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ANALYSIS OF THREE NEGATIVE EFFECTS IN THE MOTHER CELL REGULATORY NETWORK OF SPORULATING BACILLUS SUBTILIS

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Lijuan Wang

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Lee Kroos
Major Professor's Signature

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ANALYSIS OF THREE NEGATIVE EFFECTS IN THE MOTHER CELL REGULATORY NETWORK OF SPORULATING BACILLUS SUBTILIS

By

Lijuan Wang

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ABSTRACT

ANALYSIS OF THREE NEGATIVE EFFECTS IN THE MOTHER CELL REGULATORY NETWORK OF SPORULATING BACILLUS SUBTILIS

By

Lijuan Wang

During sporulation of *Bacillus subtilis*, four regulatory factors act in the order of σ^E, SpoIIID, σ^K, and GerE, to temporally control gene expression in the mother cell. SpoIIID and GerE are small sequence-specific DNA-binding proteins that activate or repress transcription of many genes. There are three negative regulatory links between different components, adding complexity to the network. First, transcriptionally active σ^K RNA polymerase inhibits early gene transcription, reducing SpoIIID late in sporulation. Second, GerE down-regulates σ^K by binding to the *sigK* promoter. Third, repression by SpoIIID causes *cotC* to be expressed at least one hour later than other *cot* genes, such as *cotB* and *cotD*.

In this study, to understand the importance of the negative effects in the mother cell regulatory network of sporulating *B. subtilis*, we showed that by maintaining SpoIIID at a higher level late in sporulation, less heat- and lysozyme-resistant spores are produced. Transmission electron microscopy showed structural defects in the spore coat. Reporter fusions to the σ^K-dependent *gerE*, *cotD*, and *cotC* promoters showed altered expression patterns. Interestingly, the expression patterns and spore resistance properties were restored when GerE negative regulation of *sigK* was simultaneously eliminated. On the other hand, eliminating only GerE negative regulation of *sigK* (without maintaining SpoIIID at a higher level late in sporulation) caused no defect in spore resistance properties, and a different pattern of altered reporter fusion expression. A strain engineered to express *cotC* two hours

earlier than normal during sporulation showed no defect in spore resistance properties. These results demonstrate that the *B. subtilis* mother cell regulatory network was robust to the perturbations we tested in terms of the number of resistant spores produced, although maintaining SpoIIID at a higher level late in sporulation caused a defect that was reversed by eliminating GerE negative regulation of *sigK*. The mechanism of this suppression can be rationalized in terms of the opposing effects of σ^K RNA polymerase and SpoIIID on expression of genes late in sporulation.

Spore coat protein analysis revealed no significant differences between the strains that are constructed for the study of negative effects and wild-type *B. subtilis*, although some of the engineered strains showed defects in spore resistance properties. This implies that structural, not compositional, differences cause the spore defects.

In order to elucidate the mechanism by which σ^K RNA polymerase negatively feeds back on earlier gene expression, a genetic screen was carried out to identify possible intermediate genes. Eleven candidates were identified. Among them, 16S rRNA was identified twice by independent transposition events. Most of the candidate genes affect metabolic pathways, implying a connecting between the regulation of *spoIIID* expression by the σ^K RNA polymerase negative feedback loop and metabolism of the sporulating cell.

A preliminary study revealed that the level of aconitase, which can bind to *gerE* mRNA for GerE and affect GerE synthesis, is different when cells are induced to sporulate by resuspension in starvation medium rather than by nutrient exhaustion in growth medium. This may explain the observation that the spore defect caused by persistence of SpoIIID late during sporulation can only be observed when cells are induced to sporulate by resuspension, but not by nutrient exhaustion.

To my husband, Zhiwei, my parents, Junsheng Wang and Shuqing Li, and my son, Evan, with love.

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LIST OF ABBREVIATIONS

Ap	ampicillin
DNA	deoxyribonucleic acid
DSM	Difco sporulation medium
DTT	dithiothreitol
FS	forespore
Km	kanamycin
LB	Luria-Bertani media
ONPG	ortho-nitrophenyl-beta-D-galactopyranoside
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
MC	mother cell
mRNA	messenger ribonucleic acid
RNAP	RNA polymerase
SASP	small acid-soluble proteins
SM	sporulation medium
TSS	transcription start site

INTRODUCTION

The gram-positive bacterium *Bacillus subtilis* undergoes a process called sporulation in order to survive during nutrient limitation. During sporulation, two compartments, a larger mother cell compartment and a smaller forespore compartment, are formed. Each compartment contains a complete copy of chromosomal DNA. In the mother cell of sporulating *B. subtilis*, a regulatory network controls sequential gene expression. Four regulatory factors, in the order σ^E, SpoIIID, σ^K and lastly GerE, are major players in this network. Three negative links exist in this regulatory network and their importance was unknown.

Chapter I provides a literature review of the relevant background regarding the process of sporulation, properties of the spore formed after sporulation, theory and studies on developmental transcriptional regulatory networks.

Chapter II presents the results of a study designed to understand the significance of one of the negative links in the sporulating mother cell, that is, the importance of σ^K RNA polymerase (RNAP) negative feedback on earlier gene expression. This negative effect results in reduced accumulation of SpoIIID late during sporulation. A strain in which the SpoIIID level is maintained late during sporulation was constructed by fusing *spoIIID* to the σ^K-controlled *gerE* promoter on a multicopy plasmid. This strain showed spore defects in heat- and lysozyme-resistance. Reporter fusions to *gerE*, *cotC* and *cotD*, which are expressed late in the mother cell, showed altered expression patterns compared to wild type and control strains. Although a quantitative germination assay showed no significant defect of the spores formed by the strain with SpoIIID maintained late during sporulation, thin-section transmission electron microscopy revealed structural defects of the spores. These

results suggest that σ^K RNAP negative feedback at least partially relies on fine-tuning of the SpoIIID level in order to allow normal spore formation.

Chapter III discusses a perturbation study of the mother cell regulatory network, in which one or two negative links were removed at the same time during sporulation. GerE represses *sigK* transcription. A strain with site-directed mutations in the *sigK* promoter eliminated this GerE negative feedback. This strain did not show spore resistance or germination defects. However, when a strain was constructed that both maintained SpoIIID late during sporulation and eliminated the GerE negative feedback, it sporulated normally, demonstrating suppression of the spore resistance defects caused by only maintaining the SpoIIID level late during sporulation. Reporter assays showed opposing effects of these two perturbations, suggesting that one change to the mother cell regulatory system can be compensated for by a second perturbation.

Chapter IV presents the results of a genetic screen designed to identify intermediates in the σ^K RNAP negative feedback loop. Eleven candidates were identified. Among them, 16S rRNA seems to be most interesting and further investigation is proposed.

Appendix I document the results of spore coat protein analysis of all the strains studied in Chapter II and Chapter III. No significant difference was found between any of the strains and wild-type *B. subtilis*. Appendix II shows that the level of aconitase when cells are induced to sporulate by resuspension in SM medium than by nutrient exhaustion in DSM. This result provides a possible explanation for the observation in Chapter II that strain with late persistence of SpoIIID exhibits spore defects upon induction by resuspension, but not by DSM. Appendix III shows that in a *B. subtilis* strain containing

P_{xyl}-*sigE*, whether the *sigE* expression is successfully induced or not is inconclusive based on western blot analyses.

The summary and perspectives summarizes the significance of the results and sheds light on future directions toward a better understanding of the mother cell regulatory network during sporulation of *B. subtilis*.

CHAPTER I: Literature Review

In developmental biology, questions of how cells generate distinct cell types by utilizing different gene expression programs and how different cells communicate with each other to ensure proper development are of fundamental significance. As one of the best-studied gram-positive organisms, *B. subtilis* provides an ideal model system to address these questions experimentally. Also, the transcriptional regulatory network, which responds to stimuli during sporulation of *B. subtilis* provides a powerful model for studies of signal transduction and gene regulation (Kroos. 2007; Piggot and Hilbert. 2004). Therefore, sporulation of *B. subtilis* has been intensively studied during the last several decades.

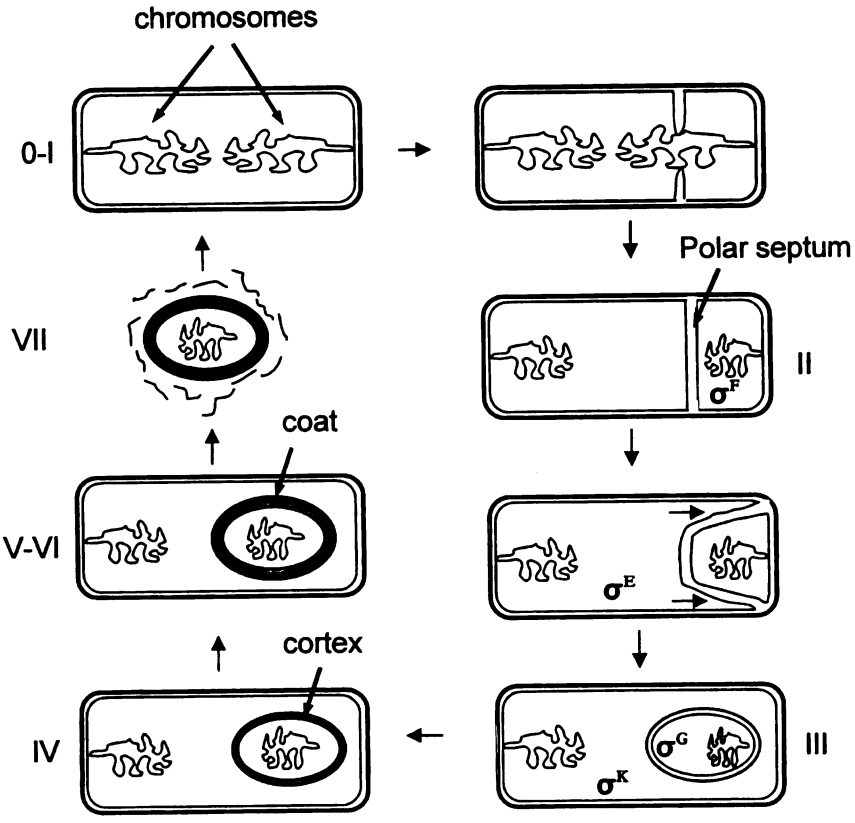
When sensing nutrient limitation, such as carbon or nitrogen depletion (White. 1999), *B. subtilis*, at a high enough cell density, will initiate a developmental process called sporulation that culminates in the formation of a dormant spore (Dawes and Mandelstam. 1970; Coote. 1972; Burbulys, *et al.* 1991; Lazazzera and Grossman. 1998). The spore is resistant to many kinds of extreme environments such as heat, UV light, lysozyme, and chemicals. As a result, the spore can survive in harsh environments, preserving the ability to germinate when nutrients are available, and resume as a vegetative cell.

Overview of Morphological Stages of Sporulation and Cell-specific σ Factors

The morphological stages of sporulation are illustrated schematically in Figure 1.1. Successive stages are identified by Roman numerals. The vegetative cell of *B. subtilis* is designated as Stage 0. The first morphological change after the initiation of sporulation is the formation of an axial filament of chromatin, which is designated as Stage I. During this stage, two copies of the chromosome from the last round of DNA replication condense and elongate to form a filament that spans across the cell's long axis, with the origin of each chromosome near to one pole of the rod-shape cell. A DNA replication checkpoint ensures

that two copies of the chromosome are present before cell division (Burkholder, *et al.* 2001). Stage II of sporulation is designated as the completion of septation. The cell is divided asymmetrically by a structure called the septum, resulting in a larger compartment called the mother cell and a small compartment called the forespore. Approximately one-third of one copy of the chromosome is present in the forespore, while the other two-thirds are quickly pumped into the forespore by a DNA translocase, SpoIIIE. σ^F is activated in the forespore shortly after the completion of the asymmetric cell division. A σ^F-controlled signal from the forespore triggers the activation of σ^E in the mother cell. Stage III is defined as the completion of engulfment of the forespore. During this stage, the asymmetric septum undergoes septal thinning, migrates around both sides of the forespore and engulfs the forespore. When the migration is complete, the forespore is pinched off and released into the mother cell as a free-floating protoplast. Two membranes are surrounding the forespore, each derived from one of the two cells. σ^G is activated in the forespore after the completion of engulfment. Then σ^G and σ^F dependent signals from the forespore trigger the activation of σ^K in the mother cell. Stage IV is complete when two peptidoglycan layers are deposited in between the two membranes surrounding the forespore, forming the germ cell wall and cell-wall-like material called cortex. The cortex renders the spore heat-resistant since it attains and also maintains the dehydrated state of the spore. During Stage V and VI, more than 60 spore coat proteins are synthesized and deposited onto the outside surface of the forespore. Maturation of the spore is achieved by gaining resistance to UV light and high temperature. UV resistance is due to the coating of the chromosomal DNA with a

Fig. 1.1. The morphological stages during sporulation of *B. subtilis*. The stages are designated by Roman numerals. The wavy circles represent chromosomal DNA. The sporangia are surrounded by cytoplasmic membrane (inner thinner circle) and cell wall (outer thicker circle). The cortex is indicated by the gray color in stages IV-VII and the spore coat by black in stages V-VII. Four sporulation-specific sigma factors become to be active in the mother cell or the forespore during different stages of sporulation as shown. Adapted from the Dissertation of H. Ichikawa, 2000. See text for references.


chromosomes
0-I
Polar septum
II
σ^F
σ^E
III
σ^G
σ^K
IV
cortex
V-VI
coat
VII

group of small acid-soluble proteins (SASP) and condensing the genome into a doughnut-like structure. Finally, at Stage VII, the mother cell lyses and releases the mature spore into the environment. The whole process of sporulation takes about six to ten hours in laboratory situations. The complex morphological changes during sporulation are accurately controlled by different σ factors, which bind to core RNAP sequentially during sporulation (Haldenwang. 1995; Stragier and Losick. 1996; Piggot and Hilbert. 2004).

Initiation of Sporulation

Factors that trigger sporulation include nutrient starvation, such as carbon or nitrogen resource limitation, DNA damage, high cell density, and the Krebs cycle (Trach and Hoch. 1993; Burkholder and Grossman. 2000; Perego and Hoch. 2002). Spo0A is the master regulator that controls entry into sporulation. Activation of Spo0A involves a phosphorelay, a more complex version of a two-component signal transduction system (Burbulys, *et al.* 1991). At least five kinases, KinA through KinE, are involved in the phosphorelay process. In response to unknown stimuli, the kinases first autophosphorylate themselves and then transfer the phosphate groups to Spo0F. Spo0F is an intermediate that donates its phosphate group to Spo0A, in a reaction catalyzed by a phosphetransferase Spo0B. KinA and KinB are the two primary kinases that are responsible for the phosphorelay. Negative effects from Spo0E and Rap protein regulators exist. They dephosphorylate Spo0A to regulate the initiation of sporulation. Spo0A-PO_4 activates genes whose products are responsible for axial filament formation and for asymmetric division. σ^A and σ^H are required for transcription of relevant genes at the initiation of sporulation (Hilbert and Piggot. 2004).

Efficient sporulation requires extracellular conditions such as high cell density and nutrient starvation. Blocking of the initiation or elongation of DNA replication, executed by a suppressor of *dnaA*, Sda, prevents initiation of sporulation (Burkholder, *et al.* 2001).

Approximately 121 genes are directly controlled by the activation of Spo0A through phosphorelay. Of them, about one-third are positively regulated and the remaining are negatively regulated. Twenty-five of them are transcription factors, so Spo0A also plays an role in transcriptional changes at the initiation of sporulation (Hilbert and Piggot. 2004).

Asymmetric Division of the Cell and Segregation of the Chromosome

One characteristic that distinguishes sporulation from vegetative cell division is the formation of an axial filament, which was identified first by electron microscopy (Aronson and Fitz-James. 1976) and then by fluorescence microscopy (Bylund, *et al.* 1993). The fact that a mutant failing to form an axial filament does not start asymmetric division suggests a checkpoint on cell division at the level of axial filament formation (Graumann and Losick. 2001). DivIVA (Graumann and Losick. 2001; Thomaides, *et al.* 2001), RacA (Ben-Yehuda, *et al.* 2003; Wu and Errington. 2003) and Soj (Ben-Yehuda, *et al.* 2003; Wu and Errington. 2003) are reported to be involved in axial filament formation. Once the axial filament is formed, asymmetric division is triggered by the relocation of the chromosomal origins to the poles of the cell. During asymmetric cell division, a prokaryotic tubulin homologue FtsZ forms a Z-ring that locates initially at mid-cell. The Z-ring is then redeployed to each pole of the cell through an intermediate helical structure (Ben-Yehuda and Losick. 2002). However, a complete septum is normally formed at only one pole of the cell. The prevention of a secondary polar septum is accomplished by the σ^E-controlled proteins SpoIID, SpoIIM and SpoIIP (Pogliano, *et al.* 1999; Eichenberger, *et al.* 2001). The

determination of which end of the cell forms the polar septum seems to be related to the age of the cell poles. Almost all forespores are formed at the older pole of the cell, but this can be disturbed by centrifugation at about 5,000 g and with 25 mM Mg^{2+} (Dunn. 1977).

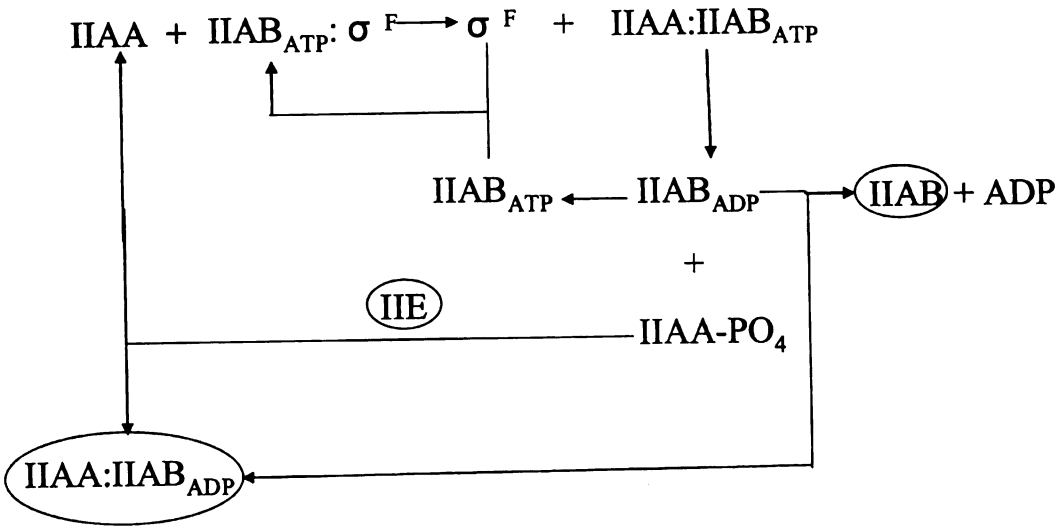
When the asymmetric septum is formed in a sporulating *B. subtilis* cell, it only traps about one-third of the origin-containing part of the chromosome in the forespore compartment, leaving approximately two-thirds in the mother cell (Wu and Errington. 1998). This genetic asymmetry is important for transient spatial gene expression in the forespore (Frandsen, *et al.* 1999; Dworkin and Losick. 2001). The origin-distal two-thirds of the chromosome left in the mother cell is transported by a DNA translocase, SpoIIIE (Wu and Errington. 1998). The question of why DNA is only translocated from the mother cell to the forespore, but not the reverse direction, remains unaddressed.

Activation and the Role of σ^F in the Forespore

After the asymmetric cell division during sporulation, both the mother cell and the forespore contain a complete copy of the chromosomal DNA. Different programs control gene expression in each compartment but communication between the mother cell and the forespore enable successive σ factor activation and couple gene expression in the mother cell and forespore.

Asymmetric division triggers the activation of σ^F, which is the first σ factor that becomes active in a specific compartment. σ^F is synthesized earlier, but it is held inactive by the anti-σ factor SpoIIAB until the completion of the polar septum formation, although it becomes active before the complete partitioning of a chromosome into the forespore(Wu and Errington. 1994; Frandsen, *et al.* 1999). The inhibition of active σ^F by SpoIIAB is released by the anti-anti-σ factor SpoIIAA. Activation of SpoIIAA is controlled

Fig. 1.2. Activation of σ^F. Shown is the network that regulates the activation of σ^F. Molecules in ovals are the three key determinants for σ^F activation: the ratio of SpoIIAA-PO_4 to SpoIIAA, which is determined by the SpoIIE phosphatase; the concentration of long-lived SpoIIAA:$SpoIIAB_{ADP}$ complex, which acts as a "sink" for SpoIIAA and SpoIIAB; and unstable SpoIIAB combined with transient genetic asymmetry. Adapted from (Kroos. 2007). See text for details and references.


IIAA + IIAB$_{ATP}$: σ ^{F} → σ ^{F} + IIAA:IIAB$_{ATP}$
IIAB$_{ATP}$ ← IIAB$_{ADP}$ → IIAB + ADP
+
IIE
IIAA-PO$_4$
IIAA:IIAB$_{ADP}$

by its phosphorylation state: it is active when dephosphorylated by SpoIIE and inactive when phosphorylated by SpoIIAB, which works both as a kinase as well as an anti-σ factor.

The activation of σ^F is coupled with asymmetric cell division and only occurs in the forespore. The mechanism is still under investigation. SpoIIE is proposed as the sensor of asymmetric cell division and in response activates σ^F. Several SpoIIE mutants have been isolated, which are able to activate σ^F independent of asymmetric cell division, but impair sporulation (Feucht, *et al.* 2002; Hilbert and Piggot. 2003; Carniol, *et al.* 2004).

Dephosphorylated SpoIIAA is believed to be sequestered in an inactive complex, called a "sink", with SpoIIAB and ADP. The "sink" plays important roles in σ^F activation: it inhibits the activation of σ^F by sequestering dephosphorylated SpoIIAA before cell division (Carniol, *et al.* 2004); it may also promote σ^F activation in the forespore by sequestering SpoIIAB (Lee, *et al.* 2001; Clarkson, *et al.* 2004). Structural studies reveal that two molecules of SpoIIAB form a complex with σ^F, but only one SpoIIAB molecule binds to σ^F directly. SpoIIAA interacts with the other SpoIIAB molecule that does not bind to σ^F and releases it by steric displacement (Ho, *et al.* 2003). The concentration of dephosporylated SpoIIAA is believed to be pivotal. Only when it reaches a threshold in the forespore is σ^F activated (Carniol, *et al.* 2004; Clarkson, *et al.* 2004).There are three factors in the forespore contributing to the activation of σ^F: the ratio of SpoIIE:SpoIIAA (Carniol, *et al.* 2004); and the instability of SpoIIAB (Pan, *et al.* 2001; Dworkin. 2003). The pathway that regulates the activation of σ^F is illustrated in Fig. 1.2.

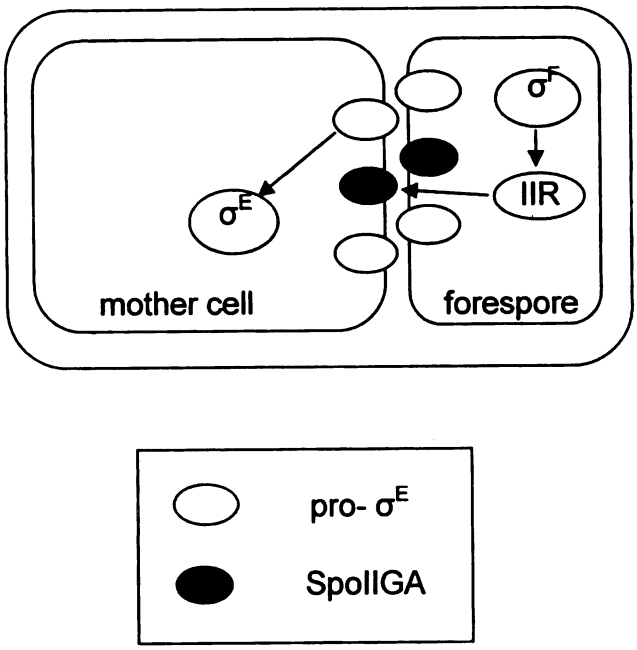
Once activated, σ^F facilitates RNAP to transcribe many genes, including *spoIIR* and *spoIIIG*. *spoIIR* encodes a signal protein that is required for σ^E activation (Karow and Piggot. 1995; Londono-Vallejo and Stragier. 1995). *spoIIIG* encodes the late forespore-

specific transcriptional factor σ^G (Sun, *et al.* 1989; Sun, *et al.* 1991). Activation of σ^G, the second specialized sigma factor in the forespore, does not occur until the completion of engulfment (Errington, *et al.* 1992; Kellner, *et al.* 1996). The σ^F regulon encodes at least three classes of genes involved in sporulation (Karmazyn-Campelli, *et al.* 1989), germination (Paidhungat. 2002), and in protection of the spore from DNA damage (Fajardo-Cavazos and Nicholson. 2000). In particular, σ^G RNAP transcribes *spoIVB*, whose product is a serine protease that mediates cleavage of SpoIVFA to provide intracellular signals for pro-σ^K processing in the mother cell (Dong and Cutting. 2003; Zhou and Kroos. 2005; Campo and Rudner. 2006). σ^G RNAP also transcribes *bofC*, whose product is a negative regulator of SpoIVB (Gomez and Cutting. 1997; Wakeley, *et al.* 2000).

Activation and the Role of σ^E in the Mother Cell

σ^E is the first sigma factor that is activated in the mother cell compartment after asymmetric cell division. The *spoIIG* operon encodes two genes that are involved in σ^E formation: *spoIIGA* and *spoIIGB*. *spoIIGA* encodes SpoIIGA, the processing enzyme for the proteolytic cleavage of pro-σ^E. *spoIIGB* encodes pro-σ^E, (Kenney and Moran. 1987; Jonas, *et al.* 1988) the precursor of σ^E, which is expressed preferentially in the mother cell (Arabolaza, *et al.* 2003; Fujita and Losick. 2003) and degraded in the forespore (Ju, *et al.* 1997; Ju, *et al.* 1998; Fujita and Losick. 2002). Pro-σ^E is subjected to proteolytic cleavage to remove 27 residues at its amino-terminal end to release the active σ^E (LaBell, *et al.* 1987; Stragier, *et al.* 1988). Time of activation of σ^E in the mother cell is closely linked to the activation of σ^F in the forespore. The trigger of processing is σ^F-controlled SpoIIR in the forespore (Karow and Piggot. 1995; Londono-Vallejo and Stragier. 1995). A strain that expresses *spoIIR* in the predivisional cell rather than the forespore only shows a mild effect

Fig. 1.3. The processing of pro-σ^E. Shown is a sporangium with a larger mother cell compartment and a smaller forespore compartment at stage II, just after polar septation. Pro-σ^E and SpoIIGA are thought to be associated with both septal membranes. Active σ^F RNAP in the forespore transcribes *spoIIR*, which encodes a signal protein that triggers the activation of a putative protease, SpoIIGA, resulting in processing of pro-σ^E to active σ^E in the mother cell. The gene for pro-σ^E and SpoIIGA are not as highly expressed in the forespore (Fujita and Losick. 2002) and σ^E made there appears to be degraded by an unknown enzyme (McBride, *et al.* 2005). Adapted from (Kroos, *et al.* 1999).


σF
σE
IIR
mother cell
forespore
pro- σE
SpoIIGA

on spore formation (Zhang, *et al.* 1996). However, premature expression of *spoIIG* from a constitutive promoter instead of its native promoter, together with constitutive expression of *spoIIR*, results in early processing of pro-σ^E and poor sporulation (Fujita and Losick. 2002). The pathway that regulates the activation of σ^E is illustrated in Fig. 1.3.

The σ^E regulon contains 272 genes that are organized in 171 transcription units. Among them, 262 genes in 163 transcription units are activated by σ^E in the absence of SpoIIID, a small mother cell specific transcription factor. Fourteen genes in 10 transcriptional units in the σ^E regulon are repressed by GerR, a recently discovered DNA-binding protein functioning as a transcriptional factor (Eichenberger, *et al.* 2004).

There are some important genes in the σ^E regulon. The *spoIID*, *spoIIM* and *spoIIP* genes are important for engulfment and to prevent a second asymmetric cell division in the mother cell (Rong, *et al.* 1986; Smith, *et al.* 1993; Smith and Youngman. 1993; Frandsen and Stragier. 1995; Pogliano, *et al.* 1999; Eichenberger, *et al.* 2001). The *spoIIIA* operon is required for the activation of the late forespore-specific sigma factor σ^G (Illing and Errington. 1991; Kellner, *et al.* 1996). The *sigK* gene encodes the precursor of the late mother cell-specific sigma factor σ^K (Stragier, *et al.* 1989; Kunkel, *et al.* 1990). The *spoIVCA* gene encodes the recombinase that generates *sigK* through a chromosomal rearrangement (Kunkel, *et al.* 1990; Popham and Stragier. 1992; Sato, *et al.* 1994). The *spoIIID* encodes a small transcription factor important for mother cell gene expression during sporulation (see below). Finally, *gerR* encodes another transcription factor that was recently discovered (Eichenberger, *et al.* 2004).

Engulfment of the Forespore

The important morphological event that follows the activation of σ^F and σ^E activation is engulfment of the forespore by the mother cell. First, the cell wall material of the septum is lost by an autolytic process. Then, the points where the septal membrane is attached to the peripheral cell membrane start to migrate toward the forespore pole. Finally, the forespore is completely engulfed into the mother cell, resulting in a free-floating protoplast (Fig. 1.1) (Illing and Errington. 1991; Londono-Vallejo, *et al.* 1997). Proteins that are either forespore-specific or mother cell-specific are required for engulfment. The *spoIID* (Abanes-De Mello, *et al.* 2002), *spoIIP* (Frandsen and Stragier. 1995; Abanes-De Mello, *et al.* 2002), *spoIIM*(Smith, *et al.* 1993), and *spoIIQ* (Londono-Vallejo, *et al.* 1997) mutants display defects during engulfment and are thought to be involved in the completion of engulfment.

Activation and the Role of σ^G in the Forespore

Completion of engulfment of the forespore by the mother cell triggers the activation of new compartment-specific sigma factors: σ^G in the forespore and σ^K in the mother cell. σ^G is encoded by the *spoIIIG* gene, which is transcribed initially by σ^F RNAP and later, after σ^G activation, by σ^G RNAP. The *spoIIIG* gene is located right after the *spoIIG* operon in the genome and is transcribed by both the *spoIIIG* promoter as well as the *spoIIG* promoter. However, the fact that moving *spoIIIG* to an ectopic locus does not impair sporulation suggests that read-through transcription from the *spoIIG* promoter is not critical for σ^G expression (Sun, *et al.* 1991). Several factors prevent the activation of σ^G despite read-through transcription. First, the comparison of β-galactosidase activities between a transcriptional and a translational fusion of *spoIIIG-lacZ* indicates that very few *spoIIIG* have been translated into σ^G (Sun, *et al.* 1991). Secondly, SpoIIAB functions as an inhibitor

of σ^G activity *in vivo* and SpoIIAB binds to σ^G *in vitro* (Kirchman, *et al.* 1993; Kellner, *et al.* 1996; Serrano, *et al.* 2004). A *B. subtilis* strain with mutant σ^G showed increased σ^G activity only in the mother cell, but not in the forespore. The mutant σ^G functions normally except it cannot bind to SpoIIAB (Kellner, *et al.* 1996; Serrano, *et al.* 2004). This result suggests that SpoIIAB prevents σ^G activation in the mother cell. Transcription of *spoIIIG* requires the activation of σ^E in the mother cell, suggesting a possible signaling pathway from the mother cell to the forespore (Partridge and Errington. 1993). The expression of *spoIIIG* also depends on the expression of *spoIIQ* in the forespore (Sun, *et al.* 2000), due to the relationship between engulfment and activation of σ^G. Finally, σ^G, when it becomes active, also directs its own transcription (Sun, *et al.* 1991). This necessitates tight control of σ^G activation.

The mechanism of σ^G activation is unclear to date. The activation of σ^G is subject to post-translational regulation, which is evident in that both the *spoIIIJ* gene and the *spoIIIA* operon are required for σ^G activation (Errington, *et al.* 1992; Kellner, *et al.* 1996; Illing and Errington. 1991). Amino-terminal sequencing confirmed that the active form σ^G does not undergo proteolytic processing like pro-σ^E, suggesting that an unknown negative regulator holds σ^G inactive in the forespore. It is speculated that σ^E-controlled SpoIIIJ modulates the insertion of membrane proteins such as SpoIIIAE, one of the eight products of the *spoIIIA* operon, into the forespore membrane, where it functions (Serrano, *et al.* 2004). All eight protein products of the *spoIIIA* operon are required for σ^G activation. They are speculated to be located in the membranes separating the mother cell and the forespore, possibly forming a protein complex to translocate unidentified signals from the mother cell to the

intermembrane space of the forespore. The signals would then release inhibition by the putative negative regulator of σ^G.

The σ^G regulon contains at least 95 genes, including a few regulatory genes and mostly structural genes for spore formation. A few genes in the σ^G regulon encode metabolic enzymes (Steil, *et al.* 2005; Wang, *et al.* 2006). The metabolic enzymes help in glucose uptake, glycolysis, or glycine metabolism, although it is believed that most of the energy and materials required for spore formation at this stage come form the mother cell.

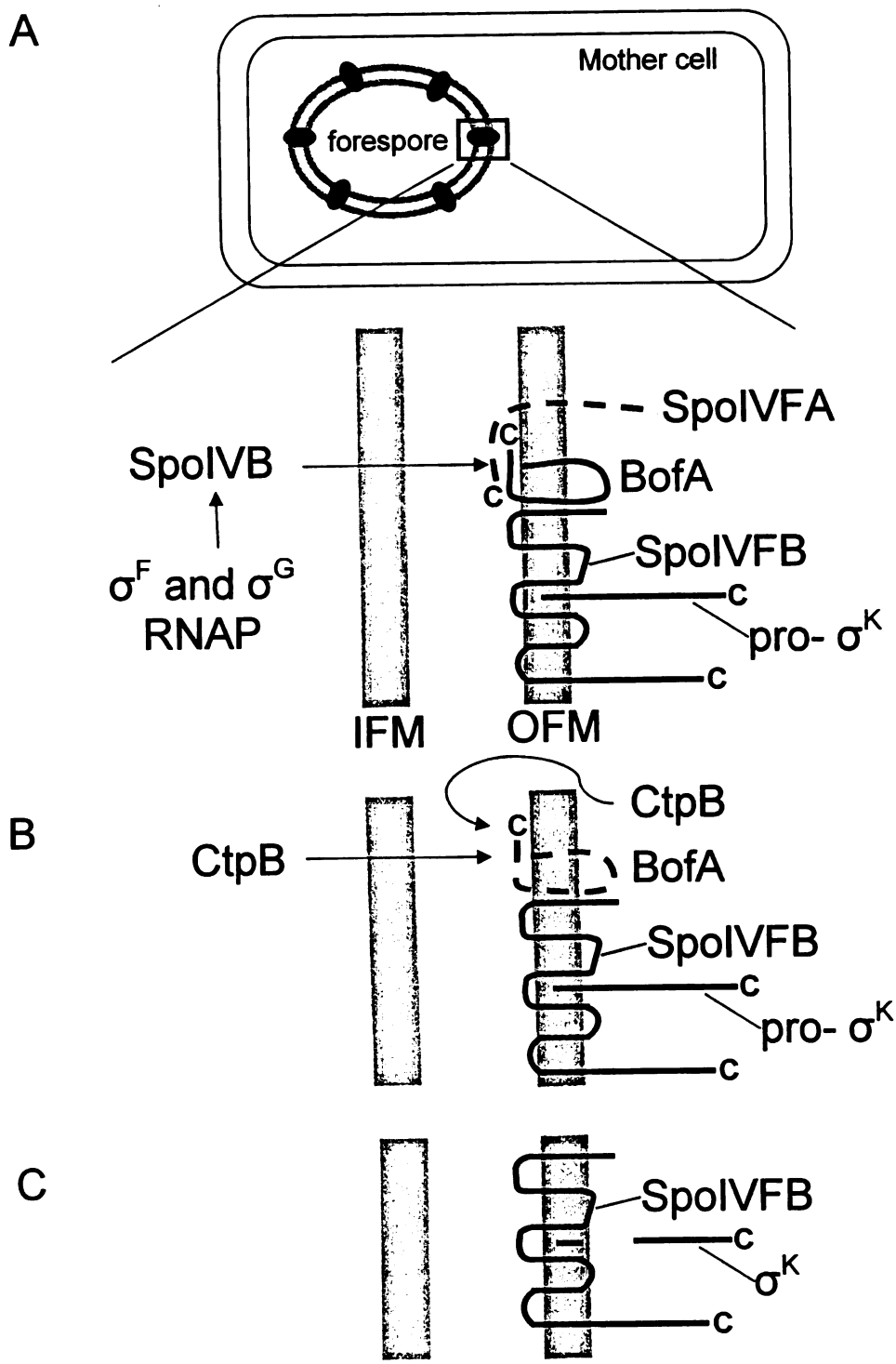
Activation and the Role of σ^K

The production of active σ^K, the last mother cell-specific sigma factor during sporulation, requires a developmental chromosomal DNA rearrangement, forespore signals coming from σ^G controlled genes, and processing of pro-σ^K.

A chromosomal DNA rearrangement event happens when *B. subtilis* cells are induced to sporulate. An approximately 48 kb DNA fragment, called the skin element, is located between *spoIVCB* and *spoIIIC* and is excised by SpoIVCB, which is a Hin family site-specific recombinase (Popham and Stragier. 1992). The resulting single, composite gene, *sigK*, encodes the precursor of σ^K, pro-σ^K (Stragier, *et al.* 1989). Transcription of *sigK* into pro-σ^K by σ^E RNAP in the mother cell starts from about 3 h after the initiation of sporulation and absolutely depends on SpoIIID (Kunkel, *et al.* 1988; Kroos, *et al.* 1989) .

The *sigK* gene product, pro-σ^K, is subject to regulated intramembrane proteolysis (RIP) to generate active σ^K (Kroos, *et al.* 1989; Stragier, *et al.* 1989) (Yu and Kroos. 2000) (Rudner, *et al.* 1999; Zhou and Kroos. 2005). Initiation of RIP requires σ^G-controlled SpoIVB coming from the forespore (Gomez, *et al.* 1995). SpoIVB contains a peptidase domain and a PDZ domain for protein-protein interaction; both are important for RIP of

Fig. 1.4. The processing of pro-σ^K. (A) The upper part shows a mother cell with a sporangium engulfed into the cytosol. Black ovals represent a protein complex that spans the double membranes of the forespore and is responsible for pro-σ^K processing. The lower part shows an enlarged view of the region including the protein complex (not all components are shown) that spans from the inner forespore membrane (IFM) to the outer forespore membrane (OFM). SpoIVB, a serine protease is made in the forespore first under the control of σ^F but its level must be boosted by σ^G RNAP. SpoIVB is believed to be secreted into the space between the IFM and the OFM. It cleaves the C-terminal part of SpoIVFA, a protector of BofA. (B) BofA exposed after the cleavage of SpoIVFA is subjected to cleavage by CtpB, a serine protease that is made in the mother cell under the control of σ^E and in the forespore under the control of σ^G. (C) SpoIVFB, after loss of BofA, cleaves the N-terminal of pro-σ^K, releasing σ^K into the mother cell. Adapted from (Kroos. 2007). See text for references.


A
Mother cell
forespore
SpoIVFA
C
SpoIVB
C
BofA
SpoIVFB
σF and σG
RNAP
c
pro- σK
c
IFM
OFM
B
CtpB
CtpB
C
BofA
SpoIVFB
c
pro- σK
c
C
SpoIVFB
c
σK
c

pro-σ^K (Wakeley, *et al.* 2000; Hoa, *et al.* 2001). The SpoIVB-mediated signal transduction pathway is called "the σ^K checkpoint", which is critical for efficient sporulation (Cutting, *et al.* 1990). σ^G-controlled *bofC* encodes an inhibitor that inhibits SpoIVB autoproteolysis and thus pro-σ^K processing (Gomez and Cutting. 1997).

In the mother cell, three loci are important for pro-σ^K processing: the σ^F and σ^E-controlled *ctpB*, and the σ^E-controlled *spoIVF* bicistronic operon and *bofA*. The two genes in the *spoIVF* operon are: *spoIVFA*, which encodes a target protein of SpoIVB; and *spoIVFB*, which encodes a membrane-embedded metalloprotease that carries out RIP of pro-σ^K. The *bofA* gene encodes an inhibitor of SpoIVFB. The *ctpB* gene encodes a serine protease with a PDZ domain, like SpoIVB, and cleaves BofA to release its inhibition of SpoIVFB (Zhou and Kroos. 2005).

It is proposed that RIP of pro-σ^K involves a three-step proteolytic cascade in which SpoIVB first cleaves SpoIVFA, CtpB then cleaves BofA, and finally SpoIVFB cleaves pro-σ^K (Zhou and Kroos. 2005). The process of RIP of pro-σ^K is schematically illustrated in Fig. 1.4.

σ^K, as the last mother cell-specific sigma factor, acts during stage IV (deposit of cortex) and stage V (coat formation) of sporulation. σ^K RNAP transcribes 144 genes that are located in 94 transcription units. Among them, 33 genes in 23 transcription units are transcribed by both σ^K RNAP and σ^E RNAP (Eichenberger, *et al.* 2004). σ^K RNAP transcribes many *cot* genes such as *cotA*, *cotD*, and *cotH*. It also transcribes *gerE*, which encodes the last mother cell specific transcription factor during sporulation (Eichenberger, *et al.* 2004).

Transcriptional Regulatory Network in the Mother Cell of Sporulating *B. subtilis*

Sporulation is an elaborate process in which gene expression is temporally and spatially controlled to form a spore. In the mother cell of sporulating *B. subtilis*, a hierarchical cascade of four transcription factors, σ^E, SpoIIID, σ^K, and lastly, GerE, sequentially control compartmental gene expression. Negative effects exist in the sporulating mother cell, adding complexity to the regulatory cascade and forming a regulatory network. The significance of these negative links to sporulation was unknown, leading to the research interests in Chapter II and Chapter III of this Dissertation. Since σ^E, SpoIIID and σ^K have been discussed above, below I introduce the other two important regulatory factors in the mother cell transcriptional regulatory network, SpoIIID and GerE.

Appearance and The Role of SpoIIID

SpoIIID is a small sequence-specific DNA binding protein that binds to DNA sequences resembling WWRRACAR-Y (W is A or T, R is purine, and Y is pyrimidine) (Halberg and Kroos. 1994; Zhang, *et al.* 1997; Ichikawa and Kroos. 2000). The SpoIIID protein contains 93 amino acids and has a molecular weight of approximately 10.8 kDa (de Lencastre and Piggot. 1979; Tatti, *et al.* 1991). Translation of the mRNA encoding SpoIIID depends on the translation of a small upstream open reading frame (*usd*), which is located in between the promoter and the coding sequence of *spoIIID* (Decatur, *et al.* 1997). The mRNA for *usd-spoIIID* contains an inverted repeat sequence and is predicted to form a stem-loop structure. The synthesis of SpoIIID is proposed to require interruption of the stem-loop by translation through the upstream open reading frame (Decatur, *et al.* 1997). Production of SpoIIID starts at approximately 3 h after the initiation of sporulation, peaks at about 5 h into sporulation, and drops sharply thereafter (Halberg and Kroos. 1992). The decrease of SpoIIID after its peak is believed to be the consequence of active σ^K RNAP-

mediated negative feedback on early gene expression (Zhang and Kroos. 1997) (see below).

SpoIIID is predicted to be in the λ phage cI superfamily of helix-turn-helix proteins (Himes and Kroos.). After *spoIIID* is transcribed by σ^E RNAP, SpoIIID works in conjunction with σ^E RNAP, and later on σ^K RNAP, as both a repressor and an activator to regulate gene expression in the mother cell of sporulating *B. subtilis.* As many as 62 transcription units are down-regulated and as least 8 transcription units, including *spoIIID* (an autoregulation loop) and *sigK*, are up-regulated by SpoIIID (Eichenberger, *et al.* 2004). SpoIIID regulates the appearance of σ^K, the last mother cell-specific sigma factor, by at least two mechanisms. First, SpoIIID is required for the chromosomal rearrangement to generate the composite gene (*sigK*) that encodes pro-σ^K (Stragier, *et al.* 1989; Kunkel, *et al.* 1990). Second, transcription of *sigK* absolutely depends on SpoIIID (Kunkel, *et al.* 1988; Kroos, *et al.* 1989). Other genes that are regulated by SpoIIID could have important roles in sporulation. For example, SpoIIID negatively regulates a σ^E–controlled polysaccharide deacetylase gene homologue (*pdaB*), which, when mutated, is deficient in spore formation (Fukushima, *et al.* 2004). The gene encoding an unusual small protein SpoVM (*spoVM*) is activated by SpoIIID and transcribed by σ^E RNAP. SpoVM is required for the asymmetric cell division and completion of forespore engulfment since it is involved in the formation of the "Z-ring". A possible mechanism is that SpoVM, as a substrate of FtsH, can save FtsZ from degradation by FtsH allowing formation of the "Z-ring" (Srinivasan, *et al.* 2007; Zhang, *et al.* 1997). Potential impact of SpoIIID on sporulation could also be influenced by the combined action of SpoIIID and GerE on regulation of some *cot* genes, such as *cotC* and *cotX* (Ichikawa and Kroos. 2000).

Appearance and the Role of GerE

GerE is encoded by *gerE* and is transcribed by σ^K RNAP at about 4 h after the initiation of sporulation. It works as both an activator and a repressor to regulate the mother cell gene expression. Approximately 36 transcriptional units are down-regulated by GerE, including the *sigK* gene. At least 27 transcriptional units are up-regulated by GerE, including the *cotC* and *cotD* genes (Eichenberger, *et al.* 2004). GerE binds to the promoter region of *sigK* and negatively regulates the production of σ^K (see below). As a consequence, genes in the σ^K regulon are generally affected by GerE appearance. It has been shown that SpoIIID and GerE compete for binding sites on promoter regions of some genes, such as *cotC* (Ichikawa and Kroos. 2000), implying interactions between transcriptional regulators in the mother cell.

Negative Links in the Mother Cell Regulatory Network

Control of mother cell gene expression in sporulating *B. subtilis* is accomplished by a cascade of regulators in the order σ^E-SpoIIID-σ^K-GerE. A second layer of control, known as negative feed-forward or feedback effects, connects different components of the cascade, increasing complexity of the network.

One example of negative feedback is that from σ^K RNAP on early sporulation genes. Transcriptionally active σ^K RNAP inhibits the transcription of the *sigE* operon, resulting in the reduced accumulation of *spoIIID* mRNA since σ^E RNAP transcribes *spoIIID* (Zhang, *et al.* 1999). Two different pathways of σ^K repression of early gene expression have been described. When σ^K is made one hour earlier than normal, it appears to exert its negative effect through the Spo0A-P-stimulated transcriptional activation of the *sigE* operon. When σ^K is expressed at the normal time, it affects the activity of σ^A RNAP, which transcribes the

sigE operon (Zhang, *et al.* 1999). This conclusion is supported by the observation that another early sporulation gene *ald* is also inhibited when σ^K is expressed normally (Zhang, *et al.* 1999). *ald* encodes an alanine dehydrogenase and is also transcribed by σ^A RNAP.

A second example of a negative feedback loop in the mother cell is provided by GerE, which, after its gene is transcribed by σ^K RNAP, can down-regulate σ^K by repressing *sigK* (Ichikawa, *et al.* 1999). GerE has been shown to inhibit *sigK* transcription *in vitro* (Zheng, *et al.* 1992). A *gerE* mutant shows a two-fold increase in expression of *sigK* during late sporulation when compared to a strain containing wild-type GerE (Ichikawa, *et al.* 1999).

A third example of negative control is exerted by SpoIIID on *cotC* expression. The combined action of SpoIIID and GerE regulates the expression of several *cot* genes (Ichikawa and Kroos. 2000). GerE activates σ^K RNAP to transcribe *cotC*, *cotB* and *cotX* by binding to two sites in each promoter region. Meanwhile, SpoIIID provides another level of control on these genes by binding to these promoter regions to repress transcription. An *in vitro* transcription assay showed that *cotC* is repressed by a low level of SpoIIID. DNase I footprinting experiments revealed two high-affinity SpoIIID binding sites in the *cotC* promoter region (Ichikawa and Kroos. 2000). These SpoIIID binding sites overlap the binding sites of GerE and σ^K RNAP, suggesting that competition for the binding sites regulates *cotC* transcription. Expression of *cotC-lacZ* is delayed one hour compared with that of *cotB-lacZ*. The mRNA of *cotC* reaches its peak two hours later than the peak time of *cotB* mRNA (Ichikawa and Kroos. 2000). The difference is believed to be that *cotC*, but not *cotB*, is repressed by SpoIIID.

The importance of these negative links in the mother cell regulatory network is the question that we address in Chapters II and III of this dissertation.

Structure and Properties of the Spore

A mature spore is composed of three substructures that play different roles in protection of the spore: the core, the cortex, and the coat. The core is defined as the interior compartment of the spore and houses small acid-soluble proteins (SASP) (Setlow. 1995) that are associated with condensed DNA. The condensed DNA structure is resistant to UV light. The core is surrounded by a cross-linked peptidoglycan layer called cortex. Small molecules such as water can pass through this woven-fabric like structure. However, the core remains dry largely because the cortex constricts the core (Driks. 2003). Finally, at least 60 proteins assemble the multilayer coat of a spore outside the membrane surrounding cortex. Two layers of the coat have been identified by thin-section microscopy: a lightly stained, fine striated inner coat and a darkly stained, coarsely layered outer coat.

The resistance properties of a spore are closely related to the physical structure of it. A series of assays have been developed to define spore defects. The heat resistance assay tests the dehydrated state of the spore. The heat-resistant property primarily depends on the maintenance of the cortex and to a lesser extent the coat of the spore (Popham, *et al.* 1996). The cortex also provides resistance against organic solvents. The lysozyme resistance property is due to the coat (Zheng, *et al.* 1988). The germination assay tests the permeability of the coat and the sieved-structure of the cortex to germinants (sugar molecules, amino acid molecules, etc.). Complementary to these assays are other techniques such as electron microscopy (Cutting, *et al.* 1991; Margolis, *et al.* 1993) and spore coat protein analysis (Henriques, *et al.* 1995). A spore morphogenetic mutant may not exhibit defects in all assays. For example, although a *cotG* mutant showed no strong effects when examined by heat resistance and germination assays, the spore of a *cotG*

mutant shows a significant defect when examined by electron microscopy (Henriques. 1998)

Development Transcription Regulatory Network

There are two types of transcriptional regulatory networks: the sensory transcription networks, which respond to external stress or nutrients, and the developmental transcription network, which guide differentiation events (Alon. 2003). Interactions within the former happen rapidly and usually are reversible, while interactions within the latter are slow and irreversible (Alon. 2003). The transcriptional regulatory network in the mother cell of sporulating *B. subtilis* belongs to the developmental transcription network and therefore shares some common characteristics of it.

First of all, all transcriptional regulatory networks are built by a set of recurring regulation patterns, called "motifs". Each motif can carry out certain functions. For example, a negative autoregulation motif can speed up the time of response for a genetic circuit and reduce cell-cell variation in protein level (Alon. 2007). In developmental transcription networks, the following types of motifs are often seen: negative autoregulation, feedforward loop, feedback loop, transcription cascade, single-input modules, and dense overlapping regulons (Alon. 2007).

Another common feature of developmental transcription networks is robustness to component tolerances (Alon. 2003). The notion of "robustness" in biology was first brought out by Michael A. Savageau in 1971(Savageau. 1971) and has been widely applied thereafter. Robustness is defined as the ability of a system to maintain performance or phenotypic stability in the face of diverse perturbations and uncertainty such as environmental changes, stochastic events, and genetic variation (Stelling, *et al.* 2004).

Feedback and feedforward controls are common elements in a robust circuit and are thought to be necessary for a robust implementation of exact adaptation. A study in *Saccharomyces cerevisiae* suggested that in a global network, a topological structure favors robustness (Maslov and Sneppen. 2002). This type of topology has a small number of nodes with very high connectivity, while most nodes have low connectivity. Connections between highly connected nodes are systematically suppressed, whereas connections between highly connected nodes and low-connected nodes are favored. This topology decreases the likelihood of crosstalk between different modules in the network and therefore enhances the overall robustness of the network by localizing perturbation effects (Maslov and Sneppen. 2002). On the other hand, no transcriptional network can tolerate all changes. Sensitivity is required for a transcriptional network to maintain its function. The balance between robustness and sensitivity optimizes the function of a transcriptional network.

In this dissertation, we explored the properties of the developmental transcription network in the mother cell of sporulating *B. subtilis*, by perturbing key nodes within this network. The system showed typical behavior of a transcription network: when a node with high connectivity, SpoIIID in our study, was engineered to maintain the SpoIIID level late during sporulation, this perturbation went beyond the capacity of the system and caused a spore defect. On the other hand, perturbation of another important node, by elimination of GerE negative feedback on *sigK*, was tolerated, demonstrating robustness of the network.

CHAPTER II: Maintaining the Transcriptional Factor SpoIIID Level Late during Sporulation Causes Spore Defects in *Bacillus subtilis*

ABSTRACT

During sporulation of *Bacillus subtilis*, four regulatory proteins act in the order σ^E, SpoIIID, σ^K, and GerE to temporally control gene expression in the mother cell. σ^E and σ^K work sequentially with core RNA polymerase to transcribe different sets of genes with certain overlapping. SpoIIID and GerE are small, sequence-specific DNA-binding proteins that activate or repress transcription of many genes. Previous studies showed that transcriptionally active σ^K RNA polymerase inhibits early mother cell gene expression, reducing accumulation of SpoIIID late in sporulation. Here, the effects of perturbing the mother cell gene regulatory network by maintaining the SpoIIID level late during sporulation are reported. Persistent expression was obtained by fusing *spoIIID* to the σ^K–controlled *gerE* promoter on a multicopy plasmid. Fewer heat- and lysozyme-resistant spores were produced by the strain with persistent *spoIIID* expression, but the number of spores resistant to organic solvents was unchanged, as was their germination ability. Transmission electron microscopy showed structural defects in the spore coat. Reporter fusions to σ^K-dependent promoters showed lower expression of *gerE* and *cotC*, and higher expression of *cotD*. Altered expression of *cot* genes, which encode spore coat proteins, may account for the spore structural defects. These results suggest that one role of negative feedback by σ^K RNA polymerase on early mother cell gene expression, is to lower the level of SpoIIID late during sporulation, in order to allow normal expression of genes in the σ^K regulon.

INTRODUCTION

Upon sensing nutrient limitation, the Gram-positive bacterium *Bacillus subtilis* initiates an adaptive process called sporulation that culminates in the formation of a dormant spore (reviewed in Driks. 2002; Kroos. 2007). During sporulation, *B. subtilis* undergoes an asymmetric division, forming a larger mother cell (MC) compartment and a smaller forespore (FS) compartment. Different genes are expressed in each compartment. Signaling between MC and the FS ensures precise temporal and spatial control of gene expression. The products of genes expressed in the two cell types cause further morphological changes. The FS is engulfed by the MC membrane, surrounding the FS with two membranes and pinching it off as a protoplast within the MC. Then, cell wall-like material called cortex is deposited between the two membranes of the FS. Next, the spore coat is assembled on the surface of the FS and its interior or core is dehydrated. The mature spore, which is resistant to environmental insults such as heat, ultraviolet light, lytic enzymes, and chemicals, is released by lysis of the MC. Under favorable environmental conditions, the dormant spore germinates and forms a growing vegetative cell.

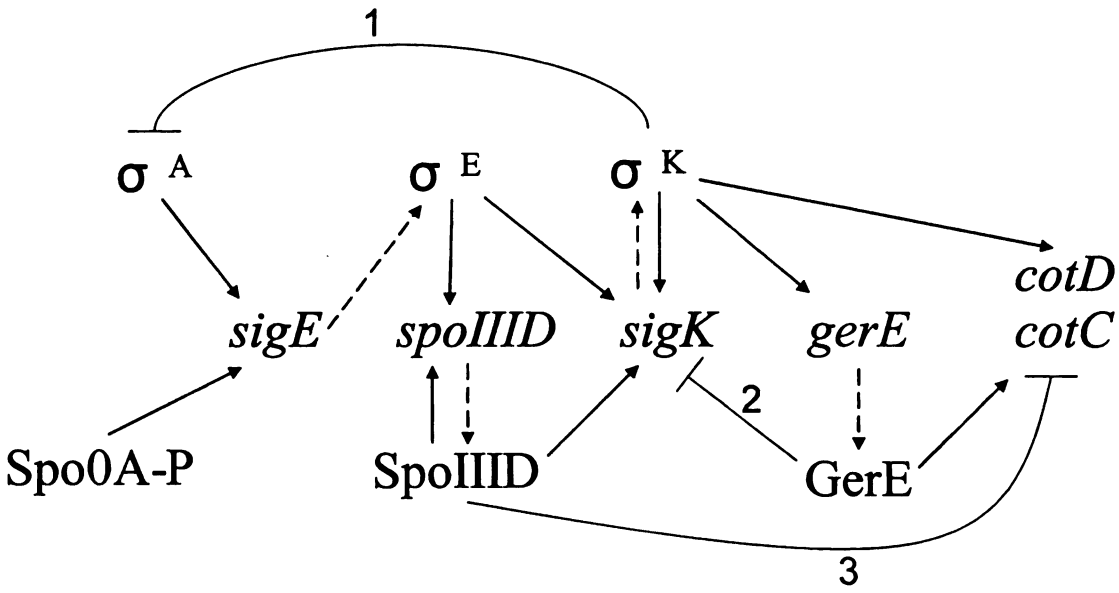
In the MC, a hierarchical cascade of four regulatory factors, σ^E, SpoIIID, σ^K and lastly GerE, governs gene expression during sporulation (Zheng and Losick. 1990; Eichenberger, *et al.* 2004) (Fig. 2.1). The *sigE* gene is transcribed by σ^A RNA polymerase (RNAP) , the major form of RNAP in growing cells, prior to asymmetric division, but initiation of transcription at the promoter of the *spoIIG* operon, which includes *sigE*, requires phosphorylated Spo0A (Spo0A-P), a key transcription factor that governs entry into the sporulation process (reviewed in Haldenwang. 1995). After formation of the asymmetric septum, Spo0A-P persists in the MC and the product of *sigE*, pro-σ^E, accumulates primarily

in the MC (Fujita and Losick. 2002; Fujita and Losick. 2003). Pro-σ^E is cleaved in response to a signal from the FS (Hofmeister, *et al.* 1995; Karow, *et al.* 1995; Londono-Vallejo and Stragier. 1995), forming active σ^E RNAP in the MC, where it transcribes more than 260 genes, including those that encode SpoIIID and pro-σ^K (Eichenberger, *et al.* 2004) (Fig. 2.1). SpoIIID is a small sequence-specific DNA-binding protein (Halberg and Kroos. 1994) that positively regulates as least eight transcription units in the σ^E regulon, including the *sigK* gene (Fig. 2.1), and negatively regulates at least 62 transcription units (Eichenberger, *et al.* 2004). SpoIIID also negatively regulates genes in the σ^K regulon, including *cotC* and *cotD* (Kroos, *et al.* 1989; Halberg and Kroos. 1994; Ichikawa and Kroos. 2000) (Fig. 2.1), which encode spore coat proteins (Donovan, *et al.* 1987). The *sigK* gene encodes pro-σ^K (Stragier, *et al.* 1989). Like pro-σ^E, pro-σ^K is activated by proteolytic cleavage in response to a FS signal (Cutting, *et al.* 1990; Lu, *et al.* 1990), although the mechanisms of signaling and proteolysis are different (reviewed in Kroos. 2007). σ^K RNAP transcribes more than 100 genes, including many *cot* genes that encodes spore coat proteins, and the gene encoding GerE (Eichenberger, *et al.* 2004) (Fig. 2.1). Like SpoIIID, GerE is a small sequence-specific DNA-binding protein (Zheng, *et al.* 1992). It positively regulates at least 27 transcription units, including the *cotC* and *cotD* genes, and negatively regulates at least 36 transcription units (Eichenberger, *et al.* 2004), including the *sigK* gene (Zheng, *et al.* 1992; Ichikawa, *et al.* 1999) (Fig. 2.1).

All of the regulatory interactions depicted in Figure 1 are direct effects based on *in vitro* studies, with one exception. The negative feedback loop from σ^K to σ^A is inferred from *in vivo* studies (Halberg and Kroos. 1992; Zhang and Kroos. 1997; Zhang, *et al.* 1999). Transcriptionally active σ^K RNAP inhibits early MC gene expression, including

transcription of *sigE* by σ^A RNAP (Zhang, *et al.* 1999). This results in reduced accumulation of σ^E and SpoIIID late during sporulation (Halberg and Kroos. 1992; Zhang and Kroos. 1997). Neither the mechanism nor the significance of this negative feedback loop is known. To access its significance, we engineered ectopic expression of *spoIIID*, such that the SpoIIID level was maintained late during sporulation. This resulted in fewer heat- and lysozyme-resistant spores being produced, and most of the spores exhibited a structural change in their coat. Perturbing the MC gene regulatory network in this way also uncovered a novel connection that was unexpected from *in vitro* studies; persistent *spoIIID* expression lowered expression of a *gerE-lacZ* fusion. These results demonstrate the importance of lowering the SpoIIID level late during sporulation, which is one consequence of the σ^K-dependent negative feedback loop.

Fig. 2.1. Gene regulatory network in the MC during sporulation of *B. subtilis*. Dashed arrows indicate gene to protein relationships. Solid arrows or lines with barred ends represent positive or negative regulation, respectively. Numbers 1, 2, and 3 represent three negative regulatory loops, respectively. See text for references.


1
σ A
σ E
σ K
cotD
cotC
sigE
spoIIID
sigK
gerE
2
Spo0A-P
SpoIIID
GerE
3

MATERIALS AND METHODS

Construction of plasmids. A DNA fragment containing the *gerE* promoter region was synthesized by the polymerase chain reaction (PCR) using primers 5'—GGGAATTCCTGGACGGATGAGGAGAAAG—3' and 5'—GGGAAGCTTGCGAATCAGAAACGAATGG—3', which contain *Eco*RI and *Hin*dIII restriction sites (underlined), respectively. This fragment spans from -96 bp to +33 bp relative to the transcriptional start site of *gerE* and does not include the ribosomal binding site (Cutting, *et al.* 1989). The PCR product was digested with *Eco*RI and *Hin*dIII, and ligated into *Eco*RI-*Hin*dIII-digested pDG364 (Karmazyn-Campelli, *et al.* 1989), resulting in plasmid pRB1 (Burri and Kroos.). The sequence of this insert was determined by the Research Technology Support Facility at Michigan State University in order to ensure that no errors occurred during the PCR. A DNA fragment containing the *spoIIID* gene, from +145 to +463 relative to the transcriptional start site of *spoIIID* (Tatti, *et al.* 1991), including the ribosomal binding site and the entire coding region, was amplified by PCR using primers 5'—GGAAGCTTAGGGAGGTCGAGTGGTGTG—3' and 5'—CGGATCCCAAGAAGGCAATGCCAGG—3', which contain *Hin*dIII and *Bam*HI restriction sites (underlined), respectively. The PCR product was digested with *Hin*dIII and *Bam*HI, and ligated with *Hin*dIII-*Bam*HI-digested pRB1, resulting in pRB2 (Burri and Kroos.). The sequence of the entire P_{gerE}-*spoIIID* fusion in pRB2 was determined as described above. Next, pRB2 was digested with *Eco*RI and *Bam*HI, the P_{gerE}-*spoIIID* fragment was purified, and it was ligated with a 6.6-kb fragment of *Eco*RI (partial digestion)-*Bam*HI-digested pDG148 (Stragier, *et al.* 1988), resulting in pJP1. To construct a control plasmid containing only P_{gerE}, pJP1 was digested with *Bam*HI and *Hin*dIII to

remove the *spoIIID* insert. The resulting 6.7-kb fragment was treated with the Klenow fragment of DNA polymerase I and the blunt ends were ligated by T4 DNA ligase, resulting in pJP2.

Bacterial strains. *Escherichia coli* strain AG115 [*araD139* Δ(*ara,leu*)*7697* Δ*lacX74* *galU galK hsr hsm*$^+$ *strA* (F′ *proAB lacI*^{q}*Z*::Tn5)] was obtained from A. Grossman (Massachusetts Institute of Technology), and used for construction and maintenance of plasmids. Luria-Bertani medium (LB) (Sambrook, *et al.* 1989) was used to grow *E. coli* and *B. subtilis*, and was supplemented with appropriate antibiotics. PY79 (Spo$^+$ prototroph) (Youngman, *et al.* 1984), BK556 (*spoIVCB23*) (Kunkel, *et al.* 1989), and OR825 (PY79 SPβ::*cotC-lacZ*) (Crater, *et al.* 2002) were provided by R. Losick (Harvard University). EUDC9901 (*trpC2 pheA1 gerE*::*kan*) (Crater and Moran. 2002) was provided by C. Moran (Emory University). pJP1 was transformed into PY79 and OR825 as described (Harwood and Cutting. 1990), with selection by addition of kanamycin sulfate (5 μg/ml) to the medium, generating strains BJP1 and BJP3, respectively. Similarly, pJP2 was transformed into PY79 and OR825 to generate strains BJP2 and BJP4, respectively. SPβ::*cotD-lacZ* and SPβ::*gerE-lacZ* have been described (Cutting, *et al.* 1989; Cutting, *et al.* 1990). Specialized transduction was used to move *lacZ* fusions into BJP1, BJP2, and PY79 as described (Harwood and Cutting. 1990). Transductants were selected on LB agar containing chloramphenicol (5 μg/ml). In each case, at least 10 candidates were toothpicked onto a DSM agar (Harwood and Cutting. 1990) plate with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 μg/ml). Three or more isolates with average blue color were saved for further analysis, excluding occasional isolates with abnormally high or low β-galactosidase activity.

Cell growth and sporulation. Sporulation was induced by resuspension of cells in SM medium as described (Harwood and Cutting. 1990). The time of resuspension is defined as the onset of sporulation (T_0).

Western blot analysis. Starting at 3 h into sporulation (T_3) and at hourly intervals thereafter until T_9, 0.5 ml samples were subjected to centrifugation (14,000 g for 1 min), the supernatants were removed and cell pellets were stored at -70°C. Preparation of whole-cell extracts, electrophoresis, and electroblotting were as described (Lu, *et al.* 1990; Halberg and Kroos. 1992). The membranes were probed with anti-SpoIIID antiserum diluted 1:10,000 (Halberg and Kroos. 1992) or polyclonal anti-pro-σ^K antiserum diluted 1:10,000 (Lu, *et al.* 1990). Immunodetection of primary antibodies was as described (Kroos, *et al.* 2002).

Analysis of β-galactosidase activity. Samples were collected during sporulation as described above. Cell pellets were stored at -70°C prior to the assay. Cells were resuspended, then treated with lysozyme and permeabilized by toluene as described (Miller. 1972). The β-galactosidase specific activity was determined as described (Miller. 1972) using *o*-nitrophenol-β-D-galactopyranoside (ONPG) as the substrate. One unit of the enzyme hydrolyzed 1 μmol of substrate per minute per unit of initial culture optical density at 595 nm.

Spore purification, and germination and resistance assays. Spores were harvested at T_{24} by centrifugation at 7,000 g for 10 min, washed with 4°C water, and stored at 4°C overnight. The next day, spores were purified on a step gradient of 20% to 50% RenoCal-76 (Bracco Diagnostics Inc.) as described (Henriques, *et al.* 1995). Purity of the spores was verified by microscopy. The germination assay was performed with purified spores using

L-alanine (10 mM) as the germinant as described (Naclerio, *et al.* 1996). Assays for resistance to heat, lysozyme, and organic solvents were performed at T_{24} without spore purification, as described (Harwood and Cutting. 1990).

Transmission electron microscopy of spores. Spores were harvested at T_{24}, washed with water, and immediately fixed as described (McPherson, *et al.* 2005).

RESULTS

Engineering *B. subtilis* to maintain the SpoIIID level late during sporulation. In wild-type PY79, the level of SpoIIID reaches a maximum at 5 h into sporulation and decreases thereafter (Halberg and Kroos. 1992) (Fig. 2.2A). To test whether the SpoIIID decrease is important, we initially constructed a *B. subtilis* strain with a single copy of a P_{gerE}-*spoIIID* fusion integrated at the *amyE* locus in the chromosome. Unlike the native *spoIIID* promoter, which is under σ^E RNAP control, the *gerE* promoter is under control of the later-acting σ^K RNAP (Fig. 2.1). However, at single copy, the P_{gerE}-*spoIIID* fusion had no apparent effect on the level of SpoIIID during sporulation (Burri and Kroos.). Therefore, we constructed pJP1, a multicopy plasmid bearing the P_{gerE}-*spoIIID* fusion. pJP1 was transformed into PY79, resulting in *B. subtilis* BJP1. As a control, a plasmid with only P_{gerE}, pJP2, was also transformed into PY79, resulting in strain BJP2. During sporulation, the SpoIIID level in BJP2 decreased after 5 h (Fig. 2.2C), as observed for PY79 (Fig. 2.2A), but in BJP1 the level of SpoIIID remained about the same at least until 9 h into sporulation (Fig. 2.2B). We conclude that BJP1 maintains the level of SpoIIID late during sporulation.

Pro-σ^K and σ^K levels are unchanged in *B. subtilis* that maintain the SpoIIID level late during sporulation. SpoIIID activates or represses many genes in the MC (Halberg and Kroos. 1994; Halberg, *et al.* 1995; Eichenberger, *et al.* 2004). One key gene that it activates is *sigK* (Fig. 2.1), which encodes pro-σ^K (Stragier, *et al.* 1989). SpoIIID activates *sigK* transcription by RNAP containing either σ^E or σ^K (Halberg and Kroos. 1994). Hence, maintaining the SpoIIID level late during sporulation might elevate the pro-σ^K and/or σ^K

Fig. 2.2. Level of SpoIIID during sporulation. (A) *B. subtilis* wild-type (WT) PY79, (B) BJP1 bearing pJP1 with the P_{gerE}-*spoIIID* insert, and (C) BJP2 bearing pJP2 with the P_{gerE} insert were induced to sporulate by resuspension in SM medium and samples are collected at hourly intervals beginning at 3 h after the onset of sporulation. Equal volumes (5 ul) of whole cell lysates were fractionated on SDS 14% polyacrlamide gels and subjected to Western blot analysis with anti-SpoIIID antiserum.

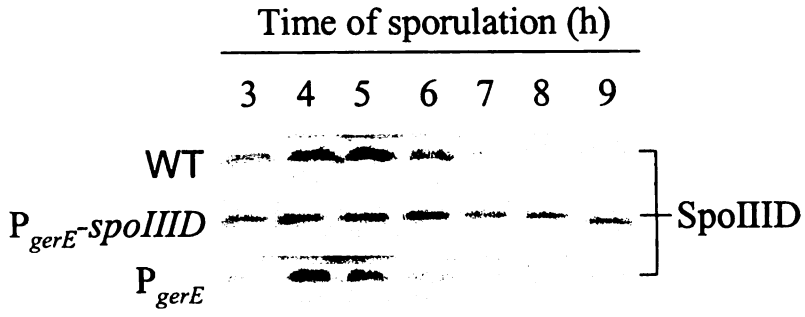

Time of sporulation (h)
3 4 5 6 7 8 9
WT
P gerE-spoIIID
P gerE
SpoIIID

Fig. 2.3. Pro-σ^K and σ^K levels during sporulation. (A) *B. subtilis* wild-type (WT) PY79, (B) BJP1, and (C) BJP2 were induce to sporulate and samples were subjected to Western blot analysis as described in the Fig. 2.2 legend except with anti-pro-σ^K serum.

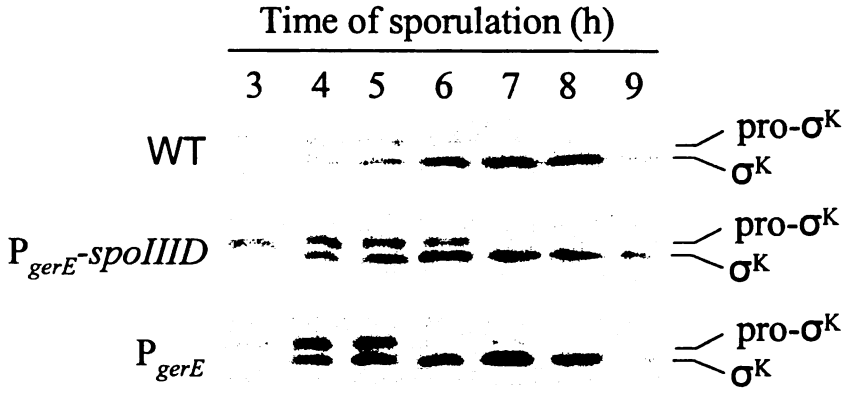

Time of sporulation (h)
3 4 5 6 7 8 9
WT
pro-σK
σK
PgerE-spoIIID
pro-σK
σK
PgerE
pro-σK
σK

Fig. 2.4. Effects on gene expression. β-galactosidase activity during sporulation after resuspension in SM medium was measured for *B. subtilis* containing *lacZ* fused to the (A) *gerE*, (B) *cotC*, or (C) *cotD* promoter in wild-type PY79 (◊) or strains bearing multicopy P_{gerE}-*spoIIID* (■) or P_{gerE} (▲). Each point is the average of three determinations and error bars show 1 standard deviation of the data.

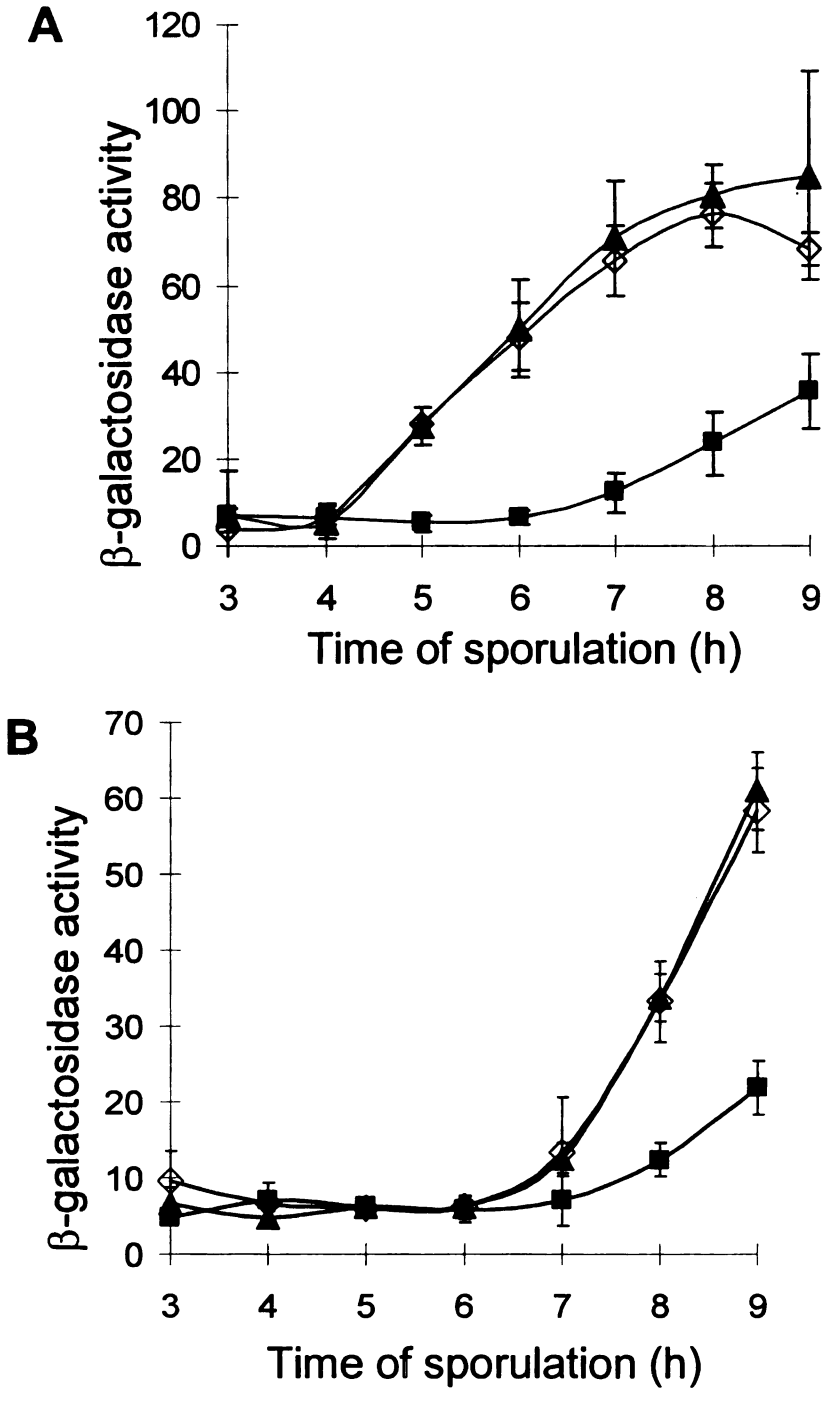
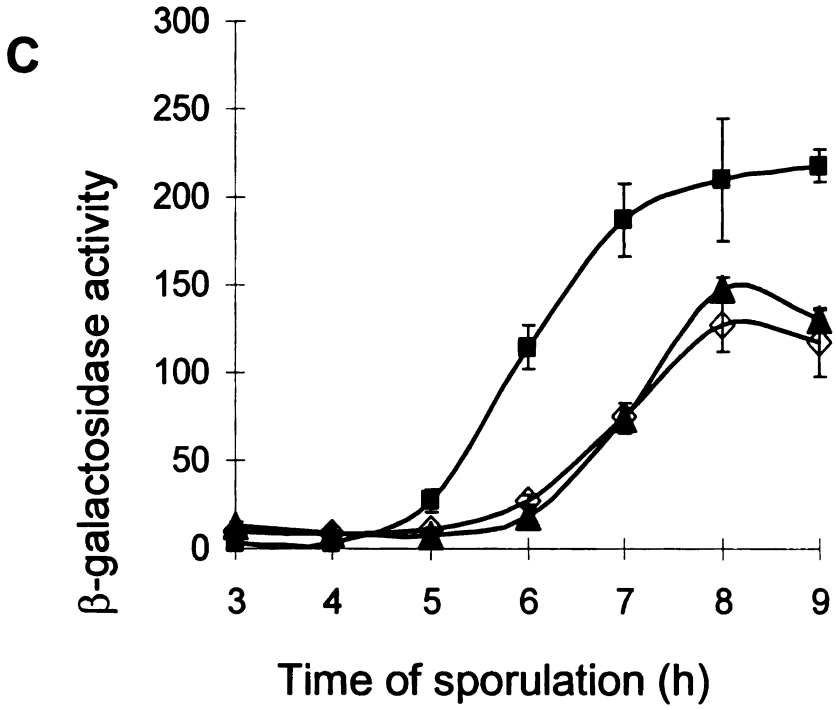

A
120
100
80
60
40
20
0
β-galactosidase activity
3
4
5
6
7
8
9
Time of sporulation (h)
B
70
60
50
40
30
20
10
0
β-galactosidase activity
3
4
5
6
7
8
9
Time of sporulation (h)

Fig. 2.4 (continued)



level at late times, if SpoIIID is limiting for transcription of *sigK* and/or for processing of pro-σ^K to σ^K.

The levels of pro-σ^K and σ^K were indistinguishable in PY79, BJP1, and BJP2 (Fig. 2.3). This result indicates that SpoIIID is not the limiting factor for pro-σ^K or σ^K production late during sporulation. Negative feedback loops in the MC network (Fig. 2.1) may limit σ^E and σ^K production late during sporulation, counteracting potential activation by SpoIIID of *sigK* transcription.

Maintaining the SpoIIID level late during sporulation alters gene expression. SpoIIID strongly represses transcription of *cotD* (Halberg and Kroos. 1994; Halberg, *et al.* 1995) and *cotC* (Ichikawa and Kroos. 2000) by σ^K RNAP *in vitro*, and weakly activates transcription of *gerE* by σ^K RNAP *in vitro* (Halberg, *et al.* 1995). Using *lacZ* reporter fusions to these three promoters, we examined the effect of persistent *spoIIID* expression late during sporulation. Surprisingly, *gerE-lacZ* expression was reduced in the strain that maintains the SpoIIID level late during sporulation, as compared with wild type or the control strain with multicopy P_{gerE}, which were indistinguishable (Fig. 2.4A). This result was unanticipated from the *in vitro* study (Halberg, *et al.* 1995). It suggests that SpoIIID can directly or indirectly inhibit *gerE* expression.

Since SpoIIID represses *cotC* transcription by σ^K RNAP *in vitro* (Ichikawa and Kroos. 2000), it was not surprising that *cotC-lacZ* expression was lower in the strain with multicopy P_{gerE}-*spoIIID* than in wild type or the control strain bearing multicopy P_{gerE} (Fig. 2.4B). Moreover, GerE activates *cotC* transcription by σ^K RNAP *in vitro* (Zheng, *et al.* 1992), so diminished *gerE* expression in the P_{gerE}-*spoIIID* strain (Fig. 2.4A) may also contribute to its diminished *cotC-lacZ* expression (Fig. 2.4B). Expression of *cotD-lacZ*

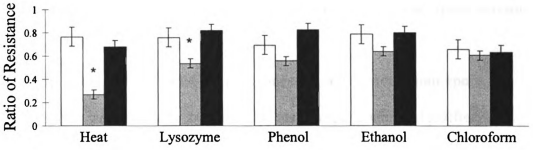
rose earlier and reached a higher level in the P_{gerE}-*spoIIID* strain than in wild type or the P_{gerE} control strain (Fig. 2.4C). This may not be surprising in light of the diminished *gerE* expression in the P_{gerE}-*spoIIID* strain (Fig. 4A), because a low level of GerE activates *cotD* transcription by σ^K RNAP *in vitro*, but at a higher level GerE represses (Ichikawa, *et al.* 1999). Although SpoIIID strongly represses transcription of *cotD* by σ^K RNAP *in vitro* (Halberg and Kroos. 1994; Halberg, *et al.* 1995), the SpoIIID level in the P_{gerE}-*spoIIID* strain may not be high enough for repression to be the dominant effect.

We conclude that maintaining the SpoIIID level late during sporulation lowers *gerE-lacZ* expression and alters the expression of other genes in the σ^K regulon, increasing or decreasing their expression depending on the effects of SpoIIID and GerE at particular promoters.

Persistent *spoIIID* expression affects certain spore resistance properties. The effects of maintaining the SpoIIID level late during sporulation on the numbers of heat-, lysozyme-, phenol-, ethanol-, and chloroform-resistant spores were measured. *B. subtilis* strain BK556 (*spoIVCB23*), which fails to make σ^K (Lu, *et al.* 1990), served as a negative control. In each assay, BK556 produced at least 10^5 less resistant spores (data not shown) than wild type (PY79 in Fig. 2.5).

About 70% of the viable cells for wild-type PY79 and the multicopy P_{gerE} control strain BJP2 at 24 h after the onset of sporulation were heat-resistant (Fig. 2.5). However, for the multicopy P_{gerE}-*spoIIID* strain BJP1, only about 30% of the viable cells were heat-resistant. Statistical analysis of the data indicated no significant difference between PY79 and BJP2, but the differences between these two strains and BJP1 were significant (*p*-values of less

Fig. 2.5. Resistance properties of spores. The ratio of colony-forming units after the indicated treatments to before the treatments was determined for *B. subtilis* wild-type PY79 (white bars), strain BJP1 bearing multicopy P_{gerE}-*spoIIID* (gray bars), and strain BJP2 bearing multicopy P_{gerE} (black bars), at 24 h after resuspension in SM medium. Bars show the average of three determinations and error bars show 1 standard deviation of the data. Asterisks indicate $p < 0.05$ in the comparison between BJP1, and both PY79 and BJP2.


Ratio of Resistance
1
0.8
0.6
0.4
0.2
0
*
*
Heat
Lysozyme
Phenol
Ethanol
Chloroform

than 0.05 in Student *t*-tests). About 80% of viable PY79 and BJP2 cells were lysozyme-resistant at 24 h after the onset of sporulation, while the number was only about 50% for BJP1 (Fig. 2.5). Again, statistical analysis indicated a significant difference (*p*-values of less than 0.05 in Student *t*-tests) between BJP1 and either PY79 or BJP2, but no significant difference between the two control strains.

Resistance to three types of organic solvents was tested: the water-immiscible solvent chloroform, the water-miscible solvent ethanol, and the organic acid phenol. On average, BJP1 exhibited slightly less resistance than the two control strains for each treatment (Fig. 2.5), but these differences were not considered statistically significant because no pairwise comparison between the data obtained for two strains treated with the same organic solvent yielded a *p*-value of less than 0.05 in Student *t*-tests.

Maintaining the SpoIIID level late during sporulation does not impair spore germination. Spores were harvested 24 h after the onset of sporulation and purified. Germination was assayed as the change in absorbance at 580 nm over time after exposure to the germinant L-alanine. A germination mutant, *B. subtilis* EUDC9901 (*gerE*), showed very little change in absorbance even after 55 min (Fig. 2.6). In contrast, the absorbance of wild-type PY79 spores decreased rapidly after exposure to L-alanine, and similar results were observed for strains bearing multicopy P_{gerE}-*spoIIID* or P_{gerE}. We conclude that spores produced by a strain with persistent *spoIIID* expression germinate normally.

Persistent *spoIIID* expression causes a defect in spore coat structure. To assess the consequences of persistent *spoIIID* expression on the assembly of cellular structures, we carried out thin-section transmission electron microscopy of spores. Spores from the wild-type (data not shown) and the control strain with multicopy P_{gerE} (Fig. 2.7A) were

Fig. 2.6. Spore germination. *B. subtilis* wild-type PY79 (□), strain BJP1 bearing multicopy P_{gerE}-*spoIIID* (●), strain BJP2 bearing multicopy P_{gerE} (○), and a *gerE* mutant EUDC9901 (■) were induced to sporulate by resuspension in SM medium, samples were collected after 24 h, spores were purified, and quantitative germination assays were performed. Each point is the average of three determinations and error bars show 1 standard deviation of the data.

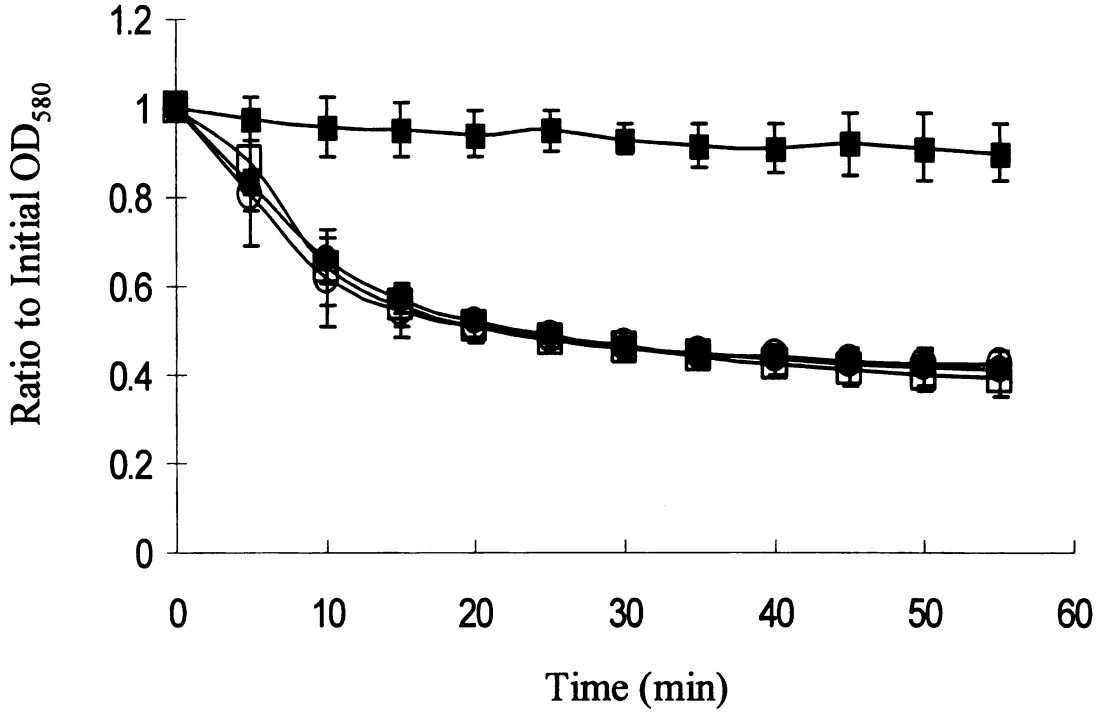
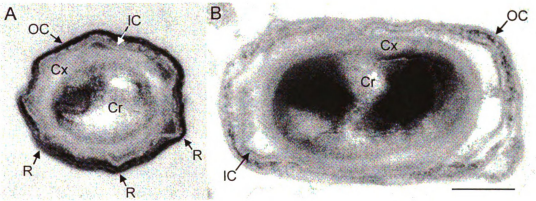

Ratio to Initial OD_{580}
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
Time (min)

Fig. 2.7. Thin-section electron microscopic analysis of spores. Spores from *B. subtilis* BJP2 bearing multicopy P_{gerE} (A) or BJP1 bearing multicopy P_{gerE}-*spoIIID* (B) were harvested 24 h after resuspension in SM medium, washed, and immediately fixed before further processing. The difference in shape of the two spores is insignificant (i.e., both strains, like wild-type PY79, showed a mixture of spherical and ovoid sections). The core (Cr), cortex (Cx), inner coat (IC), outer coat (OC) and ridges (R) are indicated. The bar indicates 200 nm.


A
OC
IC
Cx
Cr
R
R
R
B
OC
Cx
Cr
IC

indistinguishable. As is characteristic of mature spores, the core, cortex, and inner and outer coat layers, as well as ridges, were readily identified. Approximately 10% of the spores from the strain with multicopy P_{gerE}-*spoIIID* resembled wild-type spores (data not shown). The remaining 90% showed a significant defect in coat assembly. While structures resembling the inner and outer coat layers were present, these were thinner and disorganized (Fig. 2.7B). Additionally, the coat was no longer in close apposition to the cortex and ridges were less evident.

DISCUSSION

Our results demonstrate that maintaining the SpoIIID level late during sporulation alters expression of genes in the σ^K regulon, lowers the number of heat- and lysozyme-resistant spores produced, and causes a defect in spore coat assembly. Clearly, it is important that the SpoIIID level decreases in order for spore formation to proceed normally during the late stages. One contributor to the SpoIIID decrease is a negative feedback loop initiated by σ^K RNAP that inhibits early gene expression in the MC (Halberg and Kroos. 1992; Zhang and Kroos. 1997; Zhang, *et al.* 1999). Our finding that a single copy of the P_{gerE}-*spoIIID* fusion was insufficient to detectably boost the SpoIIID level late during sporulation was surprising, as was the possibly related observation that SpoIIID can negatively regulate *gerE* expression. While multiple copies of P_{gerE}-*spoIIID* did allow the SpoIIID level to be maintained late during sporulation, the levels of pro-σ^K and σ^K were unchanged, indicating that the MC regulatory network is somewhat resistant to perturbation. Such robustness is a common feature of regulatory networks (Alon. 2003; Stelling, *et al.* 2004). Our results reveal elements of both robustness and susceptibility to perturbation in the MC regulatory network.

It was surprising that a single copy of P_{gerE}-*spoIIID* did not result in perceptibly higher accumulation of SpoIIID late during sporulation. We chose the *gerE* promoter because it is strongly transcribed by σ^K RNAP *in vitro* (Zheng, *et al.* 1992) with little effect of SpoIIID (Halberg, *et al.* 1995). However, we discovered that in cells bearing multicopy P_{gerE}-*spoIIID*, the level of SpoIIID is elevated late during sporulation (Fig. 2.2) and *gerE-lacZ* expression is reduced (Fig. 2. 4A). Hence, SpoIIID might negatively autoregulate P_{gerE}-*spoIIID* expression, necessitating a high copy number to maintain the SpoIIID level late

during sporulation. Alternatively or in addition, one or more post-transcriptional mechanisms might inhibit SpoIIID accumulation late during sporulation. If so, such a mechanism(s) would presumably contribute to the decrease in SpoIIID level observed in wild-type cells late during sporulation (Halberg and Kroos. 1992).

Multicopy P_{gerE} exhibited none of the effects of multicopy P_{gerE}-*spoIIID*. Both plasmids were derived from pUB110, which is maintained at about 50 copies/cell (Maciag, *et al.* 1988). Most of these copies would presumably be in the MC (owing to its larger size) after polar septation. Considering that σ^K RNAP transcribes approximately 71 genes or operons in the MC during sporulation (Eichenberger, *et al.* 2004), the P_{gerE}-containing plasmid is expected to significantly increase the number of σ^K-dependent promoters in the MC. Yet, the strain containing this plasmid showed no differences from wild type in any of our assays. Moreover, a multicopy P_{gerE}-*cotC* fusion did not lower the number of heat- and lysozyme-resistant spores produced (Wang, *et al.*), as did multicopy P_{gerE}-*spoIIID* (Fig. 2.5). We conclude that neither P_{gerE} nor P_{gerE}-*cotC* titrates σ^K RNAP or other cellular resources sufficiently to inhibit sporulation. An earlier study showed that when σ^K is produced at a much lower level than normal, it is sufficient for considerable σ^K-dependent gene expression and sporulation (Lu and Kroos. 1994). Taken together with our results, it appears that σ^K is normally made in excess during sporulation.

Our results also suggest that the regulatory network resists elevating the levels of pro-σ^K and σ^K late during sporulation. Transcription of *sigK* depends absolutely on SpoIIID (Kunkel, *et al.* 1988). However, maintaining the SpoIIID level late during sporulation did not elevate the pro-σ^K or σ^K level (Fig. 2.3). This suggests that some other factor limits *sigK* expression late during sporulation. By that time, the level of σ^E is decreased due to

negative feedback by σ^K RNAP (Zhang and Kroos. 1997), so only σ^K RNAP can transcribe *sigK*. One factor known to limit *sigK* transcription is GerE (Zheng, *et al.* 1992; Ichikawa, *et al.* 1999). In a *gerE* mutant, *sigK* expression is elevated about twofold (Ichikawa, *et al.* 1999). A mutation in the GerE binding site in the *sigK* promoter region elevates the pro-σ^K and σ^K levels approximately twofold during sporulation (Wang, *et al.*). However, no further enhancement of the pro-σ^K or σ^K level was observed when multicopy P_{gerE}-*spoIIID* was introduced into the binding site mutant (Wang, *et al.*). Under these conditions, neither SpoIIID (acting positively) nor GerE (acting negatively) should limit *sigK* expression late during sporulation. Perhaps under these conditions, the ability to process pro-σ^K to active σ^K becomes the limiting factor.

Given the unchanged level of σ^K in cells engineered to maintain the SpoIIID level late during sporulation, we would not have predicted the observed decrease in *gerE-lacZ* expression (Fig. 2.4A), because σ^K RNAP transcribes *gerE* and SpoIIID has little effect, based on *in vitro* studies (Halberg, *et al.* 1995). The same *gerE*-promoter-containing fragment (-96 to +170) used in the *in vitro* study was used to create the *gerE-lacZ* translational fusion used here, so it is unlikely that the *gerE-lacZ* fusion has an additional SpoIIID binding site. Nevertheless, further experiments are warranted to test whether maintaining the SpoIIID level late during sporulation inhibits *gerE* expression (e.g., by measuring *gerE* mRNA and GerE protein levels), since this appears to be a previously unknown connection in the MC regulatory network, coordinating the disappearance of SpoIIID with the appearance of GerE.

Maintaining the SpoIIID level late during sporulation decreased expression of *cotC-lacZ* (Fig. 2.4B) and increased that of *cotD-lacZ* (Fig. 2.4C). These effects can be

understood from the effects of SpoIIID and GerE on transcription of *cotC* (Zheng, *et al.* 1992; Ichikawa and Kroos. 2000) and *cotD* (Halberg and Kroos. 1994; Halberg, *et al.* 1995; Ichikawa, *et al.* 1999) by σ^K RNAP *in vitro*. We predict that expression of other genes in the σ^K regulon, whose transcription is influenced by SpoIIID and/or GerE, is also altered in cells engineered to maintain the SpoIIID level late during sporulation. Only 4 genes in the σ^K regulon have been shown to be influenced by SpoIIID (Halberg and Kroos. 1994; Ichikawa and Kroos. 2000), but 53 genes or operons have been shown to be positively or negatively regulated by GerE, based on genome-wide DNA microarray expression profiling experiments (Eichenberger, *et al.* 2004).

Altered expression of genes in the σ^K regulon presumably causes the altered spore resistance properties (Fig. 2.5) and the spore coat structural defects (Fig. 2.7B) observed for spores derived from cells with persistent *spoIIID* expression. Interestingly, the coat protein composition of these spores is not detectably altered, as determined by SDS-PAGE analysis of SDS-extractable coat proteins (data not shown). We interpret this as evidence that proper assembly of the coat layers depends on proper regulation of coat protein synthesis, not just on the final protein composition. Most likely, the normal changes in the levels of SpoIIID and GerE regulate coat protein levels such that pathological interactions are suppressed. In this view, when the SpoIIID level is maintained late during sporulation, coat proteins that would not normally be present simultaneously at significant levels are able to interact inappropriately, with pathological consequences. Therefore, while a small subset of critical coat proteins play a predominant role in the assembly of the coat layers (as is argued by the current models for coat assembly) (Driks. 2002; Kim, *et al.* 2006), proper regulation of other coat proteins is also required for proper coat organization.

The nature of the spore defect caused by maintaining the SpoIIID level late during sporulation is intriguing. Resistance of the spore to environmental insults is due both to the complex structure of its cortex and coat, and to the unique physiological state of the core (reviewed in Driks. 2002; Chada, *et al.* 2003). It is believed that the cortex confines the core of the spore by forming a woven fabric-like structure, producing a highly dehydrated state that is resistant to heat. On the other hand, lysozyme resistance is largely due to the coat, which shields the cortex from the enzyme. The defect in lysozyme resistance of spores derived from cells with persistent *spoIIID* expression (Fig. 2.5) might be explained by their defect in spore coat structure (Fig. 2.7B). Although gross cortex and core defects were not evident by electron microscopic analysis of these spores, subtle defects are implied since more than half lost their ability to resist the heat treatment (Fig. 2.5).

The electron microscopic analysis of spores derived from cells with persistent *spoIIID* expression revealed that typically much of the coat did not contact the cortex and that ridges were less evident than for wild-type spores (Fig. 2.7). These observations suggest an inability of the coat to maintain the folded, contracted state that appears to be responsible for the ridges (Chada, *et al.* 2003; Driks. 2004; Plomp, *et al.* 2005; Plomp, *et al.* 2005; Plomp, *et al.* 2005). Previous work implicated CotE (possibly indirectly) in coat folding (Chada, *et al.* 2003). The present study suggests that improper regulation of coat protein levels can disrupt folding and that this phenomenon is not simply due to the presence or absence of a single protein.

The impact of maintaining the SpoIIID level late during sporulation depends to some extent on the conditions used to initiate the sporulation process. In the experiments documented in the Results, sporulation was initiated by centrifugal collection of growing

cells followed by resuspension in SM medium lacking nutrients. When sporulation was instead initiated by growth in DS medium followed by nutrient exhaustion (Harwood and Cutting. 1990), similar results as shown in Figures 2-4 were observed, but the effects of persistent *spoIIID* expression on spore resistance properties were diminished such that none of the differences were statistically significant by the criterion (p-value of less than 0.05 in a Student t-test) we used (data not shown). These results indicate that the method of sporulation initiation influences susceptibility to perturbation of the SpoIIID level late during sporulation, at least in terms of spore resistance properties. A connection between the method of sporulation initiation and expression of *cotC* late during sporulation was observed previously (Zheng and Losick. 1990). Recently, aconitase, a Krebs cycle enzyme, was shown to be required for efficient late-sporulation gene expression, apparently because it binds to the 3' untranslated region of *gerE* mRNA, facilitating its accumulation (Serio, *et al.* 2006). Aconitase provides a plausible link between early and late events during sporulation since the *citB* gene, which encodes aconitase, is induced during late exponential phase in nutrient-exhausted medium (Dingman, *et al.* 1987). Obviously, the MC gene regulatory network is more complex than depicted in Figure 1 and much remains to be learned about how entry into sporulation influences network characteristics and output.

ACKNOWLEDGMENTS

We thank R. Burri for constructing pRB1 and pRB2, and conducting preliminary studies with P_{gerE}-*spoIIID* at single copy. We also thank R. Losick, A. Grossman, and C. Moran for providing bacterial strains.

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CHAPTER III: One Perturbation of the Mother Cell Gene Regulatory Network Suppresses the Effects of Another During Sporulation of *Bacillus subtilis*

ABSTRACT

In the mother cell of sporulating *Bacillus subtilis*, a regulatory network functions to control gene expression. Four transcription factors act sequentially in the order σ^E, SpoIIID, σ^K, then GerE. σ^E and σ^K direct RNA polymerase to transcribe different regulons. SpoIIID and GerE are small sequence-specific DNA-binding proteins that activate or repress transcription of many genes in the σ^E and σ^K regulons, respectively. Several negative regulatory loops add complexity to the network. First, transcriptionally active σ^K RNA polymerase inhibits early sporulation gene expression, resulting in reduced accumulation of σ^E and SpoIIID late during sporulation. Second, GerE represses *sigK* transcription, reducing σ^K accumulation about twofold. Third, SpoIIID represses *cotC*, which encodes a spore coat protein, delaying its transcription by σ^K RNA polymerase. Circumventing the first regulatory loop by engineering cells to maintain the SpoIIID level late during sporulation causes spore defects (Chapter II). Here, the results of circumventing the second regulatory loop by mutating the GerE binding sites in the *sigK* promoter region are reported. Accumulation of pro-σ^K and σ^K increased but this did not cause a detectable spore defect. It did alter expression of σ^K-dependent reporter fusions, increasing that of *gerE-lacZ* and *cotC-lacZ*, and decreasing that of *cotD-lacZ*. Because these effects on gene expression were the opposite of those observed when SpoIIID was maintained late during sporulation, cells were engineered to simultaneously circumvent both regulatory loops. This restored expression of σ^K-dependent reporters to wild-type levels and suppressed the spore defects observed when only the first regulatory loop was circumvented. Circumventing the third regulatory loop by engineering cells to express *cotC* about 2 h earlier than normal did not cause a spore defect. This is not surprising since *cotC* is one of many targets of the mother

cell regulatory network. On the other hand, σ^E, SpoIIID, σ^K, and GerE are network nodes, each affecting the expression of many target genes. Circumventing the second regulatory loop impacted two nodes (σ^K and GerE) but did not detectably alter network output (spores). Yet, this perturbation compensated for the effects of perturbing the first regulatory loop in a way that impacts two nodes (SpoIIID and GerE) and causes spore defects. By feeding information back into the network, the first and second negative regulatory loops appear to optimize target gene expression and increase network robustness.

INTRODUCTION

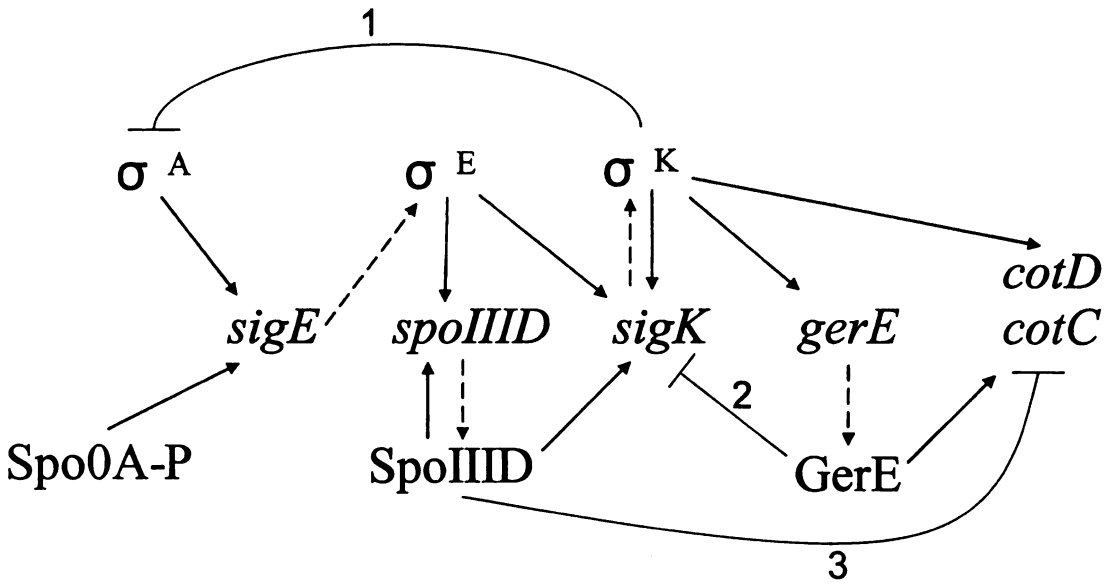
When starved, the Gram-positive bacterium *Bacillus subtilis* initiates a process called sporulation in order to form a dormant spore (Driks. 2002; Kroos. 2007). Sporulation is a complex developmental process in which morphological changes are coupled to temporal and spatial regulation of gene expression. An early morphological change is the formation of an asymmetrically-positioned septum that divides the cell into a larger mother cell (MC) compartment and a smaller forespore (FS) compartment. Completion of DNA replication ensures that a copy of the chromosome is available for each compartment. The MC engulfs the FS, pinching it off as a protoplast within the MC. Cortex, a modified peptidoglycan, is synthesized between the two membranes that surround to FS after engulfment. Proteins assemble on the surface of the FS, producing the coat. Eventually, the mature spore is released by lysis of the MC. The spore is resistant to harsh conditions such as high temperature and exposure to UV light, lytic enzymes and chemicals. The spore will germinate in the presence of nutrients and grow vegetatively.

Distinct temporal programs of gene expression occur in the two compartments, but signaling pathways between the compartments coordinate the two programs and morphogenesis. In the MC, a regulatory network controls gene expression during sporulation (reviewed in Kroos. 2007). The backbone of network consists of a cascade of four regulatory factors (σ^E, SpoIIID, σ^K, and GerE) (Zheng and Losick. 1990), but several loops are also present in the network (Fig. 3.1). Synthesis of σ^E requires σ^K RNA polymerase (RNAP), the major form of RNAP in growing cells, and phosphorylated Spo0A (Spo0A-P), a response regulator that governs initiation of sporulation (reviewed in Haldenwang. 1995). The initial product of *sigE* is inactive pro-σ^E. It is cleaved to active σ^E

in response to a signal from the FS (Hofmeister, *et al.* 1995; Karow, *et al.* 1995; Londono-Vallejo and Stragier. 1995). σ^E RNAP transcribes many genes, including *spoIIID* and *sigK* (Fig. 3.1), and others whose products contribute to engulfment and synthesis of the spore cortex and coat (Eichenberger, *et al.* 2004; Steil, *et al.* 2005). SpoIIID is a DNA-binding protein that activates transcription of *sigK* by σ^E RNAP and σ^K RNAP (Halberg and Kroos. 1994; Kroos, *et al.* 1989) (Fig. 3.1). SpoIIID also positively regulates at least 7 other transcription units and negatively regulates at least 62 transcription units in the σ^E regulon (Eichenberger, *et al.* 2004). Also, SpoIIID negatively regulates certain genes in the σ^K regulon, such as *cotC* and *cotD* (Kroos, *et al.* 1989; Halberg, *et al.* 1995; Ichikawa and Kroos. 2000) (Fig. 3.1), which encode spore coat proteins (Donovan, *et al.* 1987). σ^K, like σ^E, is first made as an inactive precursor protein that is cleaved in response to a signal from the FS (Cutting, *et al.* 1990; Lu, *et al.* 1990; reviewed in Kroos. 2007). σ^K RNAP transcribes many genes, including *gerE* (Fig. 3.1), and others whose products contribute to synthesis of the spore cortex and coat (Eichenberger, *et al.* 2004; Steil, *et al.* 2005). Like SpoIIID, GerE is a DNA-binding protein, but it activates transcription of certain *cot* genes, such as *cotC* and *cotD* (Zheng, *et al.* 1992), having the opposite effect as SpoIIID (Fig. 3.1). In addition, GerE positively regulates at least 25 other transcription units in the σ^K regulon, and negatively regulates at least 36 transcription units (Eichenberger, *et al.* 2004), including *sigK* (Zheng, *et al.* 1992; Ichikawa, *et al.* 1999) (Fig. 3.1).

Three negative regulatory loops in the MC network are shown in Fig. 3.1. First, transcriptionally active σ^K RNAP inhibits early gene expression under σ^A control, resulting in reduced accumulation of σ^E and SpoIIID late during sporulation (Halberg and Kroos. 1992; Zhang and Kroos. 1997; Zhang, *et al.* 1999). Second, GerE represses *sigK*

Fig. 3.1. Gene regulatory network in the MC during sporulation of *B. subtilis*. Dashed arrows indicate gene to protein relationships. Solid arrows or lines with barred ends represent positive or negative regulation, respectively. Numbers 1, 2, and 3 represent three negative regulatory loops, respectively. See text for references.


1
σ A
σ E
σ K
cotD
cotC
sigE
spoIIID
sigK
gerE
2
Spo0A-P
SpoIIID
GerE
3

transcription, lowering the σ^K level about twofold (Ichikawa, *et al.* 1999). Third, SpoIIID represses *cotC* transcription, delaying its expression by at least one hour compared with other genes under positive control of σ^K RNAP and GerE (Ichikawa and Kroos. 2000)

How important are the three negative regulatory loops for sporulation? To begin to address this question, we previously engineered *B. subtilis* to circumvent the decrease in the level of *spoIIID* late during sporulation, which is normally brought about by the first negative regulatory loop involving σ^K RNAP (Chapter II). We found that maintaining that SpoIIID level late during sporulation resulted in altered expression of σ^K-dependent genes, lower numbers of resistant spores, and a structural defect in the coat of most spores that were produced. Here, we report the effect of mutating the GerE binding site in the *sigK* promoter, which eliminates the second negative regulatory loop, GerE repression of *sigK* transcription (Fig. 3.1). Expression of σ^K-dependent genes was altered, but the effects were opposite those observed when the SpoIIID level was maintained late during sporulation. Elimination of GerE negative feedback on *sigK* transcription did not cause detectable spore defects. Interestingly though, it suppressed the defects caused by maintaining SpoIIID level late during sporulation. In the strain with both perturbations, expression of σ^K-dependent genes was restored to wild-type levels. We also engineered *B. subtilis* to circumvent the third negative regulatory loop, SpoIIID repression of *cotC* (Fig. 3.1). Expressing *cotC* approximately 2 hours earlier than normal did not cause detectable spore defects. Taken together, these results suggest that the second and third negative regulatory loops are less important for sporulation than the first, but that the first and second loops exert opposing effects, fine-tuning expression of target genes in order to optimize spore formation.

MATERIALS AND METHODS

Site-directed mutagenesis and construction of plasmids. A plasmid bearing the *sigK* promoter (starting at -108) and the entire coding region was constructed in several steps. First, pRG2 (Prince et al 2005) was digested with *Bam*HI and annealed oligonucleotides (5'-GATCCCACCACCACCACCACTAA-3' and 5'-GATCTTAGTGGTGGTGGTGGTGG-3') were inserted in the orientation that preserved the *Bam*HI site at the 3' end of *sigK*, generating pHP1. Second, the QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used with primers 5'-GGCTTTTGCCTACAAGCTTTTGTGGAGGTGACG-3' and 5'-CGTCACCTCCACAAAAGCTTGTAGGCAAAAGCC-3' (mutant nucleotides are underlined), and pHP1 as template, to introduce a *Hin*dIII site between the *sigK* promoter and ribosome binding site, generating pHP12, for which the *sigK* sequence was verified. Third, site-directed mutagenesis was likewise performed with primers 5'-CCCGAAAAGTGCCACCTGGTGTCTAAGAAACC-3' and 5'-GGTTTCTTAGACACCAGGTGGCACTTTTCGGG-3', using pDG364 as template to eliminate its *Aat*II site, generating pHP13. Finally, the *sigK*-containing *Eco*RI-*Bam*HI fragment from pHP12 was gel-purified and ligated into *Eco*RI-*Bam*HI-digested pHP13, generating pHP14, which due to the *Bam*HI site and vector adds four codons (specifying the amino acids GSPA) to the 3' end of *sigK*. This *sigK* allele is flanked by the 5' and 3' ends of *amyE* and it complements a *sigK* mutant when it replaces the normal *amyE* gene in the *B. subtilis* chromosome (see below). The plasmid pHP14 was the template for site-directed mutagenesis designed to eliminate the GerE binding site in the *sigK* promoter

region. . Four nucleotide changes, GG to CC at +4 and +5, and CC to GG at +12 and +13 were made using primers 5'—CCGGTCACATACATTTACATATACCCTTTTGGGTAC ATACTTTTGTGGAGG—3' and 5'—CCTCCACAAAAGTATGTACCCAAAAGGGTA TATGTAAATGTATGTGACCGG—3'. The resulting allele in pJP16 was designated *sigKmut* and was sequenced to confirm the presence of only the desired mutations.

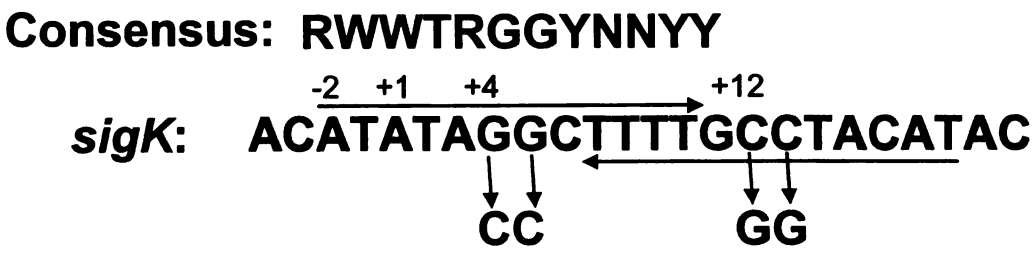
In order to express *cotC* earlier than normal, we used the *gerE* promoter to drive expression of the *cotC* gene. A DNA fragment that containing the entire *cotC* gene from slightly upstream of the *cotC* ribosomal binding site to slightly downstream of the stop codon, was synthesized using polymerase chain reaction (PCR) and primers 5'—CGAAGCTTTAAAGGAGGAGTATATATGGGTTATTAC—3' and 5'—GCGGATCCACCCGGCAATAGCCGG—3', which contain *Hin*dIII and *Bam*HI restriction sites (underlined), respectively, with chromosomal DNA from *B. subtilis* PY79 as the template. The PCR products were digested with *Hin*dIII- *Bam*HI, and ligated with *Hin*dIII-*Bam*HI digested pJP1 (Chapter II). The sequence of the entire P_{gerE}-*cotC* fusion was determined to ensure that no errors occurred during the PCR.

Bacterial strains. *Escherichia coli* strain AG115 [*araD139* Δ(*ara,leu*)*7697* Δ*lacX74* *galU galK hsr hsm*$^+$ *strA* (F′ *proAB lacI*^{q}*Z*::Tn5)] was obtained from A. Grossman (Massachusetts Institute of Technology). It was used for construction and maintenance of plasmids. Luria-Bertani medium (LB) (Sambrook, *et al.* 1989) was used to grow *E. coli* and *B. subtilis*, and was supplemented with appropriate antibiotics as needed. *B. subtilis* strains used in this study are listed in Table 3.1. The plasmid pJP4 was transformed into PY79 with selection on LB agar containing kanamycin sulfate (5 μg/ml) to generate strain BJP3. Plasmids pJP14 and pJP16 bearing wild-type *sigK* and the *sigKmut* allele,

Table 3.1. List of *B. subtilis* strains used in the study

Strain	Genotype or derivation	Source or reference
PY79	Spo^+ prototroph	(Youngman, *et al.* 1984)
OR825	SPβ::*cotC-lacZ*/Cm^r	(Crater, *et al.* 2002)
EUDC9901	*trpC2 phe*A1 *gerE*::*kan*/Km^r	(Crater and Moran. 2002)
BK556	*spoIVCB23*	(Kunkel, *et al.* 1989)
BD071	*cotC*::*cat*/Cm^r	(Donovan, *et al.* 1987)
BJP1	PY79 transformed with pJP1(P_{gerE}-*spoIIID*)/Km^r	Chapter II
BJP2	PY79 transformed with pJP2(P_{gerE})/Km^r	Chapter II
BJP3	PY79 transformed with pJP4(P_{gerE}-*cotC*)/Km^r	This study
BLW1	BK556 transformed with pHP6(*amyE*::*sigK*)/Cm^r	This study
BLW2	BK556 transformed with pJP16(*amyE*::*sigKmut*)/Cm^r	This study
BLW3	BLW1 transformed with pJP1/Km^rCm^r	This study
BLW4	BLW2 transformed with pJP1/Km^rCm^r	This study
BLW5	BLW1 transformed with pCm::Sp/Sp^r	This study
BLW6	BLW2 transformed with pCm::Sp/Sp^r	This study
BLW7	BLW3 transformed with pCm::Sp/Km^rSp^r	This study
BLW8	BLW4 transformed with pCm::Sp/Km^rSp^r	This study

Fig. 3.2. Mutation made in the GerE binding site in the *sigK* promoter region. The top line shows the consensus sequence for GerE binding (Zhang, *et al.* 1994) in which R means A or G, W means A or T, Y means C or T, and N means A, G, C, or T. The bottom line shows the sequence in the *sigK* promoter region where GerE binds, as defined by DNase I footprinting (Henriques, *et al.* 1995). Numbering is relative to the TSS. The rightward arrow indicates a perfect match to the consensus. The leftward arrow indicates a 9 out of 12 match to the consensus on the strand not shown. The downward arrows show the 4 changes in the *sigKmut* allele.


Consensus: RWWTRGGYNNYY
-2 +1 +4 +12
sigK: ACATATAGGCTTTTGCCTACATAC
CC
GG

respectively, were transformed into BK556 with selection on LB agar containing chloramphenicol (5μg/ml). Strains in which *amyE* was replaced by sigK (BLW1) or *sigKmut* (BLW2) were identified as described (Cutting and Horn. 1990). pJP1 (a multicopy plasmid with a P_{gerE}-*spoIIID* fusion, Chapter II) was transformed into BLW1 and BLW2 with selection on LB agar containing chloramphenicol (5 μg/ml) and kanamycin sulfate (5 μg/ml) to generate strains BLW3 and BLW4, respectively. The chloramphenicol resistance gene of BLW1, BLW2, BLW3 and BLW4 was replaced with the spectinomycin resistance gene of pCm::Sp as described (Steinmetz and Richter. 1994), generating strain BLW5, BLW6, BLW7 and BLW8, respectively. A lysate containing SPβ::*cotC-lacZ* was obtained by heat induction of strain OR825 as described (Cutting and Horn. 1990). SPβ::*cotD-lacZ* and SPβ::*gerE-lacZ* have been described (Cutting, *et al.* 1989; Cutting, *et al.* 1990). Specialized transduction was used to move *lacZ* fusions into BLW5, BLW6, BLW7 and BLW8 as described (Harwood and Cutting. 1990). Transductants were selected on LB agar containing chloramphenicol (5 μg/ml). In each case, at least 10 candidates of isolates were transferred onto a DSM agar (Harwood and Cutting. 1990) plate with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 μg/ml). Three or more isolates with average blue color were saved for further analysis, excluding occasional isolates with abnormally high or low β-galactosidase activity.

Cell growth and sporulation. Sporulation was induced by resuspension of cells in SM medium as described (Harwood and Cutting. 1990). The time of resuspension is defined as the onset of sporulation (T_0).

Western blot analysis. Starting at 3 h into sporulation (T_3) and at hourly intervals thereafter until T_9, 0.5 ml samples were subjected to centrifugation (14,000 g for 1 min),

the supernatants were removed and cell pellets were stored at -70°C. Preparation of whole-cell extracts, electrophoresis, and electroblotting were as described (Lu, *et al.* 1990; Halberg and Kroos. 1992). The blots were probed with polyclonal anti-SpoIIID antiserum diluted 1:10,000 (Halberg and Kroos. 1992), polyclonal anti-pro-σ^K antiserum diluted 1:10,000 (Lu, *et al.* 1990), or polyclonal anti-CotC antibodies diluted 1:10,000 (Isticato, *et al.* 2004). Immunodetection of primary antibodies was as described (Kroos, *et al.* 2002).

Analysis of β-galactosidase activity. Samples were collected during sporulation as described above. Cell pellets were stored at -70°C prior to the assay. Cells were resuspended, then treated with lysozyme and permeabilized by toluene as described (Miller. 1972). The β-galactosidase specific activity was determined as described (Miller. 1972) using *o*-nitrophenol-β-D-galactopyranoside as the substrate. One unit of the enzyme hydrolyzed 1 μmol of substrate per minute per unit of initial culture optical density at 595 nm.

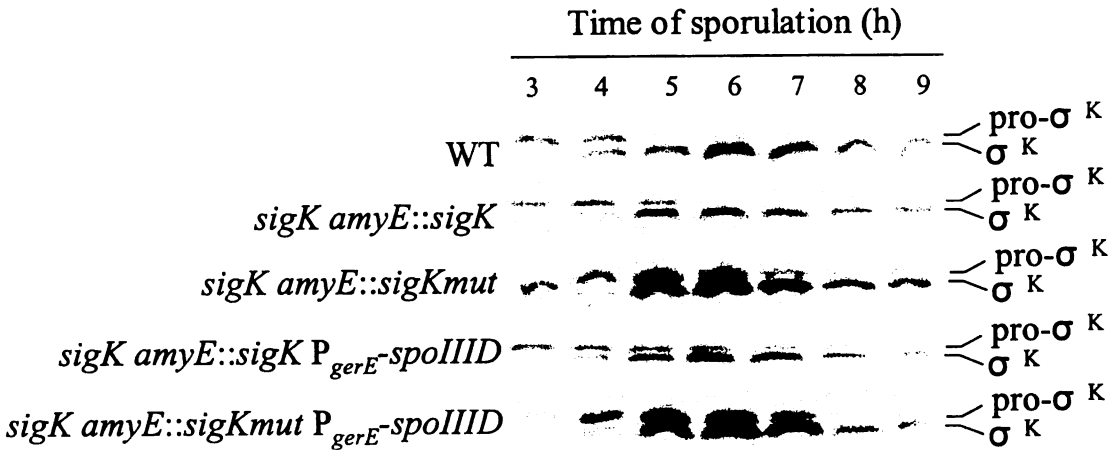
Spore purification, and germination and resistance assays. Spores were harvested at T_{24} by centrifugation at 7,000 g for 10 min, washed with 4°C water once, and stored at 4°C overnight. The next day, spores were purified on a step gradient of 20% to 50% RenoCal-76 (Bracco Diagnostics Inc.) as described (Henriques, *et al.* 1995). Purity of the spores was verified by microscopy. The germination assay was performed with purified spores using L-alanine (10 mM) as the germinant as described (Naclerio, *et al.* 1996). Assays for resistance to heat, lysozyme, and organic solvents were performed at T_{24} without spore purification, as described (Harwood and Cutting. 1990).

Transmission electron microscopy of spores. Spores were harvested at T_{24}, washed with water, and immediately fixed as described (McPherson, *et al.* 2005).

RESULTS

Engineering *B. subtilis* to circumvent GerE repression of *sigK*. GerE binds to a site overlapping the *sigK* TSS and represses *sigK* transcription about twofold (Ichikawa, *et al.* 1999). Within the GerE binding site are two sequences that match the consensus sequence for GerE binding (Fig. 3.2). To assess the importance of *sigK* repression by GerE, we mutated two nucleotides in each GerE binding site in *sigK* promoter region (Fig. 3.2). A previous study showed that changing GG to TT near the center of the GerE binding site in the *cotH* promoter region impaired GerE repression of *cotH* transcription (Baccigalupi, *et al.* 2004). We changed GG at +4 and +5 to CC, and CC at +12 and +13 to GG in the *sigK* promoter region (Fig. 3.2). This mutant allele of *sigK*, including the promoter and the entire coding region, was integrated at the *amyE* locus of *sigK* mutant *B. subtilis* BK556, creating BLW2 (*sigK amyE*::*sigKmut*). As a control, a wild type *sigK* allele was integrated at the *amyE* locus of the *sigK* mutant, creating BLW1 (*sigK amyE*::*sigK*). BLW1 and BLW2 were grown and induced to sporulate along with *B. subtilis* wild type PY79 as an additional control. Samples were collected during sporulation and whole-cell extracts were subjected to Western blot analysis to detect pro-σ^K and σ^K. The levels of pro-σ^K and σ^K were higher in the *sigK amyE*::*sigKmut* strain than in either control strain, starting at 5 h into sporulation (Fig. 3.3A-C). The pro-σ^K and σ^K levels were similar in the two control strains. The effect of the *sigKmut* allele on *sigK* expression was similar to the effect of a *gerE* null mutation (Ichikawa, *et al.* 1999), suggesting that GerE repression of *sigK* is eliminated in BLW2.

Fig. 3.3. Levels of pro-σ^K and σ^K during sporulation. *B. subtilis* wild-type (WT) PY79, BLW1 (*sigK amyE*::*sigK*), BLW2 (*sigK amyE*::*sigKmut*), BLW3 (*sigK amyE*::*sigK* P_{gerE}-*spoIIID*), and BLW4 (*sigK amyE*::*sigKmut* P_{gerE}-*spoIIID*) were sporulated by resuspension in SM medium. Samples were collected at hourly intervals beginning at 3 h after the initiation of sporulation. Equal volumes (5 ul) of whole-cell lysates were fractionated on SDS 14% polyacrlamide gels and subjected to Western blot analysis with anti-pro-σ^K antiserum.


Time of sporulation (h)
3
4
5
6
7
8
9
WT
sigK amyE::sigK
sigK amyE::sigKmut
sigK amyE::sigK P gerE -spoIIID
sigK amyE::sigKmut P gerE -spoIIID
pro-σ K
σ K

Effects of circumventing GerE repression of *sigK*. Because σ^K acts after SpoIIID in the MC gene regulatory network (Fig. 3.1), we did not expect the SpoIIID level during sporulation to be altered in the *sigK amyE*::*sigKmut* strain. Indeed, it was indistinguishable from that in wild type and the *sigK amyE*::*sigK* strain (Fig. 3.4).

To test whether expression of σ^K-dependent genes was affected by the elevated level of σ^K in BLW2, we measured β-galactosidase activity from *lacZ* fusions to *gerE*, *cotC* and *cotD* during sporulation. In order to introduce *lacZ* fusions with associated Cm^r markers into strains BLW2 and BLW1, we replaced their Cm^r cassettes with Sp^r cassettes, creating BLW6 and BLW5, respectively (Table 3.1). Expression from *gerE-lacZ* and *cotC-lacZ* was elevated in BLW6 relative to the BLW5 and PY79 control strains (Fig. 3.5A-B). Apparently, elevated σ^K in sporulating cells can increase transcription of σ^K-dependent genes. Interestingly, expression from *cotD-lacZ* was reduced in BLW6 relative to the control strains (Fig. 3.5C). This might be due to an elevated level of GerE since a high level of GerE repressed *cotD* transcription by σ^K RNAP *in vitro* (Ichikawa, *et al.* 1999).

Circumventing GerE repression of *sigK* compensates for persistent *spoIIID* expression. The effects on expression of the three σ^K-dependent reporters (Fig. 3.5) were the opposite of the effects observed previously when cells were engineered to maintain the SpoIIID level late during sporulation (Chapter II). In that study, SpoIIID was expressed ectopically from a multicopy plasmid bearing a P_{gerE}-*spoIIID* fusion. Introduction of multicopy P_{gerE}-*spoIIID* into the *sigK amyE*::*sigKmut* background (creating BLW4 initially, then replacing its Cm^r cassette with a Sp^r cassette to create BLW8 for introduction of *lacZ* fusions; Table 3.1) restored expression of *gerE-lacZ*, *cotC-lacZ*, and *cotD-lacZ* to

Fig. 3.4. Levels of SpoIIID during sporulation. *B. subtilis* wild-type (WT) PY79, BLW1 (*sigK amyE*::*sigK*), BLW2 (*sigK amyE*::*sigKmut*), BLW3 (*sigK amyE*::*sigK* P_{gerE}-*spoIIID*), and BLW4 (*sigK amyE*::*sigKmut* P_{gerE}-*spoIIID*) were sporulated by resuspension in SM medium. Samples were collected and subjected to Western blot analysis as described in the Fig. 3.3 legend except anti-SpoIIID antiserum was used. Numbers indicate the h into sporulation.

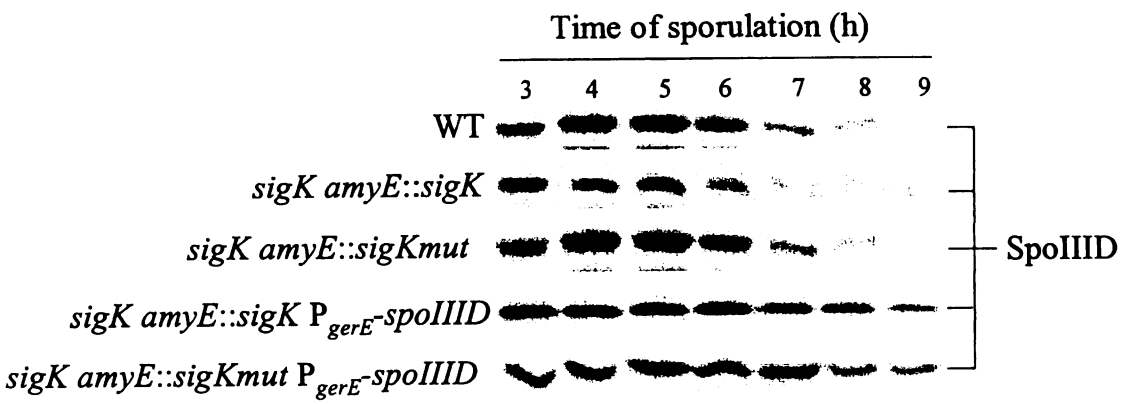
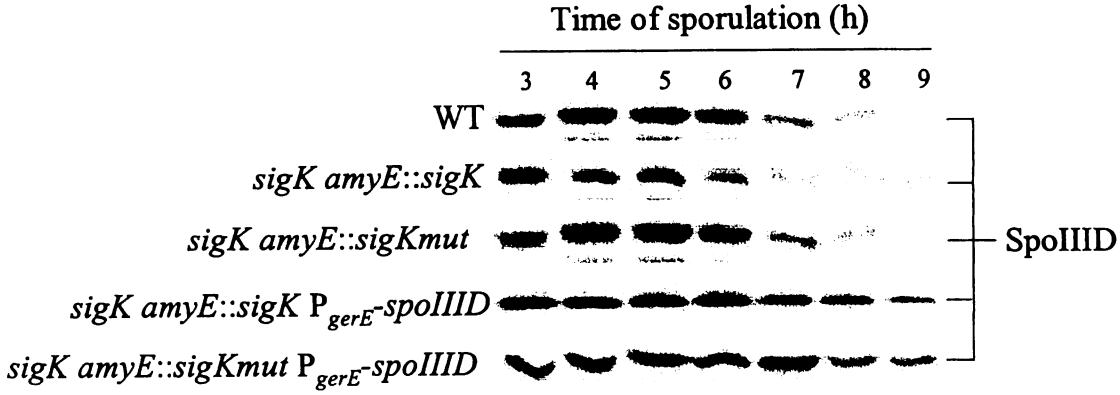

Time of sporulation (h)
3
4
5
6
7
8
9
WT
sigK amyE::sigK
sigK amyE::sigKmut
sigK amyE::sigK P gerE -spoIIID
sigK amyE::sigKmut P gerE -spoIIID
SpoIIID

Fig. 3.4. Levels of SpoIIID during sporulation. *B. subtilis* wild-type (WT) PY79, BLW1 (*sigK amyE::sigK*), BLW2 (*sigK amyE::sigKmut*), BLW3 (*sigK amyE::sigK* P_{gerE}-*spoIIID*), and BLW4 (*sigK amyE::sigKmut* P_{gerE}-*spoIIID*) were sporulated by resuspension in SM medium. Samples were collected and subjected to Western blot analysis as described in the Fig. 3.3 legend except anti-SpoIIID antiserum was used. Numbers indicate the h into sporulation.


Time of sporulation (h)
3
4
5
6
7
8
9
WT
sigK amyE::sigK
sigK amyE::sigKmut
sigK amyE::sigK P gerE -spoIIID
sigK amyE::sigKmut P gerE -spoIIID
SpoIIID

the levels observed in the wild-type PY79 and *sigK amyE::sigK* BLW5 control strains (Fig. 3.5). In contrast, introduction of multicopy P_{gerE}-*spoIIID* into the *sigK amyE::sigK* background (creating BLW3 initially, then replacing its Cm^r cassette with a Sp^r cassette to create BLW7 for introduction of *lacZ* fusions; Table 1) decreased expression of *gerE-lacZ* and *cotC-lacZ*, and increased that of *cotD-lacZ* (Fig. 3.5), similar to the effects of multicopy P_{gerE}-*spoIIID* in an otherwise wild-type background (Chapter II). As expected, both BLW3 and BLW4 exhibited a higher level of SpoIIID late during sporulation than strains without P_{gerE}-*spoIIID* (Fig. 3.4). Also as expected, the levels of pro-σ^K and σ^K were higher starting at 5 h into sporulation in BLW4 and BLW8 than in strains without *amyE::sigKmut* (Fig. 3.3). Elevated σ^K in BLW4 appears to compensate for elevated SpoIIID, restoring *gerE*, *cotC*, and *cotD* expression to wild-type levels.

Does circumventing GerE repression of *sigK* with the *sigKmut* allele compensate for the other effects of persistent *spoIIID* expression from P_{gerE}-*spoIIID*, which includes defects in spore resistance properties and coat assembly (Chapter II)? Figure 3.6 shows that BLW4 produced heat- and lysozyme-resistant spores as efficiently as wild-type. These spores germinated normally in response to L-alanine (Fig. 3.7) and were indistinguishable from wild-type PY79 spores when examined by thin-section transmission electron microscopy (data not shown). By the criteria we tested, elevated σ^K in BLW4 appeared to fully suppress the sporulation defects caused by maintaining the SpoIIID level during the late stages.

In contrast to strain BLW4 and as expected from our previous study (Chapter II), strain BLW3 in which the SpoIIID level was maintained late during sporulation without elevated

Fig. 3.5. Effects on gene expression. β-galactosidase activity during sporulation after resuspension in SM medium was measured for *B. subtilis* containing *lacZ* fused to the (A) *gerE*, (B) *cotC*, or (C) *cotD* promoter in wild-type PY79 (▵), BLW5 (*sigK amyE::sigK*) (♦), BLW6 (*sigK amyE::sigKmut*) (■), BLW7 (*sigK amyE::sigK* P_{gerE}-*spoIIID*) (○), and BLW8 (*sigK amyE:: sigKmut* P_{gerE}-*spoIIID*) (□). Each point is the average of three determinations and error bars show 1 standard deviation of the data.

A

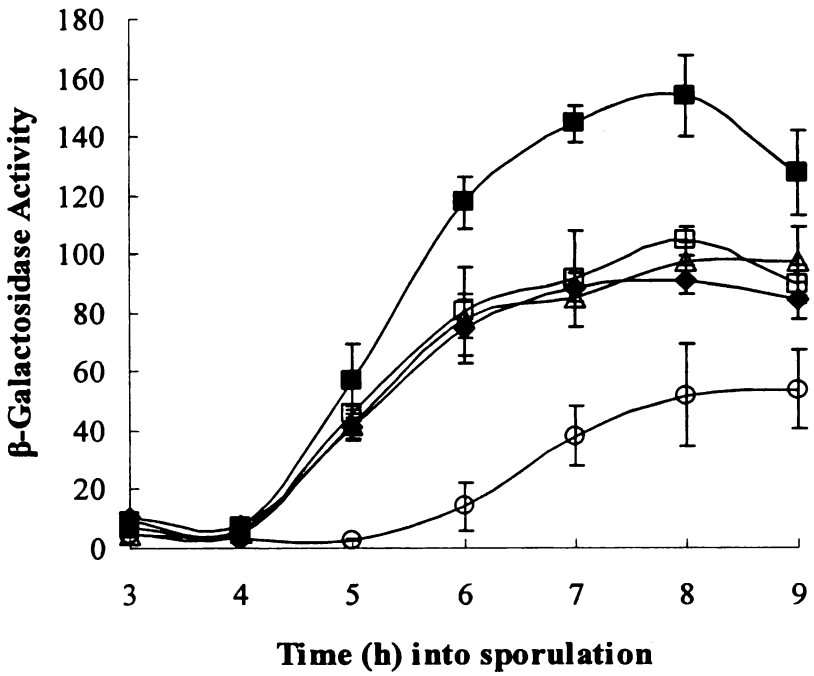

β-Galactosidase Activity
180
160
140
120
100
80
60
40
20
0
3
4
5
6
7
8
9
Time (h) into sporulation

Fig. 3.5 (continued)

B

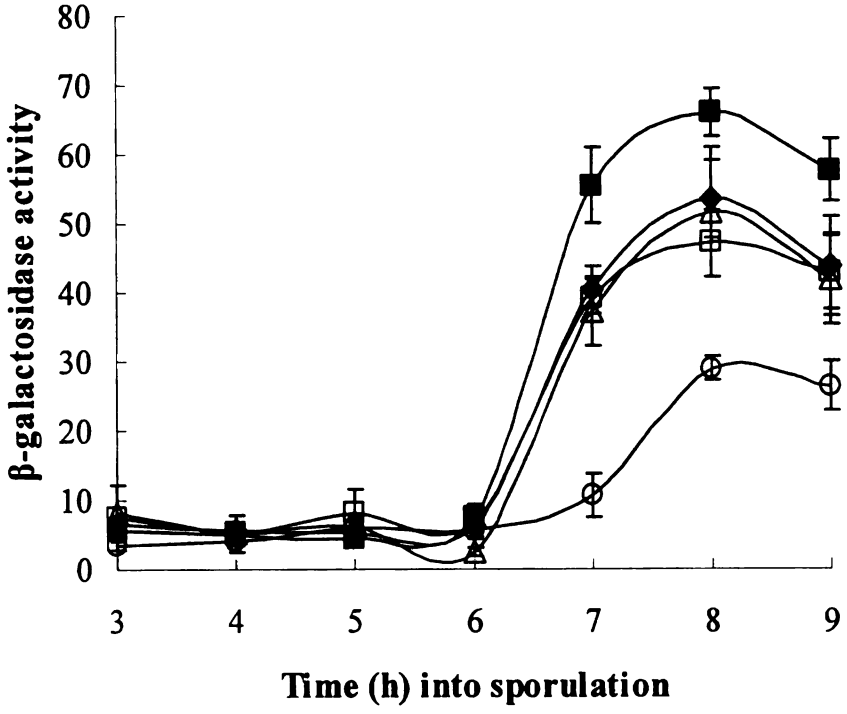
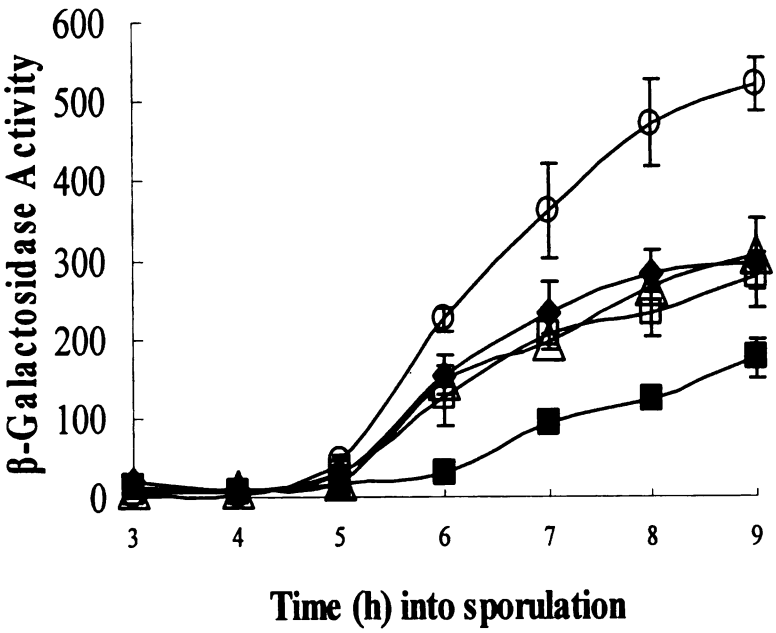


Fig. 3.5 (continued)

C



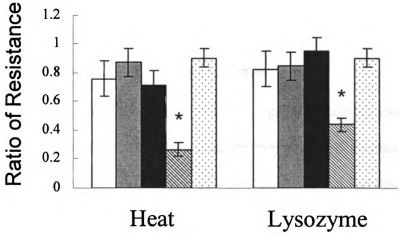
σ^K produced significantly less heat- and lysozyme-resistant spores (Fig. 3.6). Also as expected (Chapter II), these spores exhibited similar defects in coat assembly as strain BJP1 (containing P_{gerE}-*spoIIID* in an otherwise wild-type background; data not shown), but nevertheless germinated normally (Fig. 3.7).

Despite elevated σ^K (Fig. 3.3) and altered expression of σ^K-dependent genes (Fig. 3.5), strain BLW2 produced heat- and lysozyme-resistant spores as efficiently as the control strain BLW1 and wild-type PY79 (Fig. 3.6). The spores from all three strains germinated similarly (Fig. 3.7) and were indistinguishable when examined by electron microscopy (data not shown).

We also measured the ability of strains BLW1, BLW2, BLW3, and BLW4 to produce spores resistant to phenol, ethanol, and chloroform. None of the strains differed significantly (*p*-value of less than 0.05 by a Student *t*-test) from wild-type PY79 (data not shown).

Effects of circumventing the delay in CotC production. Expression of *cotC-lacZ* is delayed by 2 h relative to *gerE-lacZ* expression during sporulation (Fig. 3.5). To determine whether the delay is important for sporulation, we fused expression of *cotC* to the *gerE* promoter in a plasmid that can be maintained in *B. subtilis* in multiple copies. The plasmid was transformed into *B. subtilis* wild-type PY79, creating BJP4 with multicopy P_{gerE}-*cotC*. As a control, a plasmid with P_{gerE} but lacking *cotC* was transformed into PY79, creating BJP2. Western blot analysis of whole-cell extracts from sporulating cells showed that in BJP4, CotC accumulates to a low level at 2-4 h into sporulation, perhaps due to read-through transcription from a constitutive promoter on the plasmid, and the level of

Fig. 3.6. Resistance properties of spores. The ratio of colony-forming units after the indicated treatments to before the treatments was determined for *B. subtilis* wild-type PY79 (white), BLW1 (*sigK amyE::sigK*) (Grey), BLW2 (*sigK amyE::sigKmut*) (Black), BLW3 (*sigK amyE::sigK* P_{gerE}-*spoIIID*) (stripes), and BLW4 (*sigK amyE::sigKmut* P_{gerE}-*spoIIID*) (dots), at 24 h after resuspension in SM medium. Bars show the average of three determinations and error bars show 1 standard deviation of the data. Asterisks indicate $p < 0.05$ in the comparison between BLW3, and both PY79 and BLW4.


Ratio of Resistance
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Heat
Lysozyme

CotC raises beginning at 5 h into sporulation, presumably due to transcription from P_{gerE} (Fig. 3.8). As expected, the CotC level began to rise about 2 h later, at 7 h into sporulation, in BJP2 and PY79. These results demonstrate that multicopy P_{gerE}-*cotC* in BJP4 circumvents the 2-h delay in CotC accumulation.

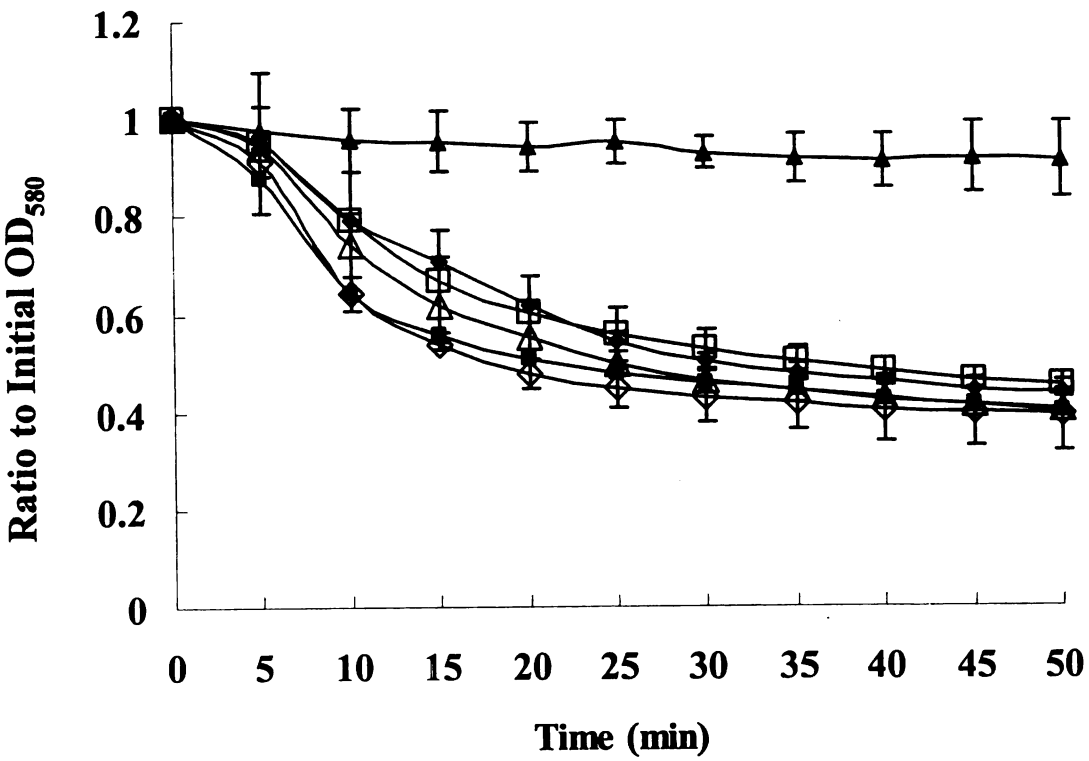
We measured the ability of strains BJP4 and BJP2 to produce spores resistant to heat, lysozyme, phenol, ethanol, and chloroform. Neither strain differed significantly (*p*-value of less than 0.05 by a Student *t*-test) from wild-type PY79 (data not shown). Also, the germination kinetics in response to L-alanine was similar for spores produced by strains BJP4 and BJP2 as compared to PY79 (data not shown). We conclude that accumulating CotC 2 h earlier than normal did not detectably alter spore resistance or germination properties.

DISCUSSION

Our study resulted in two novel findings about a negative regulatory loop in the MC gene regulatory network. First, circumventing GerE repression of *sigK* elevated the σ^K level and probably the GerE level (since expression of a *gerE-lacZ* translational fusion increased) but this did not detectably alter spore resistance, germination, or structure. This reveals robustness in the network. Second, circumventing GerE repression of *sigK* suppressed the effects on sporulation of persistent *spoIIID* expression. This suggests that negative regulatory loops with opposing effects enhance network robustness and presumably optimize target gene expression.

The results of a previous study (Baccigalupi, *et al.* 2004) aided our effort to circumvent GerE repression of *sigK*. Initially, we deleted from +5 to +15 relative to the *sigK*

Fig. 3.7. Spore germination. *B. subtilis* wild-type PY79 (■), BLW1 (*sigK amyE::sigK*) (◇), BLW2 (*sigK amyE::sigKmut*) (△), BLW3 (*sigK amyE::sigK* P_{gerE}-*spoIIID*) (◆), BLW4 (*sigK amyE:: sigKmut* P_{gerE}-*spoIIID*) (□), and EUDC9901(*gerE*) (▲) were induced to sporulate by resuspension in SM medium, samples were collected after 24 h, spores were purified, and quantitative germination assays were performed. Each point is the average of three determinations and error bars show 1 standard deviation of the data.

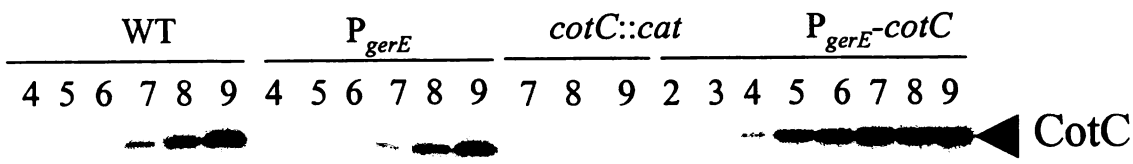

1.2
1
0.8
0.6
0.4
0.2
0
Ratio to Initial OD580
0
5
10
15
20
25
30
35
40
45
50
Time (min)

transcriptional start site but this did not result in elevated pro-σ^K and σ^K levels (data not shown), perhaps due to interference with promoter utilization by RNAP. After learning that a mutation in the GG sequence within the GerE binding site in the *cotH* promoter region increased *cotH-lacZ* expression (Baccigalupi, *et al.* 2004), we made the *sigKmut* allele shown in Figure 2. This allele resulted in similar levels of pro-σ^K and σ^K accumulation as observed previously in a *gerE* null mutant, which accumulated about twofold more total pro-σ^K/σ^K than wild type (Ichikawa, *et al.* 1999).

What limits total pro-σ^K/σ^K accumulation to about twofold more than in wild type? SpoIIID activates *sigK* transcription by σ^E RNAP (Halberg and Kroos. 1994) or σ^K RNAP (Kroos, *et al.* 1989). Late in sporulation, the SpoIIID and σ^E levels decrease due to negative feedback by σ^K RNAP (Halberg and Kroos. 1992; Zhang and Kroos. 1997; Zhang, *et al.* 1999). This suggested that SpoIIID might become limiting for *sigK* transcription by σ^K RNAP; however, this is not the case because introduction of P_{gerE}-*spoIIID* into the *sigK amyE*::*sigKmut* background did not change its pro-σ^K or σ^K level (Fig. 3.3). Perhaps processing of pro-σ^K to active σ^K, or degradation of σ^K, limits accumulation of *sigK* products under these conditions. Such posttranslational mechanisms would contribute to network robustness by limiting *sigK* autoregulation in the event that negative regulatory loops (i.e., GerE repression and loss of the SpoIIID activator) that operate at the transcriptional level fail.

The elevated σ^K level due to the *sigKmut* allele did not detectably alter the final product of the MC gene regulatory network, the spores. Elevated σ^K did not significantly alter the number of heat-, lysozyme-, phenol-, ethanol-, or chloroform-resistant spores produced

Fig. 3.8. Levels of CotC during sporulation. *B. subtilis* wild-type (WT) PY79, BJP2 (P_{gerE}), BD071 (*cotC*), and BJP4 (P_{gerE}-*cotC*) were sporulated by resuspension in SM medium. Samples were collected and subjected to Western blot analysis as described in the Fig. 3.3 except anti-CotC antiserum was used. Numbers indicate the h into sporulation.


WT
P_{gerE}
cotC::*cat*
P_{gerE}-*cotC*
4 5 6 7 8 9 4 5 6 7 8 9 7 8 9 2 3 4 5 6 7 8 9
CotC

(Fig. 3.6 and data not shown), nor did it alter spore germination kinetics in response to L-alanine (Fig. 3.7). Moreover, we detected no difference in spore structure as compared to wild type upon examination of thin sections by electron microscopy. By these criteria, the network is robust to elevated σ^K in terms of its output. On the other hand, expression of all three σ^K-dependent genes tested was altered (Fig. 3.5). Increased expression of *gerE* and *cotC* can be understood in terms of their dependence on σ^K RNAP and, in the case of *cotC*, activation by GerE (Zheng, *et al.* 1992). Transcription of *cotD* also depends on σ^K RNAP (Kroos, *et al.* 1989), but a high level of GerE represses *cotD* transcription *in vitro* (Ichikawa, *et al.* 1999), so elevated GerE in the strain with elevated σ^K might account for the observed decrease in *cotD* expression (Fig. 3.5C). Based on these three genes, it seems likely that expression of many of the 108 other genes in the σ^K regulon (Eichenberger, *et al.* 2004) is altered by elevated σ^K. In terms of gene expression, the network seems quite susceptible to perturbation of the negative regulatory loop involving GerE repression of *sigK*. This provides one rationale for retention of the regulatory loop during evolution; mutations in loop components may subtly alter MC gene expression, optimizing it for a particular ecological niche.

A second rationale for evolutionary retention of GerE repression of *sigK* in *B. subtilis* is its ability to suppress sporulation defects caused by persistent *spoIIID* expression. Preliminary results suggest that transposon insertion mutations in several genes elevate expression of a *spoIIID-lacZ* fusion (Chapter II). Hence, the MC gene regulatory network appears to be quite susceptible to mutational perturbation leading to elevated SpoIIID, which results in spore defects (Chapter II). We have shown here that mutating the GerE binding site in the *sigK* promoter elevates the σ^K level and probably the GerE level (since

expression of a *gerE-lacZ* translational fusion increased), compensating for persistent *spoIIID* expression by restoring MC gene expression (including that of *gerE-lacZ*) and formation of spores with normal resistance properties and coat structure. Undoubtedly, other mutations could elevate the σ^K level as well, but the negative regulatory loop involving GerE repression of *sigK* increases network robustness by providing additional targets for mutations that can compensate for changes in the SpoIIID level.

Is GerE repression of *sigK* likely present in sporeformers related to *B. subtilis*? A search for orthologs of MC transcription factors revealed that σ^K is present among *Bacillus* and *Clostridium* species, but GerE is absent from *Clostridium* (Eichenberger, *et al.* 2004). Among *Bacillus* species, we searched for a GerE binding site in the *sigK* promoter region. As shown in Supplemental Fig. 3SA, *Bacillus licheniformis* differed from *B. subtilis* at only one position, which did not affect either match to the GerE consensus binding sequence (Fig. 3.2). However, most strains of *Bacillus cereus*, *Bacillus weihenstephanensis*, all strains of *Bacillus anthracis*, and one strain of *Bacillus thuringiensis* have two changes that create mismatches to the TRGGY core of the GerE consensus binding sequence (Supplemental Fig. 3SB). One of these changes is a G to A transition at a position predicted to interact with Lys41 in *B. subtilis* GerE (Ducros, *et al.* 2001). This Lys residue is perfectly conserved among GerE homologs of the organisms shown in Supplemental Fig. 3S. Moreover, the strains in Supplemental Fig. 3SB do not exhibit a second match to the GerE consensus binding sequence, as does *B. subtilis* (Fig. 3.2). Therefore, GerE is likely to bind more weakly, if at all, to the corresponding position in the *sigK* promoter region of the strains in Supplemental Fig. 3SB. For the strains in Supplemental Fig. 3SC, it seems even less likely that GerE represses *sigK* transcription.

On the other hand, the strains in Supplemental Fig. 3SD retain more characteristics of the GerE binding site in the *B. subtilis sigK* promoter region, suggesting that GerE represses *sigK* transcription in these species. We speculate that about half of the distinct species shown in Supplemental Fig. 3S have GerE repression of *sigK* transcription as a negative feedback loop in their MC gene regulatory network.

Elevated σ^K in the *B. subtilis sigKmut* strain did not detectably hasten the decrease in the level of SpoIIID during sporulation (Fig. 3.4). We infer from this result that σ^K is not the rate limiting factor in the regulatory loop by which σ^K RNAP leads to a decrease in the SpoIIID level (Halberg and Kroos. 1992; Zhang and Kroos. 1997; Zhang, *et al.* 1999). As depicted in Figure 3.1, the evidence suggests that one or more genes transcribed by σ^K RNAP inhibit activity of σ^A RNAP, decreasing transcription of *sigE* and other early genes, including *spoIIID*, but the mechanism of inhibition remains a mystery.

Expression of *cotC* 2 h earlier than normal, from the heterologous *gerE* promoter, did not detectably alter spore resistance or germination properties. Why, then, is *cotC* expression normally delayed? Perhaps repression of *cotC* by SpoIIID prevents wasteful expression before CotC can assemble into the spore coat. CotH is required for assembly of CotC into the outer coat (Naclerio, *et al.* 1996; Isticato, *et al.* 2004). The *cotH* gene is expressed by 5 h into sporulation (Baccigalupi, *et al.* 2004) and CotH accumulates by 6 h (Zilhao, *et al.* 1999), but earlier times have not been examined. Under our conditions, *cotC-lacZ* was expressed by 7 h into sporulation (Fig. 3.5B) and CotC began to accumulate at that time (Fig. 3.8). Earlier expression of *cotC* from P_{gerE}-*cotC* on a multicopy plasmid may be inconsequential because CotH availability limits CotC assembly. Interestingly, GerE represses *cotH* expression, but circumventing this negative regulatory loop by

mutating the GerE binding site in the *cotH* promoter region had no detectable effect on spore structure or function, although it did allow accumulation of CotC in the MC (Baccigalupi, *et al.* 2004). Normally, CotC does not accumulate in the MC, probably because it assembles immediately into the spore coat (Isticato, *et al.* 2004). In cells with GerE-independent expression of *cotH*, elevated CotH appears to stabilize CotC in the MC, suggesting that the spore has a limited capacity to incorporate CotC (Baccigalupi, *et al.* 2004).

In summary, circumventing GerE repression of *sigK* elevated the σ^K level and probably the GerE level, two nodes in the MC gene regulatory network, likely altering expression of many genes in the σ^K regulon but not detectably changing spore resistance, germination, or structure. However, this perturbation compensated for the effects of persistent *spoIIID* expression, which also appears to alter two nodes in the network, maintaining the SpoIIID level late during sporulation (when it normally decreases) and probably reducing the GerE level (since expression of a *gerE-lacZ* translational fusion is diminished) (Chapter II). We infer that the decrease in SpoIIID normally brought about, at least in part, by an unknown mechanism that requires σ^K RNAP activity (Zhang, *et al.* 1999), promotes *gerE* expression. In contrast, GerE repression of *sigK* normally inhibits further *gerE* expression because less σ^K RNAP is produced. It appears that two negative feedback loops with opposing effects on *gerE* expression, and different effects on other nodes [σ^K RNAP activity reduces σ^E as well as SpoIIID (Halberg and Kroos. 1992; Zhang and Kroos. 1997; Zhang, *et al.* 1999) and GerE reduces σ^K (Ichikawa, *et al.* 1999) (Fig. 3)], enhance robustness of the MC network and optimize expression of target genes.

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Supplemental Figure

Fig. 3S. Alignment of predicted *sigK* promoter regions of *Bacillus* species and comparison with the GerE binding site in the *B. subtilis sigK* promoter region. (A) The top line shows the *sigK* promoter -35 and -10 region consensus sequences followed by the GerE consensus binding sequence (Eichenberger, *et al.* 2004). The italicized *gg* sequence is predicted to interact with Lys41 of GerE. The second line shows the *B. subtilis sigK* promoter (Kunkel, *et al.* 1988) followed by a gap introduced to facilitate alignment with sequences below. The gap is followed by the GerE binding site, based on DNase I footprinting (Ichikawa, *et al.* 1999), which is aligned with the GerE consensus binding sequence above and in which the bold sequence indicates the match on the complementary strand (not shown) to the TRGGY core of the GerE consensus binding sequence. This is followed by the number of nucleotides to the ATG translational start codon of *sigK*. Subsequent lines show predicted *sigK* promoters from other *Bacillus* species, followed by a gap where necessary to facilitate the alignment with the GerE consensus binding sequence. (B-D) Underlined nucleotides indicate mismatches to the GerE consensus binding sequence. In panels B and C, no match to the TRGGY core of the GerE consensus is evident on the complementary strand, whereas in panel D, the match is shown in bold with mismatches underlined.

A aca 16 catannnt rwwtrggynnyy
aca 16 catacatt tacatataggcttttgcctacatac 16 ATG *Bacillus subtilis*
aca 16 catacatt tacatataggcttctgcctacatac 20 ATG *Bacillus licheniformis* ATCC 14580

B aca 16 catacact tagtaaagaccactattttggcgg 31 TTG *Bacillus cereus* E33L
aca 16 catacact tagtaaagaccactattttggcgg 38 ATG *Bacillus cereus* G9241
aca 16 catacata tagtaaagaccactattttggcgg 11 TTG *Bacillus cereus* subsp. cytotoxis NVH 391-98
aca 16 catacaca taataaagaccactattttggcgg 2 GTG *Bacillus cereus* ATCC 14579
aca 16 catacaca tagtaaagaccactattttggcgg 11 TTG *Bacillus weihenstephanensis* KBAB4
aca 16 catacact tagtaaagaccactattttggcgg 2 GTG *Bacillus anthracis* str. A2012
aca 16 catacact tagtaaagaccactattttggcgg 11 TTG *Bacillus anthracis* str. Ames
aca 16 catacact tagtaaagaccactattttggcgg 11 TTG *Bacillus anthracis* str. Sterne
aca 16 catacact tagtaaagaccactattttggcgg 11 TTG *Bacillus thuringiensis* serovar konkukian str. 97-27

C cca 18 cgtagtga agctttatgaaacaactattatgc 10 ATG *Bacillus cereus* ATCC 10987
ata 16 catattca tatataaataacaattttatataat 26 ATG *Bacillus thuringiensis* str. Al Hakam
ata 16 catattca tatataaataacaatttcttttaat 26 ATG *Bacillus thuringiensis serovar israelensis* ATCC 35646
aca 16 catagata taaccattcttgtggaggtgagct 1 ATG *Bacillus clausii* KSM-K16

D aca 18 cataccta taaaggtaacgaactgatcat 12 GTG *Bacillus halodurans* C-125
aca 18 cataaagt gttacataggctaatacctagctac 38 TTG *Oceanobacillus iheyensis* HTE831
aca 18 cataggaattagttataggcaatagtcctagaaa 12 ATG *Bacillus* sp. NRRL B-14911
aca 18 catataca taacgataggcggtttccgccgatg 21 ATG *Geobacillus kaustophilus* HTA426
aca 18 catataca tacggtagggcggtttccgccgatg 21 ATG *Geobacillus thermodenitrificans* NG80-2

Fig 3S (continued)

tgagaatctcctccgtgtgattttgacgtgtacagacacgaccaagcccaagtcgacatacctataaaggtaacgaact
gatcatgggggtgatgtcgtg *Bacillus halodurans* C-125

tatagccacgcaaccggaccgtcagcaaaacattccttcgtacagacacaaccaagcccagtgttgcatagatataacc
attcttgtggaggtgagcttATG *Bacillus clausii* KSM-K16

ttggaagtgttgagttttataaataaactgtacagacacaaacaacctcacgcccacata
cacttagtaaagaccactattttggcggaggtgaaaggtTTG *Bacillus thuringiensis serovar konkukian* str. 97-27

attggaagtgttgagttttataaataaactgtacagacacaaacaacctcacgcccacatacacttagta
aagaccactattttggcggaggtgaaaggtTTG *Bacillus anthracis* str. Sterne

atattggaagtgttgagttttataaataaactgtacagacacaaacaacctcacgcccacatacacttagtaaa
gaccactattttggcggaggtgaaaggtttg *Bacillus anthracis* str. Ames

taaaatattttaaatattggaagtgttgagttttataaataaactgtacagacacaaacaacctcacgcccacata
cacttagtaaagaccactattTTGgcggagGTGaaaggtTTG *Bacillus anthracis* str. A2012

tcgatctaatgtaatcatgaatagtgtccccttaatagcatattcatatataaataacaatttcttttaattataat
ataacaataaaatattttaaATG *Bacillus thuringiensis serovar israelensis* ATCC 35646

ttgttacatttaataatcgatctaatgtgagcatgaatagtgtccccaaaaatagcatattc
atatataaataacaattttatataattataatataacaataaaatattttaaATG *Bacillus thuringiensis* str. Al Hakam

CHAPTER IV: A Genetic Screen to Identify Possible Components of the σ^K RNAP Negative Feedback Loop in *B. subtilis*

ABSTRACT

In sporulating *Bacillus subtilis*, a regulatory network controls gene expression temporally in the mother cell. Several negative regulatory loops exist between different components of the network. Among them, transcriptionally active σ^K RNAP negatively regulates early gene expression during sporulation, including that of *sigE* and *spoIIID*. The mechanism of this σ^K RNAP negative feedback loop was investigated using a genetic screen to identify its putative components. Random mutagenesis with mini-Tn10 was followed by screening for elevated *spoIIID-lacZ* expression as an indicator of interrupted σ^K RNAP negative feedback. Eleven candidates were identified with *spoIIID-lacZ* expression higher than the wild-type. Four insertions were at the same location in a 16S rRNA gene and one insertion was at a different location in a 16S rRNA gene. The six other insertions were in genes encoding: N-acetylglucosamine 6-P deacetylase (*nagA*); a protein functioning in DNA mismatch repair (*mutL*), a nucleoside diphosphate kinase (*ndk*), an hypothetical protein (*yvkC*), a σ^{54} family-specific transcriptional regulator (*levR*), and a serine protease, belonging to the heat-shock family of proteins (*ydkA*). Further studies are needed to confirm that null mutations in these genes elevate *spoIIID-lacZ* expression and investigate the mechanisms involved.

INTRODUCTION

Sporulation is a process that is initiated by the gram-positive soil bacterium *Bacillus subtilis* when it encounters starvation. Four regulators function in the order σ^E, SpoIIID, σ^K and GerE, form a regulatory cascade that controls gene expression in the mother cell of sporulating *B. subtilis*. Several negative regulatory loops have been discovered in the mother cell gene regulatory network. One of these involves σ^K RNAP negative feedback on early gene expression. The *ald* gene, which encodes alanine dehydrogenase, and *sigE*, which encodes σ^E, are both over-expressed in a *sigK* mutant, suggesting that σ^K negatively regulates genes in the σ^A regulon (Zhang and Kroos. 1997; Zhang, *et al.* 1999). The *spoIIID* gene is also overexpressed in a *sigK* mutant, presumably due to the higher concentration of σ^E RNAP, which transcribes *spoIIID* (Halberg and Kroos. 1992; Zhang and Kroos. 1997; Zhang, *et al.* 1999). It is particularly interesting that transcriptional activity of σ^K RNAP is required to carry out this negative effect. A single amino acid substitution in σ^K, C109R in subregion 2.4, allows the mutated σ^K to bind to the core RNAP, but fails to direct *gerE-lacZ* expression (Zhang, *et al.* 1999). When fractionated by gel filtration chromatography, σ^{KC109R} is co-eluted with core RNAP (Zhang, *et al.* 1999). Expression of *ald-lacZ* and *sigE-lacZ* is higher late during sporulation in the *sigKC109R* mutant than in wild type (Zhang, *et al.* 1999), suggesting loss of the negative effect on early gene expression in the *sigKC109R* mutant. It is possible that wild-type σ^K RNAP transcribes genes whose products function in the negative feedback loop. Alternatively, limitation of resources (e.g., nucleotides) during sporulation might cause the negative effect through transcriptional active σ^K RNAP. To elucidate the mechanism by which σ^K RNAP negatively feeds back on early gene expression during sporulation, we carried out a genetic

screen experiment designed to identify genes involved. Eleven transposon insertion mutants were identified and found to be inserted in 7 different genes. Each insertion elevates *spoIIID-lacZ* expression, mimicking the effect of interrupting the σ^K RNAP negative feedback loop.

MATERIALS AND METHODS

Bacterial strains. BK533 (spo+ SPβ::*spoIIID-lacZ*/Cm^r), a *B. subtilis* strain that is isogenic with wild-type strain PY79, and contains a translational fusion of *spoIIID-lacZ* (Kunkel, *et al.* 1989), was transformed with pCm::Sp (Steinmetz and Richter. 1994) in order to replace the *cat* gene with a gene encoding spectinomycin (*spc*) resistant. Six transformants were streaked to obtain single colonies and these were transferred with toothpicks to DSM plates containing 40 μg/ml X-gal. The transformant with the most consistent blue color, BLW110, was used as the recipient strain for the genetic screen. A cell lysate containing the phage SPβ:: *spoIIID-lacZ* was obtained from BK533 as described (Cutting and Horn. 1990). Transduction of SPβ:: *spoIIID-lacZ* into a *B. subtilis* strain BK556 (*spoIVCB23*) (Kunkel, *et al.* 1989) was as described (Cutting and Horn. 1990). The resulting strain, BLW100, was used as a control to mimic the effect of interruption of the σ^K RNAP negative feedback loop.

Preparation of mini-Tn10 mutated chromosomal DNA library. Both the suicide plasmid pHV1249 (Petit, *et al.* 1990), which carries the mini-Tn10 transposon, and the recipient strain, BLW110, carry an erythromycin-resistance gene. To avoid undesired homologous recombination between the BLW110's chromosome and pHV1249, Tn10 mutagenesis was first performed in the *B. subtilis* wild-type strain PY79 as described (Petit, *et al.* 1990). A pilot experiment showed that approximately 20% of the colonies that

were selected by adding chloromphenicol (5 μl/ml) at 51°C were also erythromycin-resistant and should still contain pHV1249. The other 80% of colonies were chloramphenicol-resistant and erythromycin-sensitive, as expected for transposition of mini-Tn10 into PY79's chromosome. Serial dilutions of the mixture resulting from the mutagenesis procedure were made to generate plates with approximately 1000 Cm^r colonies each. Fifteen of these plates were grown overnight at 51°C. Colonies from each plate were collected by scraping and a separate chromosomal DNA library was prepared from each plate as described (Cutting and Horn. 1990).

Transformation of the mini-Tn10 libraries into *B. subtilis* with the *spoIIID-lacZ* reporter. The mini-Tn10 mutagenized chromosomal DNA libraries were transformed into BLW110 as described (Cutting and Horn. 1990), with selection for resistance to spectinomycin (100 μg/ml) and chloromphenicol (5 μg/ml) on LB agar plates. After 37 °C overnight incubation, transformants were picked onto DSM agar containing 40 μg/ml X-gal. BLW100 and BLW110 were picked onto the same plate as controls. Colonies with blue color significantly darker than BLW110 and similar to BLW100, were cultured in LB liquid with appropriate antibiotics and made into frozen stocks.

Analysis of β-galactosidase activity. A single colony of each candidate strain and three isolates of BLW110 were induced to sporulate by resuspension in SM medium (10 candidates except for BLW200) or DS medium (BLW200 and BLW110 only) as described (Sterlini and Mandelstam. 1969). The onset of sporulation (T_0) is defined as the time of resuspension for cultures in SM medium and the point of departure from exponential growth for culture in DS medium. One ml samples were collected at 4 h and 5 h into sporulation. Pellets of cells were stored at -70 °C prior to the β-galactosidase activity assay.

Before the assay, cells were permeabilized with toluene and treated with lysozyme (Miller. 1972). The β-galactosidase specific activity was determined by as described (Miller. 1972) using o-nitrophenol-β-D-galactopyranoside (ONPG) as the substrate. One unit is defined as the amount of enzyme needed to hydrolyze 1 μmol of substrate per minute per unit of initial cell optical density at 595 nm. Candidates with a specific activity higher than that of BLW110 plus two standard deviations of the data (generated from three replicates of BLW110) were selected for further analysis.

Preparation of ultra-pure genomic DNA. Candidates that were selected after the β-galactosidase activity assay were grown in 14 ml LB containing spectinomycin (100 μg/ml) and chloromphenicol (5 μg/ml). Cells were harvested when the OD_{600} reached 2.0. Chromosomal DNA from these strains was prepared using the genomic-tip 500/G kit (QIAGEN), eluted to a final volume of 500 μl. Chromosomal DNA was then subjected to a second purification. First, 250 ul of phenol and 250 ul of chloroform were added to the chromosomal DNA, mixed gently and centrifuged at 4,000 rpm for 10 minutes at 4 °C. Second, an equal volume of chloroform was added to the aqueous phase from the first preps, then mixed gently and centrifuged in the same way. To the aqueous phase, 1/9 volume of 3 M sodium acetate was added and mixed by inverting the tube, then 2.5 volumes of ice cold 100% ethanol were added, mixed by inversion, and the sample was incubated at -20 °C for at least 1 hour (or overnight) to participate DNA. Samples were centrifuged at 12,000 rpm for 10 minutes and pellets were air-dried at room temperature for 5 minutes, then resuspended in 100 ul of distilled water, and stored at -20°C.

Identification of mini-Tn10 interrupted genes. Direct sequencing of ultra-pure genomic DNA was used to identify the gene interrupted for 10 out of 11 of the candidate

strains (except BLW224). Primer 1, 5'-ccgttagttgaagaaggtttt-3', which matches a sequence downstream of the *cat* gene but upstream of the inverted repeat of the mini-Tn10 in pHV1249, was used for genomic DNA sequencing. The primer (3.2 pmol) and chromosomal DNA (2 ug) in 12 ul total volume were subjected to sequencing using a BigDye3.1 Kit (ABI Prism) and an ABI Prism 310 sequencer at the Research Technology Support Facility of Michigan State University.

An inverse PCR was used to amplify the DNA fragment that contains the mini-Tn10 and adjacent interrupted gene in BLW224. Chromosomal DNA from BLW224 was digested with *Hin*dIII and ligated with T4 DNA ligase. Primer 1 and primer 2, 5'-gagcgtagcgaaaaatcct-3', which matches a sequence upstream of *cat* gene but downstream of the inverted repeat in the mini-Tn10 in pHV1249, were used to amplify the ligated BLW224 chromosomal DNA. A 1.5 kb DNA fragment was amplified and was used as the template for sequencing with primer 1 to identify the gene interrupted in BLW224.

RESULTS

Thirty-one candidates were identified by screening on X-gal-containing plates. Approximately 4000 transformants were obtained by transforming mini-Tn10 mutagenized chromosomal DNA from *B. subtilis* PY79 into *B. subtilis* strain BLW110, which has the SPβ::*spoIIID-lacZ* translational fusion as a reporter. Transformants, along with BLW110 and BLW100, a *sigK* (*spoIVCB23*) mutant bearing SPβ::*spoIIID-lacZ* in order to mimic the effect of interrupting of the σ^K RNAP negative feedback loop, were inoculated onto DSM plates containing 40 μg/ml X-gal. Colonies with deeper blue color than BLW110 and similar color as BLW100 were identified for further study. Thirty-one such candidates were

Table 4.1. Origin of candidate strains from genetic screen

No. of mini-Tn10 library	Strain name
1	BLW 200, 201, 202, 203, 204
2	BLW 205, 206, 207, 208, 224, 225, 227
3	BLW 209, 210
4	BLW 229, 230
5	BLW 220, 221, 222, 223
6	BLW 216, 217, 218, 219
7	BLW 226, 228
8	BLW 211, 212, 213, 214, 215

identified and named BLW200 to BLW230. The origins of these strains from the different mini-Tn10 chromosomal DNA libraries are described in Table 4. 1.

Candidates were narrowed down to 11 by β-galactosidase assays. To further test for higher *spoIIID-lacZ* expression in the candidate strains identified on X-gal-containing plates, β-galactosidase assays were performed to quantify *spoIIID-lacZ* expression. The expression of *spoIIID-lacZ* in BLW200 peaked at 5 hours into sporulation in DS medium (Fig. 4.1 A). The other 30 candidates were induced to sporulate in SM medium. Expression peaked at 4 or 5 hours into sporulation. Eleven of these candidates showed significantly elevated expression at T_4 (Fig. 4.1B) or T_5 (Fig. 4.1C). Expression was more than the average of three isolates of the recipient strain BLW110 plus two standard deviations (significantly elevated expression at the 95% confidence level). Therefore, 12 candidates were confirmed as having significantly higher expression than the recipient.

Identification of genes interrupted in the strains with elevated *spoIIID-lacZ* expression. Direct sequencing of genomic DNA identified 10 out of 12 interrupted genes. The gene interrupted by mini-Tn10 in BLW224 was identified by sequencing a PCR product derived from genomic DNA that had been digested with *Hin*dIII and ligated. Gene interrupted in BLW214 was accidentally missed for sequencing. The results of DNA

Fig 4.1. Analysis of β-galactosidase activity. (A) *B. subtilis* strains BLW200 (■) and BLW110 (▲) were sporulated by DSM method. β-galactosidase activities from both strains were analyzed. Standard deviation is generated from three biological replicated of each strain. (B) *B. subtilis* strains BLW201 to BLW230 (numbered as 1 to 30 in the Y axis) were sporulated by resuspension in SM medium and β-galactosidase activities from each strain was analyzed from samples at 4 h into sporulation. *B. subtilis* strain BLW110, which is wild type *B. subtilis* with the same *spoIIID-lacZ* fusion was used as negative control. Numbers above zero indicates a β-galactosidase activity that is higher than BLW110 plus two standard deviations. (C) The same experiment as in (B) except for those samples were collected at 5 h into sporulation.

(A)

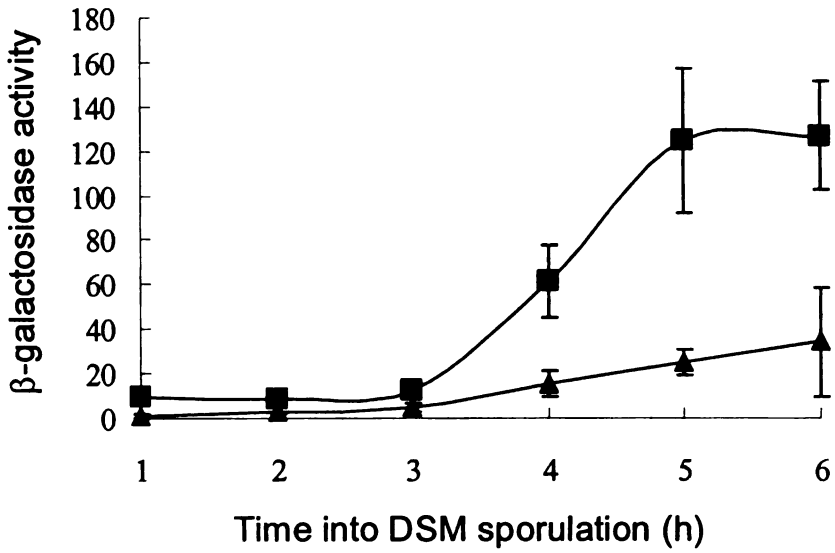
β-galactosidase activity
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
Time into DSM sporulation (h)

Fig 4.1 (continued)

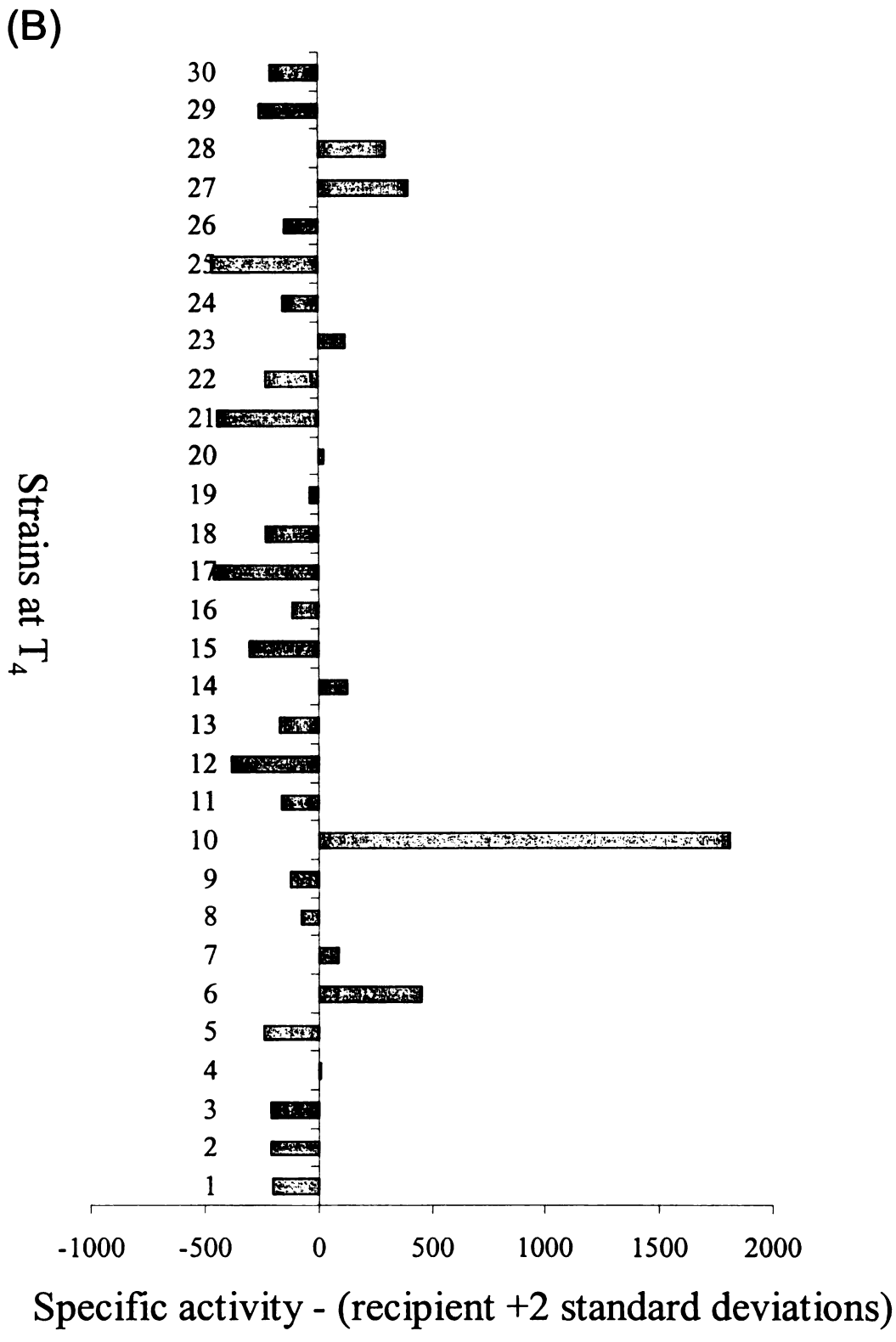

Fig 4.1 (continued)

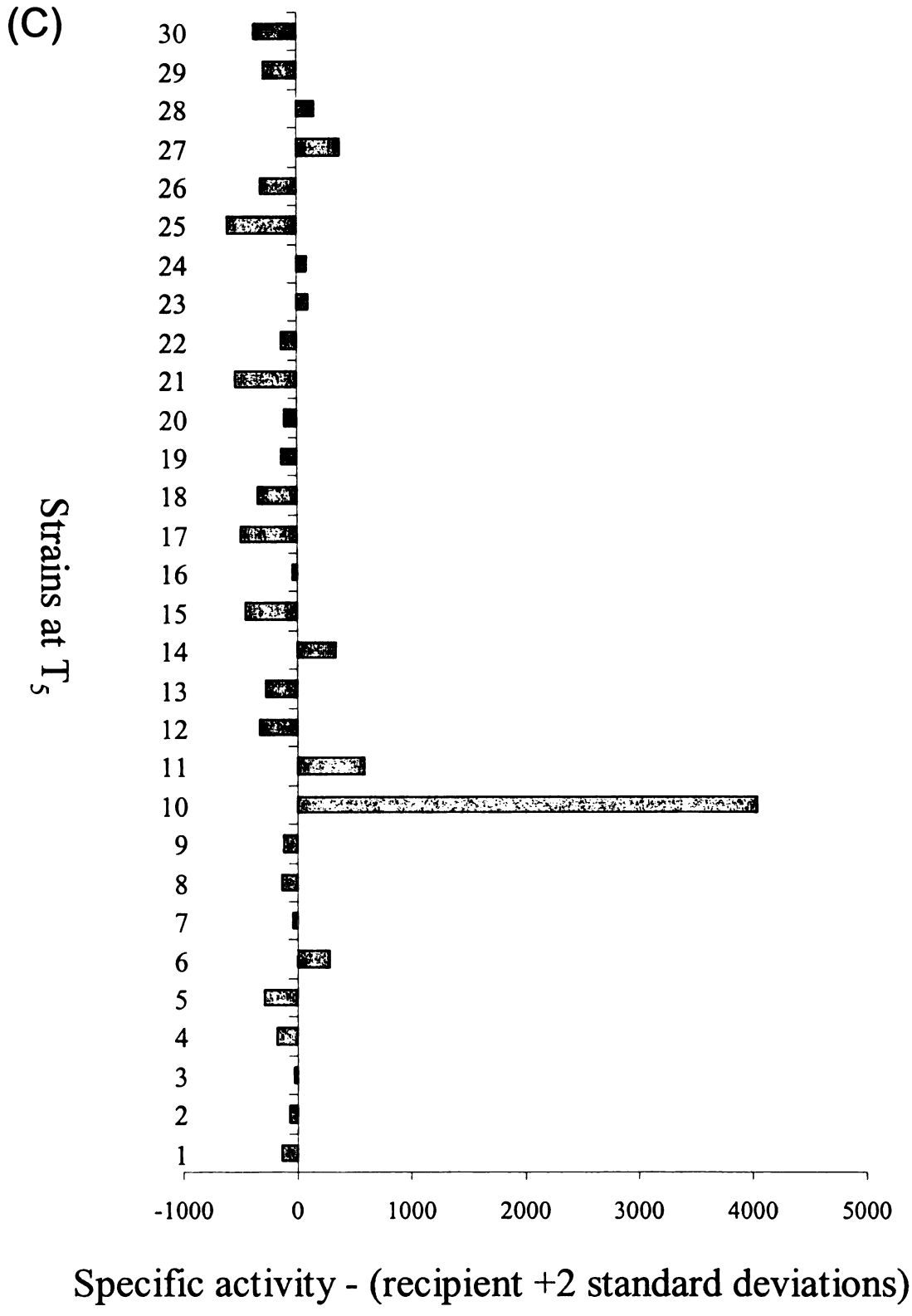

Table 4.2. Identification of genes interrupted by mini-Tn10 mutagenesis

Strain	Sequence quality[a]	Gene mapped	Annotation	Matched length (% identical)	Insertion position[c]
BLW200	120	*nagA*	N-acetylglucosamine 6-P deacetylase	238(86%)	329/461
BLW204	90	16S rRNA	Ribosomal RNA-16S	302 (95%)	840/1555
BLW206	516	16S rRNA[b]	Ribosomal RNA-16S	271(100%)	830/1555
BLW207	355	16S rRNA[b]	Ribosomal RNA-16S	136(100%)	830/1555
BLW210	80	*mutL*	DNA mismatch repair	93(88%)	78/628
BLW211	586	*ndk*	Nucleoside diphosphate kinase	320(100%)	54/150
BLW220	654	*yvkC*	Unknown. Similar to pyruvate, water dikinase	408(100%)	131/832
BLW223	517	*levR*	Transcriptional regulator LevR of *Bacillus subtilis* has domains homologous to both sigma 54- and phosphotransferase system-dependent regulators	302(100%)	777/957
BLW224	723	16S rRNA[b]	Ribosomal RNA-16S	415(100%)	830/1555
BLW227	639	16S rRNA[b]	Ribosomal RNA-16S	341(100%)	830/1555
BLW228	740	*htrA* (*ykdA*)	Serine protease Do (heat shock protein)	450(100%)	88/450

[a] Sequence quality of the number of continuous nucleotides identified with high confidence by the ABI Prism 310 sequencer.
[b] The mini-Tn10 is at the same location in a 16S rRNA gene in four strains.
[c] Codon into with mini-Tn10 inserted/total codons for protein-encoding genes or nucleotide into with mini-Tn10 inserted/total nucleotides for 16S rRNA.

sequencing are indicated in Table 4.2. DNA fragments with good qualities are indicated by "trimmed length", which provides the number of nucleotides that have been identified with

good confidence by the ABI Prism 310 sequencer. Junctions between mini-Tn10 and the interrupted genes were identified and all were located within the region of high quality sequence except for BLW210. Three strains, BLW200, BLW214, and BLW210, showed less than 100% identity to a *B. subtilis* gene, perhaps due to lower sequence quality. The poor sequence quality seemed to be strain-dependent. For example, for BLW210, chromosomal DNA was extracted three times and sent for sequencing in parallel with a positive control. The positive control yielded high quality sequence each time but BLW210 chromosomal DNA never gave high quality sequence. Also, except for BLW224, chromosomal DNA from the other 10 strains could not be successfully digested with *Hin*dIII and ligated to generate a PCR product for sequencing.

DISCUSSION

A gene for 16S rRNA was interrupted in five strains (Table 4.2). Four of these have mini-Tn10 inserted at exactly the same location in a 16S rRNA gene. There are 10 copies of 16S rRNA in the genomes of *B. subtilis*. Due to the limited lengths of good sequence and high similarity of the 16S rRNA genes, our data do not identify which 16S rRNA gene has been interrupted. The four strains with mini-Tn10 at the same location in a 16S rRNA gene might represent a single transposition event since all four strains arose from transformation with chromosomal DNA library 2 (Table 4.1). On the other hand, BLW214 represents an independent transposition event since this strain arose from transformation with a different chromosomal DNA library (Table 4.1) and mini-Tn10 is inserted at a slightly different location within a 16S rRNA gene. The identification of at least two independent insertions in one or more genes for 16S rRNA implies an important role for rRNA synthesis in the level of *spoIIID* expression during sporulation.

We speculate that disruption of an operon for rRNA frees σ^A RNAP and nucleotides for expression of other genes. In wild type, the σ^K RNAP negative feedback loop might, at least in part, reflect competition between σ^K and the earlier-acting mother cell sigma factors, σ^A and σ^E, for nucleotides and engagement of core RNAP in transcription of their regulons. Disruption of a highly transcribed rRNA operon might relieve the competition, resulting in higher *spoIIID* expression. Deletion of multiple rRNA operons might result in even higher spoIIID expression. Deletion of one rRNA operon occurs spontaneously with little or no effect on viability (Gottlieb, *et al.* 1985; Loughney, *et al.* 1983) but it is unknown whether additional rRNA operons can be deleted. This would be a possible direction of future studies, but first it would be important to verify that the mini-Tn10 causes the observed increase in *spoIIID-lacZ* expression by isolating chromosomal DNA from an insertion strain and transforming the original recipient strain BLW110.

Among the other genes interrupted by mini-Tn10, only *levR* encodes a transcription factor (Table 4.2). However, *levR* is a transcriptional activator of the levanase operon, which encodes a fructose-specific phosphotransferase system and the extracellular enzyme levanase that hydrolyzes fructose polymers and sucrose (Martin-Verstraete, *et al.* 1998) LevR has an N-terminal domain similar to σ^{54}-dependent activator proteins and a C-terminal domain similar to anti-terminator proteins like BglG and LicT. It seems unlikely the LevR is a direct repressor of *spoIIID* transcription. How disruption of *levR* increases *spoIIID* expression during sporulation (assuming this result is confirmed) is a mystery.

The same can be said for the other mini-Tn10 insertions. Like LevR, NagA is involved in carbohydrate catabolism. It is N-acetylglucosamine-6-phosphate deacetylase, which catalyzes the conversion of GlcNAc6P to glucosamine-6-phosphate (GlcN6P)

(Barnhart, *et al.* 2006). The *nagA* gene is likely in an operon with *nagB* (also involved in GlcN6P utilization) and possibly *yvoA*, a GntR family transcriptional regulator. Conceivably, the mini-Tn10 in *nagA* has a polar effect on *yvoA*, and YvoA is a repressor of *spoIIID*. This hypothesis could be tested by making a mutation in *yvoA*. Alternatively, elevated *spoIIID* expression might be an indirect effect of elevated intracellular GlcN6P due to *nagA* disruption, as deletion of *nagA* alters transcription from the curli-specific promoters of the *csgBA* and *csgDEFG* operons in *E. coli* (Barnhart, *et al.* 2006).

Two mini-Tn10 insertions interrupted genes whose products appear to be involved in nucleotide metabolism. Ndk is nucleoside diphosphate kinase, which catalyzes reactions that convert NDP to NTP (N could be A, T, G, C). YvKC is similar to pyruvate, water dikinase. This enzyme catalyzes the following reaction: ATP + pyruvate + H_2O = AMP + phosphoenolpyruvate + phosphate. Disruption of *yvkC* might increase the ATP level; increasing *spoIIID* transcription, but disruption of *ndk* would be expected to decrease NTP levels, so it is unclear how this could cause increased *spoIIID* transcription. As *yvkC* is likely in an operon with *yvkB*, which encodes a TetR/AcrR family transcriptional regulator, the possibility of a polar effect exists as described above for the insertion in *nagA*. On the other hand, *ndk* appears to be followed by a transcriptional terminator, so a polar effect is unlikely.

The *htrA* gene also appears to be followed by a transcriptional terminator. It encodes a serine protease induced by heat shock or secretion stress, and is probably involved in processing of extracellular enzymes (Noone, *et al.* 2001). Conceivably, its disruption stabilizes the *spoIIID*-β-galactosidase chimera produced from the *spoIIID-lacZ* fusion.

The interrupted gene in BLW210 was not identified with confidence due to low sequence quality (Table 4.2). Genomic DNA of BLW210 was prepared in parallel with other strains that yielded high quality sequence. The $OD_{260/280}$ of the BLW210 DNA suggested high purity. The reason for difficulty in sequencing is unknown. The junction between the mini-Tn10 and the interrupted gene is not located within the good sequence. However, a short stretch of *mutL* could be identified, although there were some mismatches (Table 4.2). MutL functions in DNA mismatch repair. Without proper DNA mismatch repair, the genome is prone to mutagenesis. The very high *spoIIID-lacZ* expression in BLW210 (Fig. 4.1 B and C) might be due to one or more mutations in the *spoIIID* promoter region. This hypothesis predicts that the mini-Tn10 in BLW210 will not result in high-level *spoIIID-lacZ* expression upon transformation into BLW110.

Obviously, this study is incomplete. As mentioned above, each mini-Tn10 must first be transformed into the original recipient strain BLW110 to test whether the mini-Tn10 insertion is responsible for elevated *spoIIID-lacZ* expression. Some additional ideas for further studies are also mentioned above. These studies might reveal the mechanistic basis of the σ^K RNAP negative feedback loop, However, the preliminary results presented here suggest that mutations in many genes can cause increased *spoIIID-lacZ* expression during sporulation. In terms of this single-gene output, the regulatory network seems quite susceptible to perturbation. It would be interesting to see whether any of the mini-Tn10 insertions perturb other network outputs, such as heat-resistant spore formation.

We hoped to identify one or more genes under σ^K control whose products carry out the negative feedback loop. Several genes were identified, but none of these have been reported to be under σ^K control. This suggests that σ^K RNAP negative feedback on early

gene expression during sporulation might stem from competition between different sigma factors for nucleotides and engagement of core RNAP in transcription of their regulon.

ACKNOWLEDGEMENTS

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APPENDIX I

Title: SDS-PAGE of Spore Coat Proteins form *B. subtilis* Strains with Perturbations in the Mother Cell Regulatory Network

ABSTRACT

Chapters II and III of this thesis describes *B. subtilis* strains that were constructed and studied in order to understand the importance of several negative regulatory loops in the mother cell network of gene expression during sporulation. Spores from a strain with SpoIIID maintained late during sporulation showed heat- and lysozyme-resistance defects. A strain in which GerE negative feedback on *sigK* was eliminated did not show spore defect, but expression of at least two spore *cot* genes (*cotC* and *cotD*) was altered. These and other strains in Chapters II and III were examined by SDS-PAGE to compare their spore coat proteins. None of the strains differed significantly difference from wild-type *B. subtilis*. This result suggests that structural, rather than compositional, differences cause the spore defects in the strain with persistent *spoIIID* expression.

INTRODUCTION

In the mother cell of sporulating *B. subtilis*, a regulatory network controls gene expression. Four transcriptional regulators, in the order σ^E-SpoIIID-σ^K-GerE, form a cascade of gene expression in which each regulator controls expression of the next regulator. Three negative regulatory effects, σ^K RNAP inhibition of early gene expression, GerE repression of *sigK* transcription, and SpoIIID repression of *cotC* transcription were studied (Chapters II and III). Strains that circumvent one or two of these negative regulatory loops were constructed. Here, the strains were induced to form spores and the composition of the spore coat was analyzed by SDS-PAGE. Such analysis can reveal gross changes in spore coat composition. For example, when *cotE*, encoding a protein that coordinates assembly of the inner and the outer coats, was expressed at a different time

than normal, the composition of total coat protein was altered (Little and Driks. 2001). Coat protein analysis displayed by SDS-PADE electrophoresis gels showed alternative pattern than that of the wild-type *B. subtilis*. Applying the same analysis to the strains described in Chapters II and III showed no significant differences from wild-type *B. subtilis* PY79.

MATERIALS AND METHODS

Bacterial strains. Strains used in this assay are listed in Table I.

Cell growth and sporulation. Sporulation was induced by resuspension of cells in SM medium as described (Cutting and Horn. 1990). The time of resuspension is defined as the onset of sporulation (T_0). Spores were harvested at T_{24} by centrifugation at 7,000 g for 10 min, washed with 4°C water once, and stored at 4°C. Spores were purified on a step gradient of 20% to 50% RenoCal-76 (Bracco Diagnostics Inc.) as described (Henriques, *et al.* 1995)

SDS-PAGE of spore coat proteins. Coat proteins were extracted from purified spores as described (Henriques, *et al.* 1995). 25 µl of solubilized spore coat proteins were subjected to SDS-PAGE as described (Henriques, *et al.* 1995).

RESULTS AND DISCUSSION

A representative result from several experiments is shown in Fig. I. The pattern of spore coat protein bands for wild type is similar to that observed in a previous study, but some of the band intensities are slightly different (Henriques, *et al.* 1997). Only one band was observed with a molecular weight of about 60 kDa, which might be CotA and/or CotB. A previous study showed two bands in this region of the gel (Henriques, *et al.* 1997). The

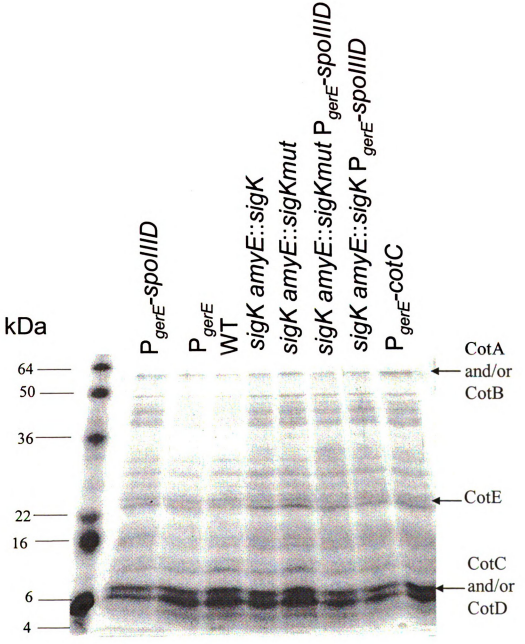
Table I. List of *B. subtilis* strains used in the study

Strain	Genotype or derivation	Source or reference
PY79	Spo$^+$ prototroph	(Youngman, *et al.* 1984)
BJP1	PY79 transformed with pJP1(P_{gerE}-*spoIIID*)/Km^r	Chapter II
BJP2	PY79 transformed with pJP2(P_{gerE})/Km^r	Chapter II
BJP3	PY79 transformed with pJP4(P_{gerE}-*cotC*)/Km^r	Chapter III
BLW1	BK556 transformed with pHP6(*amyE*::*sigK*)/Cm^r	Chapter III
BLW2	BK556 transformed with pJP16(*amyE*::*sigKmut*)/Cm^r	Chapter III
BLW3	BLW1 transformed with pJP1/Km^{r}Cm^r	Chapter III
BLW4	BLW2 transformed with pJP1/Km^{r}Cm^r	Chapter III

reason for only one band on my gel might be the shorter length of the gel, or possibly degradation of CotA or Cot B. Similar explanations might apply for the band labeled CotC and/or CotD. The other strains showed a similar pattern of bands as wild type. The intensity of bands for wild type and BJP2 (P_{gerE}) appears to be less than for the other strains in Fig. I but this was not observed consistently in several experiments. Rather, the pattern and abundance of spore coat proteins appeared to be similar for all the strains.

Two of the strains analyzed in Fig. I showed altered patterns of spore coat gene expression that nevertheless did not detectably change the composition of the spore coat. BJP1 (P_{gerE}-*spoIIID*) exhibited lower expression of *cotC-lacZ* and higher expression of *cotD-lacZ* (Chapter II). Because CotC and CotD were not resolved by SDS-PAGE (Fig. I), it is possible that BJP1 spore coats contain less CotC and more CotD than wild type spore coats. However, given the differences in resistance properties and spore structure (Chapter II), it was surprising that other differences in the spore coat protein profile were not observed (Fig. I). This suggests that structural, rather than compositional, differences in the spore coat primarily account for the observed defects in BJP1 spores. Perhaps altered spore coat gene expression impairs assembly of the spore coat. In contrast, BLW2 (*sigK*

Fig. I. Spore coat proteins. *B. subtilis* wild-type (WT) PY79, BJP1(P_{gerE}-*spoIIID*), BJP2 (P_{gerE}), BJP3 (P_{gerE}-*cotC*), BLW1 (*sigK amyE*::*sigK*), BLW2 (*sigK amyE*::*sigKmut*), BLW3 (*sigK amyE*::*sigK* P_{gerE}-*spoIIID*), and BLW4 (*sigK amyE*::*sigKmut* P_{gerE}-*spoIIID*) were inducted to sporulate by resuspension in SM medium. Spores were collected at 24 h after the initiation of sporulation. Spores were purified and spore coat proteins were extracted. Coat proteins from equal amounts of spores were fractionated on SDS 15% SDS polyacrylamide gels and subjected to Coommassie staining. Assignment of coat protein identification is based on comparison of the pattern of bands with previous SDS-PAGE analysis (Henriques, *et al.* 1997).


kDa
64
50
36
22
16
6
4
P gerE -spoIIID
P gerE WT
sigK amyE::sigK
sigK amyE::sigKmut
sigK amyE::sigKmut P gerE -spoIIID
sigK amyE::sigK P gerE -spoIIID
P gerE -cotC
CotA
and/or
CotB
CotE
CotC
and/or
CotD

amyE::*sigKmut*) exhibited higher *cotC-lacZ* expression and lower *cotD-lacZ* expression than wild type but no difference in spore resistance or structure by electron microscopy (Chapter III). As for BJP1 spores, we cannot rule out a difference in the amounts of CotC and CotD in BLW2 spore coats, since these proteins were not resolved (Fig. I), but if there is a difference, it appears to have little or no effect on assembly and function of the spore coat.

Further investigation of spore coat composition could be done using High Performance Liquid Chromatography (HPLC), which can provide higher resolution and better quantification.

APPENDIX II

Title: The level of Aconitase in Sporulating *B. subtilis* Depends on the Method of Starvation

ABSTRACT

A strain with persistent *spoIIID* expression yielded spores with heat- and lysozyme-resistance defects (Chapter II). However, these defects were only observed when cells are induced to sporulate by resuspension in starvation medium, not when cells where induced to sporulate by nutrient exhaustion in DSM. We hypothesized that this difference might be due to a difference in the GerE and aconitase levels, depending on the method of sporulation induction. Aconitase is an enzyme whose C-terminal domain has been reported to enhance accumulation of the *gerE* mRNA and GerE protein. To begin to investigate our hypothesis, we measured aconitase levels by western blot analysis of *B. subtilis* induced to sporulate by the two methods. The level of aconitase is significantly higher during sporulation when cells are induced to sporulate by nutrient exhaustion than when cells are induced to sporulate by resuspension. We speculate that the lower aconitase during sporulation by resuspension leads to a lower level of GerE, which is insufficient for normal spore formation when SpoIIID level is maintained late during sporulation. Further, the higher aconitase and presumably GerE levels during sporulation by nutrient exhaustion might compensate for detrimental effects of persistent SpoIIID.

INTRODUCTION

In the mother cell of sporulating *B. subtilis*, GerE is an important transcription factor that temporally governs gene expression. It functions as the last regulator in a cascade in the order σ^E-SpoIIID-σ^K-GerE. GerE is both a repressor (55 genes) and an activator (36 genes) of genes in the σ^K regulon (Eichenberger, *et al.* 2004). Recently, a connection between GerE and aconitase was elucidated (Serio, *et al.* 2006). Aconitase, which is

encoded by the *citB* gene, is an enzyme that catalyzes the reversible isomerization of citrate and isocitrate in the tricarboxylic acid cycle. The structure of *B. subtilis* aconitase is similar to that of eukaryotic IRP-1(iron regulatory protein 1). IRP-1 is a mRNA binding protein that regulates the expression of iron receptor, utilization, and storage proteins post-transcriptionally (Basilion, *et al.* 1994) (Gray, *et al.* 1993). The structural similarity between aconitase and IRP-1 implied that aconitase might bind to mRNA. Indeed, the C-terminal domain of *B. subtilis* aconitase binds to the 3'-intranslated region of *gerE* mRNA (Serio, *et al.* 2006). This appears to allow efficient GerE synthesis since mutations in the C-terminal region of the *citB* gene product delayed accumulation of *gerE* mRNA and GerE production, and caused defective sporulation.

In Chapter II we reported that the strain BJP1, which contains multicopy P_{gerE}-*spoIIID* insertion, produces spores with heat- and lysozyme-resistance defects when induced to sporulate by resuspension but not when induced to sporulate by nutrient exhaustion. We hypothesized that this difference might be due to a difference in the GerE and aconitase levels. In this appendix, we show that in BJP1, the level of aconitase is significantly higher when cell were induced into sporulation by nutrient exhaustion in DSM than when cells are induced to sporulate by resuspension in SM medium. This result provides a possible explanation for the absence of detectable spore defects when BJP1 is induced to sporulate by nutrient exhaustion.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* strain BJP1 was obtained by transformation of the wild-type PY79 with a multicopy plasmid bearing P_{gerE}-*spoIIID*. This strain was used in the

experiments in Chapters II and III. MAB160 (*trpC2 pheA1 ΩcitB::spc*) (Craig, *et al.* 1997) was used as a negative control in order to identify the aconitase bands in Western blots.

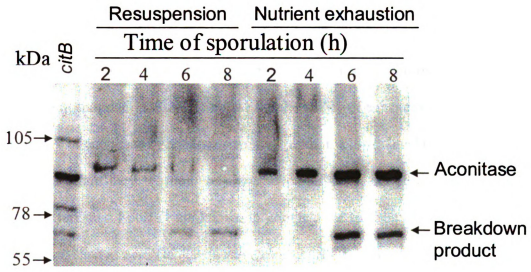
Cell growth and sporulation. Sporulation was induced by resuspension of cells in SM medium as described (Cutting and Horn. 1990). The time of resuspension is defined as the onset of sporulation (T_0). Strains were also induced to sporulate by nutrient exhaustion in DSM (Cutting and Horn. 1990). Onset of sporulation is defined as the time when cells enter into stationary phase. Samples (0.5 ml) were collected at 2, 4, 6 and 8 h into sporulation for BJP1 and at 4 h into sporulation for MAB160. Samples were subjected to centrifugation (14,000 g for 1 min), the supernatants were removed and cell pellets were stored at -70°C.

Western blot analysis. Preparation of whole-cell extracts, electrophoresis, and electroblotting were as described (Halberg and Kroos. 1992; Lu, *et al.* 1990), except that SDS 8% polyacrylamide gels were used for electrophoresis. Also the MAB160 sample was prepared by boiling cells in SDS sample buffer. Blots were probed with anti-aconitase antibodies (kindly provided by A. Sonenshein, Tufts University) diluted 1:3000. Immunodetection of primary antibodies was as described (Kroos, *et al.* 2002).

RESULTS AND DISCUSSION

A representative result of the Western blot analysis is shown in Fig. II. Aconitase migrated between 105 and 78 kDa marker proteins (Invitrogen SeeBlue2), faster than expected (120 kDa) from a previous study (Serio and Sonenshein. 2006). The difference could be due to the gel system, the marker proteins, and/or degradation of aconitase.

Fig. II. Levels of aconitase during sporulation by different methods. *B. subtilis* strain BJP1 (P_{gerE}-*spoIIID*) was induced to sporulate by resuspension or by nutrient exhaustion in DSM. Samples were collected at 2, 4, 6, and 8 h into sporulation. *B. subtilis* MAB160 (*citB*::*spc*) was induced to sporulate by resuspension and a sample was collected at 4 h into sporulation as a negative control. Equal amounts (10 ug) of BJP1 samples and 5 ul of MAB160 sample were loaded on SDS 8%-polyacrylamide gels and subjected to Western blot analysis with anti-aconitase antibodies. The positions of marker proteins, aconitase, and a putative breakdown product are indicated.


Resuspension
Nutrient exhaustion
Time of sporulation (h)
kDa
citB
2
4
6
8
2
4
6
8
105
78
55
Aconitase
Breakdown product

A putative breakdown product of aconitase was seen at about 70 kDa. The intensity of band increases at 6 and 8 h both for resuspension and DSM-induced sporulation. Neither the putative aconitase breakdown product nor the band we think corresponds to intact aconitase is observed in the *citB* mutant, although cross-reacting bands are more intense, indicating that an adequate amount of protein was loaded.

Except at 2 h, the level of aconitase in BJP1 samples was higher for cells induced to sporulate in DSM than by resuspension. For resuspension, the level of aconitase decreased from 2 to 8 h, whereas for DSM, the level increased.

The lower level of aconitase in resuspension samples of BJP1 as compared to DSM samples suggests a possible reason for the spore defect of this strain only being observed by resuspension sporulation. Since aconitase has the ability of enhance GerE accumulation, the lower level of aconitase by resuspension may result in a lower level of GerE. Under this condition, the strain may be more sensitive to perturbation of the SpoIIID late during sporulation. GerE and SpoIIID are known to compete for binding to overlapping sites at some promoter, such as *cotC* (Ichikawa and Kroos. 2000). To extend this study, the level of GerE should be compared in cells induced to sporulate by the two different methods.

APPENDIX III

Title: A Strain with P_{xyl}-*proless-sigE* Showed Inconclusive Results for *sigE* Induction

ABSTRACT

A *B. subtilis* strain bearing a plasmid containing a P_{xyl}-*proless-sigE* fusion was induced with xylose 1 hour after cells were induced to sporulate by nutrient exhaustion in DSM. The levels of pro-σ^E from the native *sigE* locus and σ^E were examined by Western blot analysis. The results were inconclusive in terms of *sigE* induction from the plasmid since some of the isolates showed a higher level of σ^E after induction while others not. Further experiments need to be done to evaluate whether this strain is useful for maintaining the σ^E level late during sporulation.

INTRODUCTION

In sporulating *B. subtilis*, the second sigma factor that becomes active in the mother cell is σ^K. Transcriptionally active σ^K negatively regulates early gene expression. Two different pathways of σ^K inhibition of early gene expression have been described. When σ^K is made one hour earlier than normal, it appears to exert its negative effect through Spo0A-P, which activates transcription of the *sigE* operon (Halberg and Kroos. 1992; Zhang and Kroos. 1997; Zhang, *et al.* 1999). When σ^K is expressed at the normal time, it affects the activity of σ^A RNAP, which transcribes the *sigE* operon. This conclusion is supported by the observation that expression of another early sporulation gene, *ald*, is also inhibited when σ^K becomes active (Halberg and Kroos. 1992; Zhang and Kroos. 1997; Zhang, *et al.* 1999). The *ald* gene encodes alanine dehydrogenase and *ald* is also transcribed by σ^A RNAP.

Why does a negative feedback loop from σ^K exist? This question has been at least partially answered by the study in Chapter II of this thesis. When σ^K RNAP negatively regulates early gene expression, the level of SpoIIID goes down late during sporulation.

This decrease of SpoIIID is important for sporulation since a strain engineered to maintain SpoIIID late during sporulation exhibits spore defects. However, if σ^K RNAP negatively affects σ^A RNAP, expression of many early genes in addition to *spoIIID* is expected to be reduced late during sporulation. As mentioned above, this is indeed the case for *sigE* and *ald*. Is it important to turn down *sigE* expression late during sporulation?

In order to answer this question, we made an effort to try to express *sigE* late during sporulation. If sporulation were more defective than for the strain with persistent *spoIIID* expression, it would indicate that turning down other σ^E-controlled genes in addition to *spoIIID* is important for sporulation.

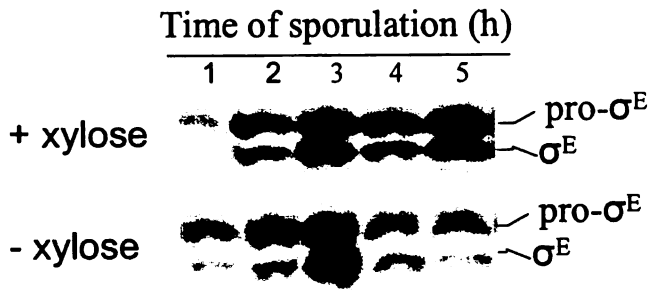
A *B. subtilis* strain with P_{xyl}-*proless-sigE* was obtained and induced by adding xylose after cells had been induced to sporulate by nutrient exhaustion in DSM. Western blot analysis failed to show conclusive evidence of increasing σ^E accumulation late during sporulation.

MATERIAL AND METHODS

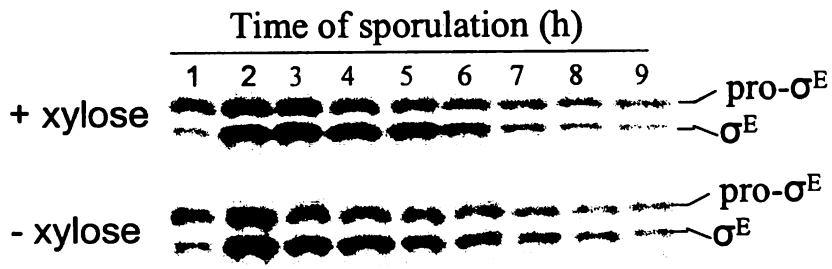
Strain, sporulation and induction. A *B. subtilis* strain (*amyE*::pMF20::*proless-sigE*/Cm^r) was obtained from Dr. Tsutomu Sato (Tokyo University of Agriculture and Technology). The portion of pMF20 containing the xylose-inducible P_{xyl} promoter fused to *sigE* lacking the region encoding the pro-sequence (amino acid 2-27 of pro-σ^E) was used to replace the *amyE* gene in the chromosome of otherwise wild-type *B. subtilis*. The strain was induced to sporulate by nutrient exhaustion in DSM twice. Each time three medium-sized colonies from freshly grown overnight DSM plates were selected. Each colony was

Fig. III. Level of pro-σ^E and σ^E. *B. subtilis* strain (*amyE*::pMF20::*proless-sigE*/Cm^r) was induced to sporulate by nutrient exhaustion in DSM and the P_{xyl} promoter was induced by adding xylose (0.1%) at 1 hour into sporulation (T_1). Samples collected at the indicated times during sporulation were subjected to Western blot analysis to detect pro-σ^E and σ^E. (A) A representative isolate from the first experiment. (B) A representative isolate from the second experiment.

(A)


Time of sporulation (h)
1 2 3 4 5
+ xylose
pro-σE
σE
- xylose
pro-σE
σE

(B)


Time of sporulation (h)
1 2 3 4 5 6 7 8 9
+ xylose
pro-σE
σE
- xylose
pro-σE
σE

inoculated into two flasks containing DSM liquid. One flask of each pair was induced by adding 0.1 ml of 10% xylose to the 10 ml DSM culture at 1 hour into sporulation. The other flask received no xylose addition as a control. 0.5 ml aliquots were collected every hour into sporulation. Samples were centrifuged (1 min at 14,000 g) and cell pellets were stored at -70 °C.

Western blot analysis. Preparation of whole-cell extracts, electrophoresis, and electroblotting were as described (Lu, *et al.* 1990; Halberg and Kroos. 1992). Samples (5 µl) were electrophoresed on SDS 14%-polyacrylamide gels. The blots were probed with anti-pro-σ^E antiserum diluted 1:1000. Immunodetection of primary antibodies was as described (Kroos, *et al.* 2002).

RESULT AND DISCUSSION

Representative Western blots from the two experiments shown in Fig. III. Panel A shows one isolate from the first experiment. This isolate appeared to have a higher level of σ^E when xylose was added. However, the pro-σ^E level also appeared to be higher in those samples, suggesting that protein extraction might have been more efficient for those samples. Two-out-of-three isolates from the first experiment showed this result. The other isolate showed similar pro-σ^E and σ^E levels at all time regardless of xylose addition, as did all three isolates from the second experiment (One is shown in Fig. III, panel B). Although I believe that I treated all the isolates the same way, inconsistent results were generated. For further investigation, I think it might be worthwhile to sporulate the cells by resuspension in SM medium and load an equal amount of protein form each sample. Also, testing different xylose concentrations might be worthwhile. It is possible that the P_{xyl} promoter

cannot be induced well after cells have been induced to sporulate. Dr. Daisuke Imamura, who created this strain, said that he had only induced σ^E production in this strain during vegetative growth, not during sporulation previously (personal communication).

SUMMARY AND PERSPECTIVES

Gene expression in the mother cell of sporulating *B. subtilis* is controlled by a regulatory network, consisting of a major cascade of four regulatory factors, in the order σ^E-SpoIIID-σ^K-GerE, and several feed back and feed forward loops between different components of the network. In this thesis, experiments have been performed in order to understand the importance of several negative links in the regulatory network. These negative effects are: transcriptionally active σ^K RNAP negatively regulates early gene expression, GerE represses *sigK* expression, and SpoIIID represses *cotC* expression. Finally, a genetic screen was performed in order to understand the mechanism by which transcriptionally active σ^K RNAP negatively regulates early gene expression.

To circumvent the σ^K RNAP negative feedback loop, which causes a decrease in SpoIIID accumulation late during sporulation, a *B. subtilis* strain was constructed that bears a multicopy plasmid containing a P_{gerE}-*spoIIID* fusion (Chapter II). This strain showed persistent expression of SpoIIID late during sporulation. Spores formed from this strain showed heat- and lysozyme-resistance defects in comparison to wild-type *B. subtilis* and a control strain with multicopy P_{gerE}. Thin-section microscopy revealed a defective coat structure of spores from the strain with P_{gerE}-*spoIIID*. This strain also exhibited altered expression of *lacZ* reporters; lower *gerE-lacZ*, lower *cotC-lacZ* and higher *cotD-lacZ* expression than wild type or the control strain during sporulation. The lower *gerE-lacZ* expression observed in the strain with persistent SpoIIID late during sporulation revealed a novel regulatory interaction. It would be interesting to perform gel mobility shift assay *in vitro* in order to test whether SpoIIID directly affects *gerE* expression. The fact that maintaining SpoIIID late during sporulation caused spore defects suggests that at least one

important reason for σ^K RNAP negative feedback on early gene expression is to fine-tune *spoIIID* expression in order to form normal spores.

To circumvent GerE repression of *sigK* transcription, site-directed mutagenesis of the GerE binding site in *sigK* promoter region was performed and this mutant *sigK* allele was integrated in the *amyE* locus of a *B. subtilis* strain with a null mutation at its native *sigK* locus (Chapter III). GerE negative feedback on *sigK* was eliminated in this strain. The strain exhibits higher expression of *gerE-lacZ* and *cotC-lacZ*, and lower expression of *cotD-lacZ*, which is exactly opposite of that observed in the strain bearing the P_{gerE}-*spoIIID* fusion (Chapter II). Although the strain with the mutant *sigK* allele does not exhibit spore defects, this allele suppressed the heat- and lysozyme-resistance defects caused by the P_{gerE}-*spoIIID* fusion. In the strain with the mutant *sigK* allele and P_{gerE}-*spoIIID*, the *gerE-lacZ*, *cotC-lacZ* and *cotD-lacZ* reporters showed similar levels of expression as in wild-type *B. subtilis*. One perturbation in the mother cell regulatory network appeared to compensate for the other perturbation. A strain engineered to express *cotC* at least 2 hours earlier than normal showed no spore defects in heat-, lysozyme-, or organic solvent-resistance assays, or in a quantitative germination assay. These results suggest a balance between sensitivity and robustness in the mother cell regulatory network of sporulating *B. subtilis*. The network can resist certain perturbations without detectable change in the overall output, a resistant spore. However, other perturbations, such as maintaining SpoIIID late during sporulation, exceed network tolerance and result in spore resistance defects. Messenger RNA of individual genes appears to be more susceptible to perturbation than the overall output. For example, the strain with the mutant *sigK* allele exhibited altered gene expression but normal spores. Also, many different mutations caused increased *spoIIID*-

lacZ expression during sporulation (Chapter IV). These strains have yet to be tested for spore defects.

We screened for transposon insertions that increase *spoIIID-lacZ* expression during development in order to understand the mechanism by which transcriptional active σ^K RNAP negatively feeds back on early gene expression (Chapter IV). We hoped to identify one or more genes under σ^K control whose products carry out the negative feedback loop. Sever genes were identified, but none of these have been reported to be under σ^K control. At least two independent insertions occurred in one or more of the 10 genes for 16S rRNA. As these genes are the first in rRNA operons that are highly transcribed by σ^A RNAP, the insertions might free nucleotides and/or core RNAP for utilization by σ^E in transcription of *spoIIID-lacZ*. This hypothesis suggests that σ^K RNAP negative feedback on early gene expression during sporulation might stem from competition between different sigma factors for nucleotides and engagement of core RNAP in transcription of their regulon. The other six insertions were in protein-encoding genes with no known connection to sporulation. The next step in analysis of all the insertions is to test whether the insertion is responsible for increased *spoIIID-lacZ* expression. If so, subsequent analysis for several of the insertions would involve testing whether the effect is due to a polar effect on expression of a downstream transcription factor. Another question for subsequent analysis would be whether the effect is specific for *spoIIID-lacZ* expression, or whether expression of other early sporulation genes is also elevated. Interestingly, the variety of insertions obtained hint at connections between cellular metabolism and the mother cell regulatory network. Ultimately, one would like to understand the molecular mechanisms of these connections, as this aspect of developmental regulatory networks is under explored.

Among them the 16S rRNA has been identified twice. Asides from the hypothetical gene *yvkC*, which might function similarly to pyruvate and water dikinase, none of other genes has been reported to show direct effects on sporulation. However, the functions of some gene products such as nucleoside diphosphate kinase, encoded by *ndk*, and 16S rRNA seems to point to the direction of cellular resource utilization and power supply during sporulation. N-acetylglucosamine 6-P deacetylase, encoded by *nagA*, and the serine protease Do, a family member of heat shock proteins which is encoded by *ykdA*, both seem to have something to do with the initiation of sporulation.

In the future study, it would be interesting to understand the mechanisms of how these gene products affect sporulation, in particular, how to affect the σ^K RNAP negative feedback.

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