerE binding site in a promoter region.

The 5′ deletion we created that eliminates the more upstream GerE binding site in the *cotX* promoter may prove to be useful for investigating the mechanism of GerE transcriptional activation at this promoter. A recent study suggests that GerE may interact with σ^K at the *cotX* promoter and facilitate the initial binding of σ^K RNAP to the promoter (36). Certain amino acid substitutions in σ^K reduced expression of a *cotX-lacZ* fusion but not expression of a *gerE-lacZ* fusion, which also depends upon σ^K RNAP but not on GerE. The authors speculated that GerE bound to the more downstream site centered at −37.5 contacts σ^K, enhancing binding of σ^K RNAP to the *cotX* promoter. To explain the observation that the substitutions in σ^K did not eliminate *cotX-lacZ* expression, the authors proposed that GerE bound to the more upstream site centered at −60.5 makes a different contact with σ^K RNAP (*e.g.* with the C-terminal domain of the α subunit). This model predicts that expression of the *cotX-lacZ* fusion we made, lacking the GerE site centered at −60.5, would be abolished in the mutants with amino acid substitutions in the σ^K region thought to interact with GerE.

Our results shed more light on how SpoIIID can function as a transcriptional repressor. SpoIIID footprints in the *bofA*, *cotD*, and *spoVD* promoter regions have been published previously (7, 8). In each case, SpoIIID binds to sites centered at +1 and/or −35, presumably preventing RNAP from binding to the promoter or hindering a subsequent step in transcription initiation. In the *cotC* promoter region, SpoIIID binds to a site centered at −67.5 (Fig. 6), which presumably prevents GerE from binding to its site centered at −68.5 (4), and SpoIIID binds to a site centered at −36.5 (Fig. 6), which presumably interferes with RNAP binding or a subsequent step in initiation. Likewise, SpoIIID binding to a site centered at −16.5 in the *cotX* promoter (Fig. 7) probably interferes with RNAP function.

Why are certain *cot* genes subject to dual regulation by SpoIIID and GerE? One possibility is that fine-tuning of *cot* gene expression allows optimal levels of Cot proteins to be

synthesized at the proper times for assembly into the spore coat. Our results suggest that expression of *cotC* is delayed relative to expression of other *cot* genes. We plan to test whether the delay in *cotC* expression is important by engineering cells to produce CotC earlier and measuring spore resistance properties. Fine-tuning of *cot* gene expression may also allow the spore coat to be suitably tailored in response to environmental conditions. Expression of a *cotC-lacZ* translational fusion was shown to depend strongly on whether sporulation was induced by sudden or gradual nutritional shift-down (19). Whether this regulation involves one of the previously known *cotC* transcription factors (*i.e.* GerE or σ^K RNAP), the newly discovered *cotC* repressor reported here (*i.e.* SpoIIID), or some other mechanism remains to be elucidated.

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CHAPTER VI

Conclusions

The goal of my research was to understand the role of GerE and SpoIIID in gene regulation during *Bacillus subtilis* sporulation. Experiments were performed to characterize GerE as a DNA-binding protein, to determine the regulatory role of GerE in the σ cascade during sporulation, and to study coordinate gene regulation by GerE and SpoIIID.

Genetic studies and the primary structure of GerE suggested that it is a small DNA-binding protein. This was tested directly by gel-purifying GerE from an *E. coli* strain that was engineered to overexpress *gerE*. GerE bound to the *cotB* and *cotC* promoter regions in a sequence-specific manner and activated transcription *in vitro*. GerE also inhibited transcription of *sigK* and *cotA in vitro*. The ability of GerE to activate transcription was further demonstrated in transcription of the *cotVWXYZ* gene cluster. GerE bound to and activated transcription from specific sequences in the *cotVWX*, *cotX*, and *cotYZ* promoters. The GerE binding site consensus sequence was established as RWWTRGGY--YY(R means purine, W means A or T, and Y means pyrimidine).

Many transcriptional activators that bind upstream of the -35 region of promoters are unable to activate transcription by RNA polymerase that lacks the C-terminal domain of the α subunit. GerE binding sites are located upstream of the -35 region of the *cotB*, *cotC*, and *cotX* promoters, but a SpoIIID binding site is located within the -35 region of the *sigK* promoter. *In vitro* transcription experiments with heterologous RNA polymerases consisting of *B. subtilis* σ^K and *E. coli* core RNA polymerase with or without the C-terminal domain of the α subunit suggested that GerE interact with αCTD to activate transcription of *cotB*, *cotC*, and *cotX*, but SpoIIID does not use this mechanism to activate *sigK* transcription (Appendix A).

GerE inhibited *sigK* transcription *in vitro*; however, when the *sigK* promoter

region was fused to the *E. coli lacZ* gene, there was no indication that GerE inhibited *sigK-lacZ* expression *in vivo*. This paradox was solved by mapping a GerE binding site near the *sigK* transcriptional start site, because the result showed that the GerE binding site was not included in the *sigK-lacZ* construct. A new *sigK-lacZ* fusion that included the GerE binding site exhibited β-galactosidase activity during sporulation that was two fold higher in *gerE* mutant cells than in wild-type cells. This showed that σ^K RNA polymerase transcription of *gerE* initiates a negative feedback loop in which GerE represses *sigK* transcription. Another example of repression by GerE was demonstrated with the *cotD* gene. Two GerE binding sites centered at -47.5 and -25.5 were mapped in the *cotD* promoter by DNase I footprinting. *In vitro* transcription assays indicated that GerE activates *cotD* transcription when present at a low concentration, but represses transcription by binding the site centered at -25.5 when present at high concentration. The existence of these negative effects by GerE clearly indicates that gene expression at the later stages of sporulation is still tightly regulated.

The relationship between GerE activation of transcription and promoter structure was investigated by performing deletion studies. There are two GerE binding sites in each of several promoters, including *cotB*, *cotC*, and *cotX*. GerE binding sites in the *cotB* promoter are centered at -73.5 and -47.5. A GerE binding site centered at or very near -47.5 is also found in the *cotD*, *cotYZ*, and *cotVWX* promoters. Upon deletion of the GerE binding site centered at -73.5 in the *cotB* promoter, activity was reduced by approximately 80 %, demonstrating that the site centered at -47.5 is sufficient for some activation by GerE, but that the site centered at -73.5 contributes greatly to activation. GerE binding sites in the *cotX* promoter region are centered at -60.5 and -36.5. The GerE binding site centered at -36.5 is interesting because its orientation is the opposite of the

others. GerE was able to activate transcription from the *cotX* promoter containing only the GerE binding site centered at -36.5 about 40 % as well as from the native promoter, suggesting that GerE can activate transcription when bound to the promoter in either orientation. There is evidence that GerE activates *cotX* transcription by two different mechanisms, one acting through the C-terminal domain of the α subunit of RNA polymerase (αCTD) and the other acting through σ^K. Studies of *E. coli* DNA-binding proteins suggest that binding upstream of the promoter -35 region may activate transcription through interaction with αCTD, whereas binding within the -35 region may activate through interaction with the σ subunit. Therefore, it is plausible that GerE bound to the sites centered at -60.5 and -36.5 may activate *cotX* transcription through interaction with the αCTD and σ^K, respectively. Deletion of a GerE binding site centered at -134.5 did not affect *cotC* promoter activity, indicating that GerE binding to a site centered at -68.5 is sufficient to activate *cotC* transcription.

In the preceding study, there appeared to be differences in the pattern of *lacZ* expression from fusion to the *cotB*, *cotC* and *cotX* promoters during sporulation. Indeed primer extension analyses showed that *cotB* mRNA accumulates slightly earlier during sporulation than that of *cotX* or *cotC*, whereas the *cotC* mRNA level reaches its maximum later than that of *cotB* or *cotX*. A possible explanation for these differences was sought using *in vitro* transcription assays. An equimolar mixture of *cotB*, *cotC*, and *cotX* DNA templates was transcribed by σ^K RNA plymerase in the presence of different amounts of GerE. A smaller amount of GerE activated *cotB* transcription than for *cotC* or *cotX*. Likewise, the effect of SpoIIID on transcription of *cotB*, *cotC*, and *cotX* was tested in mixed-template *in vitro* transcription assays with a fixed amount of GerE and different amounts of SpoIIID. A very small amount of SpoIIID strongly repressed *cotC*

transcription, a larger amount of SpoIIID moderately repressed *cotX* transcription, and SpoIIID had little or no effect on *cotB* transcription. Taken together, these results suggest the following model for the differences in expression patterns of *cotB*, *cotC*, and *cotX*. As σ^K RNA polymerase becomes active, *gerE* transcription begins and the level of SpoIIID starts to decrease. GerE accumulates to a low level and activates *cotB* transcription. For *cotX* and *cotC* to be transcribed, GerE must accumulate to a higher level. As the level of SpoIIID decreases, its repressive effect on *cotX* is lost, and finally as its level becomes very low, *cotC* is fully transcribed.

An important contribution of my thesis research is the finding that regulation of both the *sigK* gene encoding σ^K and *cot* genes in the σ^K regulon are tightly controlled by GerE and SpoIIID late in sporulation. Tight regulation of *cot* gene expression may be important for proper assembly of the spore coat or for modifying coat composition in response to environmental cues. These hypotheses can be the basis for further research on *cot* gene regulation.

APPENDIX A

Requirement of the C-Terminal Region of the α Subunit of RNA Polymerase for Transcriptional Activation by GerE but not SpoIIID of *Bacillus subtilis*

ABSTRACT

GerE and SpoIIID activate transcription by σ^K RNA polymerase during *Bacillus subtilis* sporulation. GerE activated transcription *in vitro* of *cotB*, *cotC*, and *cotX* by heterologous RNA polymerase reconstituted from *E. coli* core subunits and *B. subtilis* σ^K. Likewise, SpoIIID activated transcription of *sigK* by the heterologous enzyme. When *E. coli* core containing α with a C-terminal truncation was used in similar experiments, activation by GerE at the three *cot* promoters decreased by 30 to 50%, but activation by SpoIIID at the *sigK* promoter remained the same. These results suggest that GerE interacts with the C-terminal domain of the α subunit of RNA polymerase; however, GerE also activates *cot* gene transcription by another mechanism, and SpoIIID activation of *sigK* transcription depends completely on another mechanism.

INTRODUCTION

Activation of transcription is the primary regulatory strategy used during prokaryotic developmental processes. Sporulation of the gram-positive bacterium *Bacillus subtilis* is controlled by a cascade of RNA polymerase (RNAP) σ subunits (Kroos et al. 1999). In addition, sequence-specific DNA-binding proteins activate transcription by RNAP containing sporulation-specific σ factors. For example, GerE and SpoIIID activate transcription by σ^K RNAP in the mother-cell compartment of sporulating *B. subtilis* (Halberg and Kroos 1994; Zheng et al. 1992; Zhang et al. 1994). Here, we investigate the mechanism of transcriptional activation by GerE and SpoIIID.

GerE recognizes the consensus DNA sequence RWWTRGGY-YY (R means purine, W means A or T, and Y means pyrimidine) and activates transcription of many genes whose products are spore coat proteins (Zheng et al. 1992; Zhang et al. 1994). Here, we focus on GerE-activated transcription from the *cotB*, *cotC,* and *cotX* promoters. There are two GerE binding sites in each of these promoters (Zheng et al. 1992; Zhang et al. 1994). Deletion of the upstream site centered at -73.5 bp in the *cotB* promoter resulted in a partial reduction of *cotB-lacZ* expression, suggesting that both this site and one centered at -47.5 bp participate in transcriptional activation (Ichikawa and Kroos 2000). In contrast, deletion of the upstream GerE binding site centered at -134.5 bp in the *cotC* promoter did not affect *cotC-lacZ* expression, suggesting that GerE binding to a site centered at -68.5 bp is sufficient for full activation of *cotC* transcription (Ichikawa and Kroos 2000). In the *cotX* promoter, the upstream site centered at -60.5 bp is in the same orientation as the GerE binding sites mentioned above, but the downstream site centered at -36.5 bp is in the opposite orientation. The downstream site alone is sufficient for approximately 40% as much *cotX-lacZ* expression as when both sites are present

(Ichikawa and Kroos 2000).

SpoIIID activates transcription of the *sigK* gene (encoding σ^K) by σ^E- or σ^K-containing RNAP (Kroos et al. 1989). There is a match to the consensus sequence for SpoIIID binding, WWRRACAR-Y, centered at -27.5 bp in the *sigK* promoter, and SpoIIID has been shown previously to bind to this site (Halberg and Kroos 1994).

All four core subunits of bacterial RNAP can be targets for physical interaction with transcriptional activator proteins (Ebright 1985; Niu et al. 1996; Miller et al. 1997; Lee and Hoover 1995). The C-terminal domain of the α subunit (αCTD) is most often the target for activators that bind upstream of the promoter -35 region. Many activators that bind upstream of the promoter -35 region fail to activate transcription when αCTD is absent. Other activators do not require αCTD for transcriptional activation. In many cases, these proteins bind within the promoter -35 region, and interact with σ or the N-terminal domain of α (αNTD) (Niu et al. 1996).

Because GerE binds upstream of the *cotB*, *cotC,* and *cotX* promoter -35 regions, we hypothesized that GerE might interact with αCTD to activate transcription of these genes. Conversely, since SpoIIID binds within the *sigK* promoter -35 region, αCTD might be dispensable for activated transcription of *sigK*. The primary structures of RNAP α and β subunits of *B. subtilis* and *E. coli* have high similarity (Niu et al. 1996; Boor et al. 1995). Here, we show that heterologous RNAP reconstituted from *E. coli* core and *B. subtilis* σ^K is responsive to transcriptional activation by GerE and SpoIIID. We used this system to demonstrate that GerE activation of *cotB*, *cotC,* and *cotX* transcription depends, in part, on αCTD, but SpoIIID activation of *sigK* is independent of αCTD.

MATERIALS AND METHODS

DNA templates and proteins

To facilitate preparation of template DNA for *in vitro* transcription, pHI-11 was constructed by subcloning a *Hin*dIII-*Eco*RI fragment of pHI-3 between the *Hin*dIII and *Eco*RI sites of pUC18. pHI-3 was constructed by subcloning a fragment of the *cotB* promoter region, -86 to +37, franked with a *Eco*RI and *Hin*dIII site at the upstream and downstream end of pTKlac (Kenney and Moran 1991), respectively. The DNA fragment was prepared by PCR using pBD136 (Zheng et al. 1992) as the template. The upstream primer was 5'GGGAATTCGCGTGAAAATGGGTAT3' (the underlined portion corresponds to an *Eco*RI site). The downstream primer was 5'GCGAAGCTTAATTCCTCCTAGTCA3' (the underlined portion corresponds to a *Hin*dIII site). pHI-12 was constructed by subcloning an *Eco*RI-*Hin*dIII fragment of pHI-7 between the *Eco*RI and *Hin*dIII sites of pUC19 (Yanish-Perron et al. 1985). pHI-7 was constructed by subcloning a fragment of the *cotC* promoter region, -81 to +14, franked with *Eco*RI and *Hin*dIII restriction sites at the upstream and downstream ends, respectively, of pTKlac. The DNA fragment was prepared by PCR using (Zheng et al. 1992) as the template. The upstream primer was 5'CGGAATTCTCTATCATTTGGACAG3' (the underlined portion corresponds to an *Eco*RI site). The downstream primer was 5'CGGAAGCTTTTATTTTTACTACG3' (the underlined portion corresponds to a *Hin*dIII site). The DNA template containing the *cotX* promoter region from -98 to +82 was prepared by PCR using pJZ22 as the template. The upstream and downstream primers were 5'CGGAAAAACGATAACAATTAGC3' and 5'CGGTTTGCATCAGAACATGT3', respectively. σ^K and SpoIIID were gel purified as described previously (Kroos et al. 1989). GerE was gel purified as described previously

(Zheng et al. 1992). *E. coli* core RNA polymerase was prepared as described previously (Fujita and Ishihama 1996).

In vitro transcription assay

E. coli core RNA polymerase (0.1 pmol) with or without intact αCTD was incubated with σ^K (1 pmol) on ice for 10 min. to reconstitute holoenzyme. Transcription reactions (45μl) were performed as described previously (Carter and Moran 1986), except that RNA polymerase was allowed to bind to the DNA template for 10 min. at 37°C before the addition of nucleotides (the labeled nucleotide was [α-^{32}P]CTP). Heparin (6μg) was added 2 min. after the addition of nucleotides to prevent reinitiation. After reactions were stopped, 20μl of each reaction mixture was subjected to electrophoresis in a 5% polyacrylamide gel containing 8 M urea, and transcripts were detected by autoradiography. The signal intensities were quantified using a Storm 820 Phosphor Imager (Molecular Dynamics).

RESULTS

αCTD plays a role in GerE activation of *cotB*, *cotC*, and *cotX* transcription.

To examine the requirement for αCTD in activation of *cot* gene transcription by GerE, we reconstituted heterologous RNAPs consisting of *B. subtilis* σ^K and *E. coli* core containing full-length α (*Ec*wtα σ^K RNAP) or α truncated after amino acid 257 (*Ec*ΔαCTD σ^K RNAP). As a control to measure the relative activities of the reconstituted RNAPs, each preparation was used to transcribe *gerE*, a gene with a strong σ^K-dependent promoter that can be transcribed in the absence of GerE (Zheng et al. 1992). In the experiment shown in Figure 1, *Ec*ΔαCTD σ^K RNAP was 68% as active as *Ec*wtα σ^K RNAP at transcribing *gerE*. Since the *gerE* promoter has no apparent sequence (UP element) with which αCTD would be expected to interact directly, we attributed the difference in *gerE* transcriptional activity to different efficiencies of reconstituting active holoenzyme. In the absence of GerE, *cot* gene transcription by the heterologous RNAPs was so weak that we could not quantitate the signals (Figure 1). In the presence of GerE, the signals were markedly stronger for *Ec*wtα σ^K RNAP than for *Ec*ΔαCTD σ^K RNAP. These signals were quantified. The signals obtained with *Ec*wtα σ^K RNAP were multiplied by 0.68, to take into account the higher activity of this preparation of reconstituted RNAP compared with *Ec*ΔαCTD σ^K RNAP, as judged by *gerE* transcription. Comparing these adjusted *Ec*wtα σ^K RNAP signals with the *Ec*ΔαCTD σ^K RNAP signals, it appeared that the absence of αCTD reduced GerE-activated *cot* gene transcription by 20-50%. The experiment shown in Figure 1 was repeated with different preparations of reconstituted heterologous RNAPs, and similar results were obtained. Figure 2 shows that the absence of αCTD reduced *cotB*, *cotC*, and *cotX* transcription, on

average by 45%, 29%, and 37%, respectively. These results suggest that GerE interacts with the αCTD of heterologous RNAP, increasing *cot* gene transcription. GerE activation of *cot* genes also involves another mechanism, because *Ec*ΔαCTD σ^K RNAP was stimulated by GerE, although we could not measure the fold activation because the signals in the absence of GerE were too weak to quantitate (data not shown).

αCTD is not required for activation of *sigK* transcription by SpoIIID.

The method described above was used to examine the requirement of αCTD for SpoIIID activation of *sigK* transcription. Figure 3 shows the results of one experiment. In this experiment, the reconstituted *Ec*ΔαCTD σ^K RNAP preparation was 43% as active as *Ec*wtα σ^K RNAP at transcribing *gerE*. In the absence of SpoIIID, *sigK* transcription by the heterologous RNAPs was barely detectable (Figure 3). In the presence of GerE, the signal was stronger for *Ec*wtα σ^K RNAP than for *Ec*Δα σ^K RNAP; however, this difference could be completely accounted for by the higher activity of *Ec*wtα σ^K RNAP, as judged by *gerE* transcription. Similar results were obtained when the experiment was repeated with different preparations of reconstituted heterologous RNAPs, as summarized in Figure 2. Thus, the absence of αCTD appeared to have no effect on the ability of SpoIIID to activate *sigK* transcription.

DISCUSSION

Our results suggest that αCTD is involved in transcriptional activation by GerE when the *cotB*, *cotC*, and *cotX* genes are transcribed by heterologous RNAP consisting of *E. coli* core subunits and *B. subtilis* σ^K. These *cot* genes have GerE binding sites located upstream of the promoter -35 regions, which indicated they might be analogous to class I bacterial promoters. One of the most studied class I promoters is the *E. coli lac* promoter which has a CAP (catabolite gene activator protein) binding site centered at -61.5. Genetic and biochemical evidence support the idea that transcriptional activation by CAP bound at this site involves direct physical interaction with αCTD, increasing the affinity of RNAP for the promoter (Dove and Hochschild 1998). When this CAP site was moved to different upstream positions, CAP activated transcription from sites centered at -72.5, -82.5, and -92.5, suggesting flexibility in the distance between RNAP and CAP, but rigidity with respect to the face of the DNA helix (Zhou et al. 1994). Each of the three *cot* promoters we tested has at least one GerE binding site expected to position GerE on the same helix face with respect to RNAP (*i.e.*, GerE sites centered at -68.5, -47.5, and -36.5 bp in the *cotC*, *cotB,* and *cotX* promoters, respectively). However, GerE bound at the sites centered at -47.5 bp and -36.5 bp in the *cotB* and *cotX* promoters, respectively, might be too close to the transcriptional start site to interact with αCTD. GerE bound at the other sites in the *cotB* (centered at -73.5 bp) and *cotX* (centered at -60.5 bp) promoters would not be on the same face of the DNA helix as GerE bound at the site centered at -68.5 bp in the *cotC* promoter. These considerations suggest that GerE may be more flexible than CAP in terms of distance and/or helix face requirements for interaction with αCTD. We cannot rule out the possibility that a direct interaction between αCTD and

promoter DNA contributed to the stronger *cot* gene transcription with *Ec*wtα σ^K RNAP than *Ec*ΔαCTD σ^K RNAP in the presence of GerE, because we could not reliably measure the very low levels of basal transcription of the *cot* genes by the reconstituted heterologous RNAPs in the absence of GerE. Like the *gerE* promoter that we used to gauge the activity of the reconstituted RNAP preparations, the *cot* promoters we tested have no sequence (UP element) expected to interact directly with αCTD. Therefore, it is likely that *Ec*wtα σ^K RNAP transcribed the *cot* genes more strongly than *Ec*Δα σ^K RNAP because GerE interacts with αCTD. Further experiments are needed to test this model. Regulatory protein p4 of *Bacillus subtilis* phage Φ29 activates transcription from the viral late A3 promoter by interacting with αCTD (Monsalve et al. 1996). Interestingly, p4 was incapable of interacting with *E. coli* αCTD to activate transcription. Our results suggest that GerE can interact with *E. coli* αCTD at *cot* gene promoters, but it is possible that GerE interacts more strongly with *B. subtilis* αCTD at these promoters.

GerE and SpoIIID activated transcription by the heterologous RNAP by one or more mechanisms that do not involve αCTD. Deletion of αCTD still permitted considerable GerE activation of *cot* gene transcription, and it did not diminish SpoIIID activation of *sigK* transcription.

The *sigK* promoter, in which the SpoIIID binding site is centered at -27.5 bp, may be a class II bacterial promoter. The *E.coli galP1* promoter is a well-studied class II promoter with a CAP binding site centered at -41.5 bp. With CAP positioned so close to the promoter -35 region, αCTD is thought to be located upstream of CAP in the DNA-protein complex. Activation involves direct interaction between CAP and a surface on αNTD (Niu et al. 1996). Another example of a transcriptional activator that does not

require αCTD is bacteriophage λcI protein. cI binds to a site centered at -42 bp in the λP_{RM} promoter and activates transcription by interacting directly with the σ subunit of *E.coli* RNAP (Li et al. 1994). SpoIIID bound at the sigK promoter, and GerE bound to the downstream site in the *cotB* or *cotX* promoter, might interact directly with αNTD or σ^K to activate transcription. Recently, it was demonstrated that *cotX* transcription activation by GerE was reduced with amino acid substitutions in σ^K, suggesting a direct interaction between GerE and σ^K. GerE bound to the site centered at -68.5 bp in the *cotC* promoter may be too far upstream to make these contacts, yet GerE activated transcription of this promoter by heterologous RNAP lacking αCTD, so perhaps yet another mechanism is employed.

In summary, we have shown that heterologous RNAP reconstituted from *E. coli* core subunits and *B. subtilis* σ^K can be used to investigate the mechanisms of transcriptional activation by GerE and SpoIIID. αCTD appears to play a role in GerE activation of *cot* genes, but not in SpoIIID activation of *sigK*. Thus, one mechanism used by many bacterial activators can, in part, account for activation by GerE, but much more work will be required to determine whether the other mechanisms employed by GerE and SpoIIID follow other paradigms or are novel.

Figure 1

***In vitro* transcription of *cotB*, *cotC*, *cotX* and *gerE* by heterologous RNA polymerase with or without intact αCTD.**

DNA template (0.1 pmole) was transcribed with 0.1 pmole each of reconstituted *Ec*wtα σ^K RNAP (lanes 1 and 2) or *Ec*ΔαCTD σ^K RNAP (lanes 3 and 4). GerE (25 pmole for reactions with *cotB* template and 400 pmole for reactions with *cotC* and *cotX* templates) was added immediately after σ^K RNAP (lanes 2 and 4). DNA templates were pSC146 linearized with *Hin*dIII (204-base *gerE* transcript), a *Pvu*II restriction fragment of pHI-11 (131-base *cotB* transcript), an *Eco*RI- *Pvu*II restriction fragment of pHI-12 (197-base *cotC* transcript), and a 180 bp PCR fragment prepared as described in Materials and Methods (82-base *cotX* transcript).

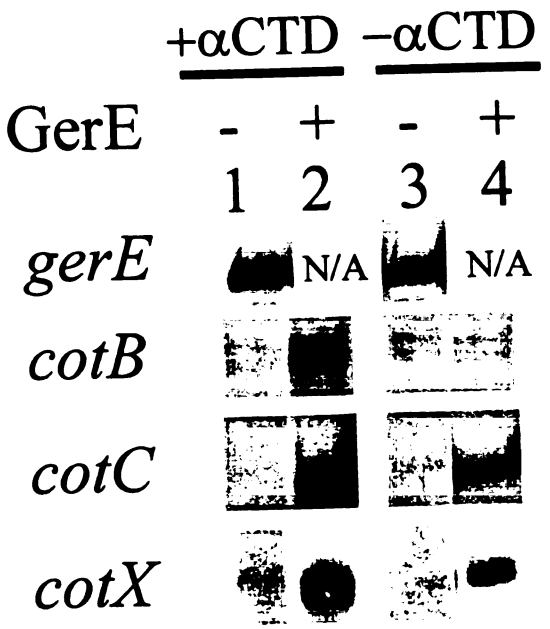

+αCTD
−αCTD
GerE
-
+
-
+
1
2
3
4
gerE
N/A
N/A
cotB
cotC
cotX

Figure 2

Effect of αCTD deletion on transcriptional activation.

In vitro transcription signals produced by heterologous RNA polymerase with or without intact αCTD in the presence of GerE were quntified from the experiments shown in Figure 1 and Figure 3, and from a second set of experiments. Signals by *Ec*wtα σ^K RNAP were adjusted as described in Results to take into account the higher activity of this reconstituted RNAP compared with *Ec*ΔαCTD σ^K RNAP. The graph shows the average ratio of the signal produced by *Ec*ΔαCTD σ^K RNAP signal, expressed as a percentage. Error bars indicate 1 standard deviation.

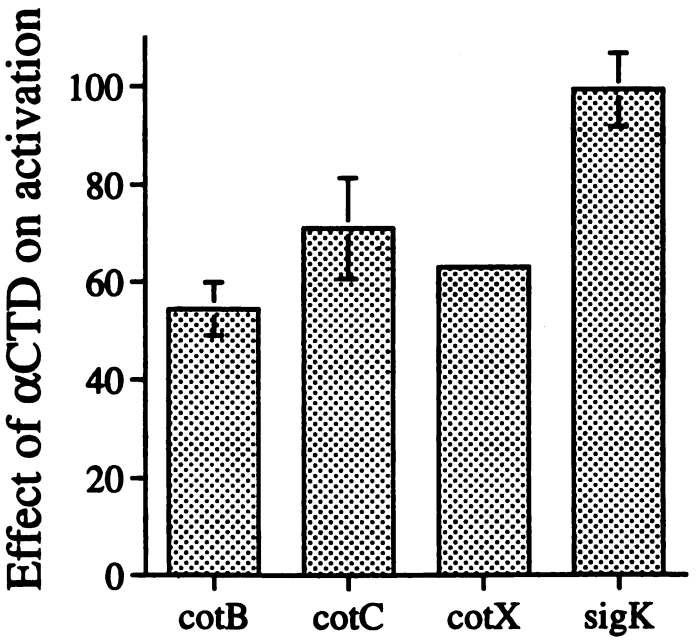
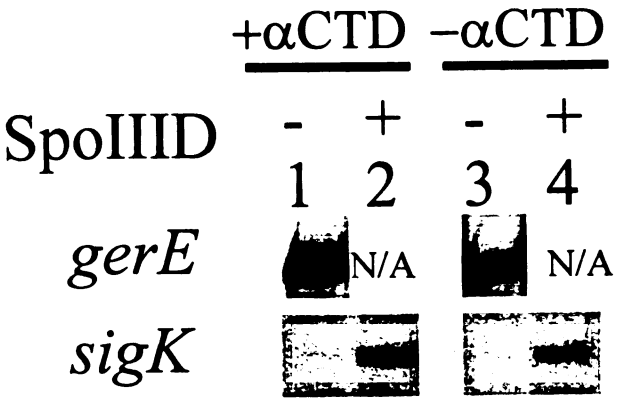

Effect of αCTD on activation
100
80
60
40
20
0
cotB
cotC
cotX
sigK

Figure 3

***In vitro* transcription of *sigK* and *gerE* by heterologous RNA polymerase with or without αCTD.**

DNA template (0.1 pmole) was transcribed with reconstituted *Ec*wtα σ^K RNAP (lanes 1 and 2) or *Ec*ΔαCTD σ^K RNAP (lanes 3 and 4). SpoIIID (400 pmole) was added immediately after σ^K RNAP (lanes 2 and 4). DNA templates were pSC146 linearized with *Hin*dIII (204-base *gerE* transcript) and pBK16 linearized with *Xba*1 (170-base *sigK* transcript).


+αCTD
−αCTD
SpoIIID
-
+
-
+
1
2
3
4
gerE
N/A
N/A
sigK

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