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DEVELOPMENTAL GENE EXPRESSION REGULATED BY A CASCADE OF SIGMA FACTORS IN BACILLUS SUBTILIS

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D degree in Biochemistry

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Date 12/17/97

DEVELOPMENTAL GENE EXPRESSION REGULATED BY A CASCADE OF SIGMA FACTORS IN *BACILLUS SUBTILIS*

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1997

ABSTRACT

DEVELOPMENTAL GENE EXPRESSION REGULATED BY A CASCADE OF SIGMA FACTORS IN BACILLUS SUBTILIS

By

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Upon starvation, *Bacillus subtilis* undergoes sporulation that culminates with the formation of a dormant spore. Initiation of sporulation is governed by the phosphorylation of Spo0A through a phosphorelay. An elevated level of Spo0A~P leads to formation of an asymmetric septum that divides the sporulating cell into two compartments of unequal size, the mother cell and the forespore. Temporal and spatial regulation of gene expression during sporulation is achieved, in part, through the sequential synthesis and activation of compartment-specific sigma factors of RNA polymerase (RNAP) and DNA-binding proteins.

Key regulators of mother cell gene expression are the sigma factors σ^E and σ^K, and the DNA-binding proteins SpoIIID and GerE. One function of SpoIIID is to switch on σ^K-dependent gene expression, because SpoIIID is required for the appearance of σ^K. SpoIIID also inhibits transcription of some late σ^K-dependent genes. A rapid decrease in the SpoIIID level is thought to be critical for the expression of these late genes. It is shown that the decrease in the level of SpoIIID is accompanied by a decrease in transcription of the *spoIIID* gene, and this depends upon the appearance of σ^K. Transcription of *spoIIID* depends on σ^E. σ^K negatively regulates transcription of *spoIIID* by inhibiting the

transcription of *sigE*, which encodes σ^E. Transcription of *sigE* is carried out by RNAP containing the vegetative sigma factor σ^A, and is activated by Spo0A~P and repressed by SinR. It is shown that σ^K does not change *sigE* expression by increasing the level or activity of SinR. Rather, it appears that σ^K affects the phosphorelay, lowering the level of Spo0A~P. σ^K may also inhibit σ^A activity, perhaps by competing directly for binding to core RNAP. Hence, the appearance of σ^K both turns on late mother cell gene expression and turns off early σ^E-directed gene expression through a negative feedback loop.

σ^K is first synthesized as an inactive precursor protein called pro-σ^K. Activation involves a proteolytic cleavage of the N-terminal pro-sequence from pro-σ^K. The putative protease is localized in the mother cell membrane surrounding the forespore. Subcellular fractionation studies show that the majority of pro-σ^K is membrane-associated in cell extracts, and is not associated with the core subunits of RNAP. Immunolocalization of pro-σ^K suggests that pro-σ^K interacts with both the membrane surrounding the mother cell and the membrane surrounding the forespore in sporulating cells. Pro-σ^K fails to bind to core RNAP in vitro under conditions that permit σ^K binding. These results suggest that the pro-sequence of pro-σ^K inhibits the core-binding activity of σ^K and promotes its association with the membrane, where processing may occur.

To

My family

ACKNOWLEDGMENTS

I am very grateful to my thesis advisor, Dr. Lee Kroos for his encouragement and guidance, both scientific and general, during the years. He has provided me with every available opportunity to become a better scientist. I enjoyed all the discussions we had over the years, which have helped me tremendously on my scientific thinking and writing.

I thank my committee members, Dr. Frans de Bruijn, Dr. Robert Hausinger, Dr. Jon Kaguni, and Dr. Steve Triezenberg, for their criticisms and advice. I also thank Dr. David Arnosti for reading my dissertation and attending my defense.

I would like to thank all the past and present members of the *Myxo* squad and *Bacillus* squad in the Kroos' laboratory, Richard Halberg, Sijie Lu, Makda Fisseha, Jannine Brandner, Dvora Biran, Michele Anderson, Hiroshi Ichikawa, Tong Hao, Greg Velicer, Nicco Yu, and many undergraduates for sharing excitement and frustrations in research and in life. They have made the lab an enjoyable place to work.

I thank Dr. Antje Hofmeister for sharing information and collaborating on immunofluorescence microscopy experiments, Dr. Pappan for help with the computers.

I would like to thank my parents and my brothers for their love, support and their faith in me, my parents-in-law for their help and encouragement. Finally, but not least, I would like to thank my wife Yusong for everything, my sons Shawn and Jason for making life more pleasant and colorful. They made this long journey through graduate school possible and meaningful.

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LIST OF ABBREVIATIONS

ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
bp	base pair
BSA	bovine serum albumin
dATP	deoxyadenosine-5'-triphosphate
DNA	deoxyribonucleic acid
DSM	Difco sporulation medium
DTT	dithiothreitol
EDTA	(ethylenedinitrilo)tetraacetic acid
FITC	fluorescein isothiocyanite
HCl	hydrochloric acid
Hepes	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
IPTG	isopropyl ß-D thiogalactopyranoside
KCl	potassium chloride
KOH	potassium hydroxide
kb	kilobases
kDa	kilodalton
LB	Luria-Bertani
M	molar
ml	milliliter
mM	millimolar

$MgCl_2$	magnesium chloride
mRNA	messanger ribonucleic acid
NaCl	sodium chloride
ng	nanogram
nM	nanomolar
ONPG	*o*-nitrophenol-ß-D-galactoside
PAGE	polyacrylamide gel electrophoresis
PI	propidium iodide
pmole	picomole
psi	pounds per square inch
PMSF	phenylmethylsulfonyl fluoride
RNAP	RNA polymerase
SDS	sodium dodecylsulfate
SM	Sterlini-Mandelstam
Tricine	N-tris(Hydroxymethyl)methylglycine
Tris	tris(hydroxymethyl)aminomethane
TSS	transcriptional start site
T_x	x hours after the onset of sporulation
µg	microgram
µl	microliter
wt/vol	weight per volume

INTRODUCTION

Sporulation of *Bacillus subtilis* in response to starvation provides an excellent model system to study the fundamental problems of gene expression and the regulation of development of a living organism. Sporulation involves a series of morphological changes. Key to the initiation of sporulation is the phosphorylation of Spo0A, an event controlled by a phosphorelay system that is regulated by multiple signals. An elevated level of Spo0A~P leads to formation of an asymmetric septum that divides the sporulating cell into a larger mother cell compartment and a smaller forespore compartment. Each compartment receives a copy of the genome, but each follows a different pathway of development. Temporal and spatial regulation of gene expression during sporulation is achieved, in part, through the sequential synthesis and activation of compartment-specific sigma factors of RNA polymerase (RNAP). DNA-binding proteins also contribute to the proper regulation of gene expression. This dissertation focuses on the regulation of key transcription factors in the mother cell.

Key regulators of gene expression in the mother cell are the sigma factors σ^E and σ^K, and the DNA-binding proteins SpoIIID and GerE. SpoIIID activates and represses transcription by both σ^E RNAP and σ^K RNAP. One function of SpoIIID is to switch on σ^K-dependent gene expression, because SpoIIID is required for the appearance of σ^K. SpoIIID also inhibits transcription of some late σ^K-dependent genes. A rapid decrease in the SpoIIID level is thought to be critical for the expression of these late genes. Chapter 2 describes a study which demonstrates that the appearance of σ^K initiates a negative

feedback loop that turns off the transcription of *sigE* and the whole σ^E regulon, including*spoIIID*. This work was published in the *Journal of Bacteriology*.

Transcription of *sigE* is carried out by RNAP containing the vegetative sigma factor σ^A, activated by Spo0A~P, and repressed by SinR. The experiments in Chapter 3 further characterize the negative effect of σ^K on *sigE* transcription. It is shown that σ^K appears to lower the level of Spo0A~P and inhibit σ^A activity. A revised version of this chapter will be submitted to the *Journal of Bacteriology*.

Experiments described in Chapter 2 and 3 contribute to our understanding of the developmental gene regulation in general by demonstrating that replacement of transcription factors acting early during development involves a feedback regulation by those acting late during development. This finding provides an alternative to the model that late-acting transcription factors simply accumulate enough amount and passively take over the earlier ones.

σ^K is first synthesized as an inactive precursor protein called pro-σ^K. Activation involves the proteolytic cleavage of the N-terminal pro-sequence from pro-σ^K in response to a forespore signal. Chapter 4 describes a study which demonstrates that the pro-sequence of pro-σ^K inhibits the core-binding activity of σ^K and promotes its association with the membrane, where processing may occur. The immunolocalization data presented in this chapter was obtained through a collaboration with A. Hofmeister at Harvard University. A manuscript based on this chapter has been submitted to the *Journal of Bacteriology*.

The Appendix describes my first project in the lab, a study which demonstrates that *spoVD*, a gene then newly cloned by R. Daniel and J. Errington at the University of Oxford, is transcribed by σ^E RNA polymerase in vitro and that SpoIIID represses *spoVD*

transcription. Two strong SpoIIID-binding sites were mapped in the *spoVD* promoter region. J. Errington and R. Daniel shared results prior to publication and provided a plasmid containing the *spoVD* promoter region for this study, which was published in the *Journal of Bacteriology*.

Chapter 1.

LITERATURE REVIEW

Understanding the temporal and spatial regulation of gene expression during the development of a living organism is a fundamental problem in developmental biology. An excellent system to address this problem is the sporulation process of the Gram-positive bacterium *Bacillus subtilis* because of its relatively simple cellular organization, its experimental tractability, and its excellent genetics.

Morphological Changes during Sporulation. The successive morphological stages of sporulation are shown in Figure 1 (27, 87, 137). Vegetative cells are defined as stage 0 with respect to the sporulation process. Entry into sporulation is characterized by the formation of a so-called axial filament in which two chromosomes from the last round of DNA replication become aligned with the long axis of the cell (stage I). The first easily observed morphological change during sporulation is the formation of an asymmetrically positioned septum (stage II) that divides the developing cell (sporangium) into two unequal compartments: the larger one is called the mother cell and the smaller one is called the forespore. Each compartment receives a chromosome. The septum then migrates around the forespore, engulfs it in a double membrane, and eventually pinches it off as a free protoplast within the mother cell (stage III). The inner membrane surrounding the forespore is referred to as "the forespore membrane". The outer membrane surrounding the forespore is called "the mother cell membrane surrounding the forespore", because it is derived from the mother cell membrane. A modified form of cell wall, known as the cortex, is synthesized between the two

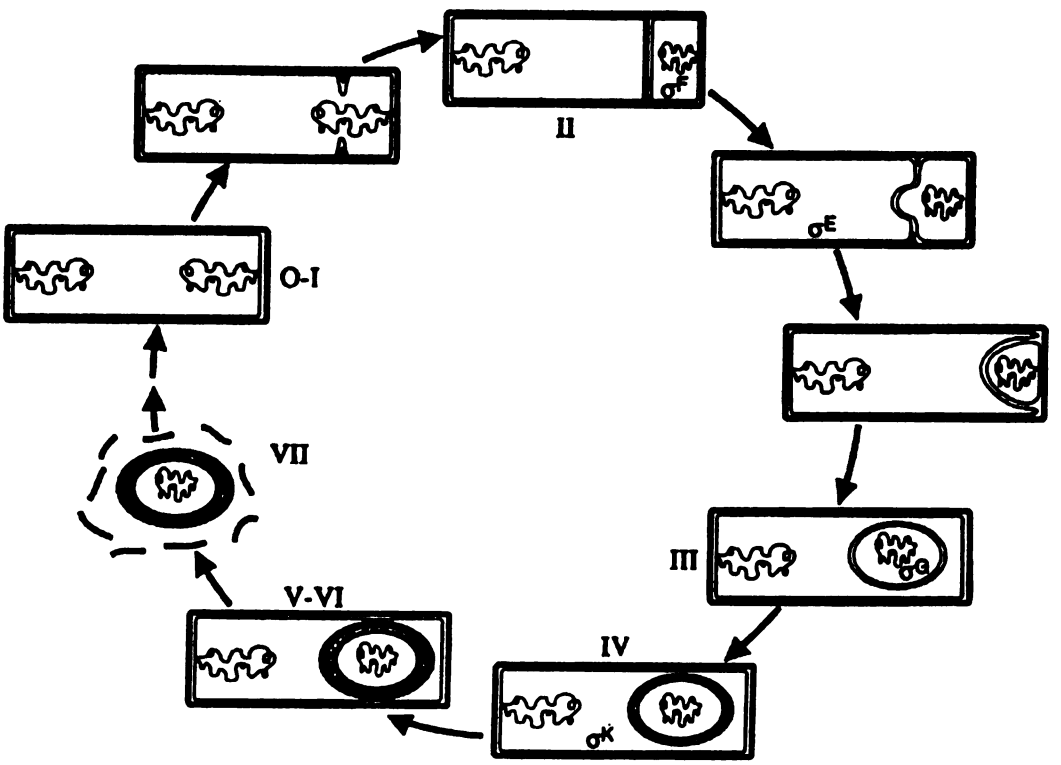


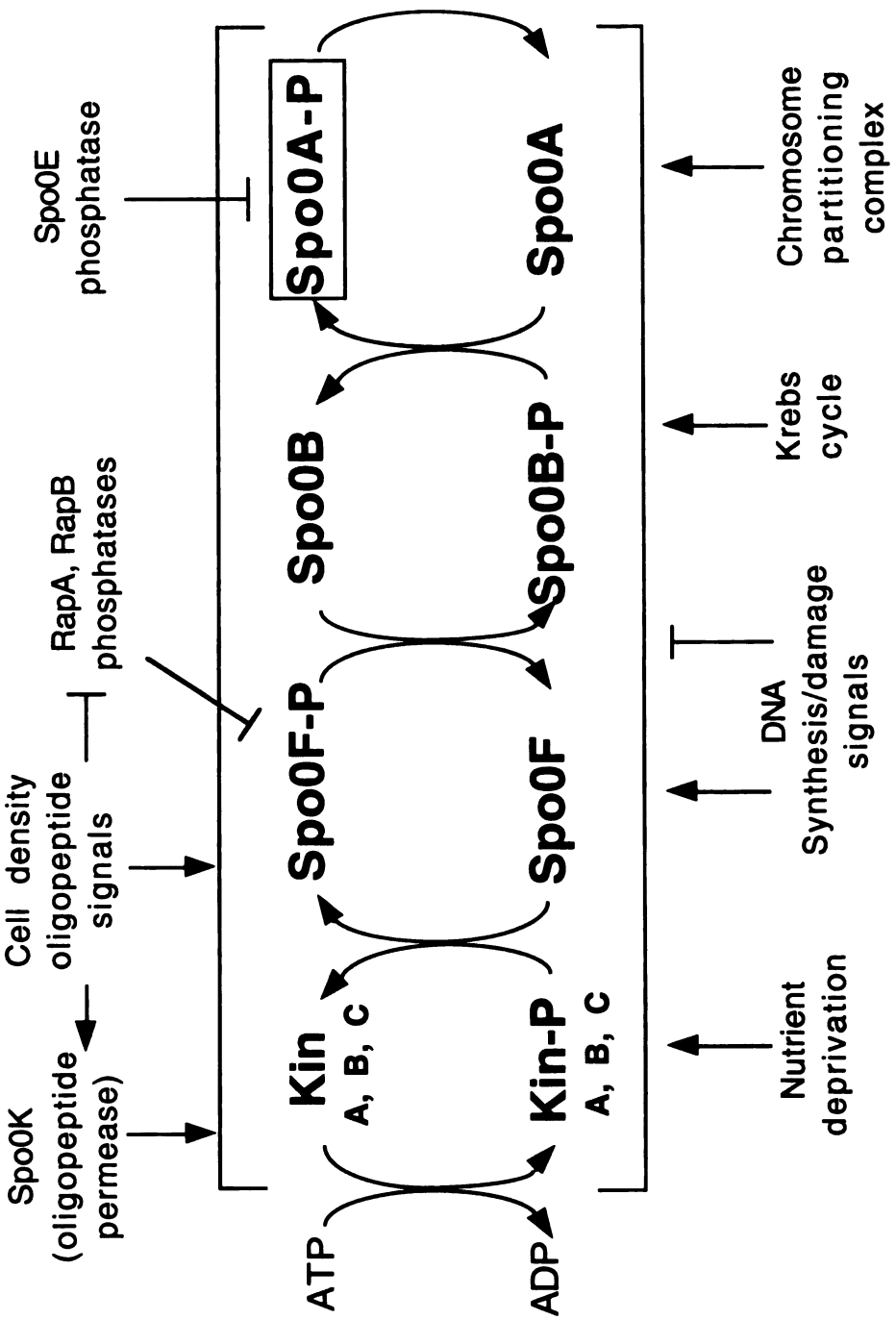
Figure 1. Morphological stages during *B. subtilis* sporulation. The compartment-specific sigma factors are shown in order of their appearance during sporulation. Reprinted from ref. 137.

membranes surrounding the forespore (stage IV). At about the time of cortex formation, more than twenty proteins are made in the mother-cell compartment and deposited around the forespore to form a multilayered protein shell called the spore coat (stage V). The final period of spore development, termed maturation (stage VI), happens with no dramatic change in morphology, but during this stage the properties of resistance, dormancy, and ability to germinate appear in sequence. The sporulation process culminates with lysis of the mother cell and release of the mature spore (stage VII). When conditions are favorable, the spore can germinate and resume vegetative growth. Over 125 genes have been identified that are induced and/or required for the sporulation process (27, 137).

Initiation of Sporulation Initiation of sporulation depends upon activation of the Spo0A transcription factor by phosphorylation, and a threshold concentration of Spo0A~P appears to be required (34, 46). Spo0A receives phosphate from three histidine protein kinases (KinA, KinB and KinC) through a multicomponent phosphorelay (Figure 2). The phosphorelay is a modified bacterial two-component signal transduction system. Two-component systems are involved in numerous adaptive responses, including chemotaxis and nitrogen utilization (101, 133). All three histidine protein kinases in the *B. subtilis* phosphorelay belong to a conserved family of proteins known as sensor kinases (2). Proteins that receive phosphate from sensor kinases belong to a conserved family of proteins known as response regulators. KinA, KinB and KinC first autophosphorylate and donate phosphate to the response regulator Spo0F. The phosphate is then transferred from Spo0F to Spo0B and finally to Spo0A (16). Among the three histidine protein kinases, KinA and KinB appear to contribute the most to the phosphorelay (144). KinC contributes the least to the phosphorelay (76, 77). KinC is required for the activation of altered forms of Spo0A (*sof1*, *rvtA11*, and *sur0B20*) (67, 76).

A major function of the phosphorelay seems to be to integrate multiple developmental signals that regulate the initiation of sporulation. Signals generated by conditions of

Figure 2. Integration of multiple signals and conditions through the phosphorelay. Sensor kinases KinA, KinB and KinC autophosphorylate and phosphate is transferred to the response regulator Spo0F, then to the phosphotransferase Spo0B and finally to an aspartate residue in the N-terminal domain of the response regulator Spo0A (16). It is not yet known which component of the phosphorelay is the direct target for a given physiological signal. Adapted from ref. 34.


Spo0K
(oligopeptide
permease)
Cell density
oligopeptide
signals
RapA, RapB
phosphatases
Spo0E
phosphatase
Kin
A, B, C
Spo0F-P
Spo0B
Spo0A-P
ATP
ADP
Kin-P
A, B, C
Spo0F
Spo0B-P
Spo0A
Nutrient
deprivation
DNA
Synthesis/damage
signals
Krebs
cycle
Chromosome
partitioning
complex

nutrient depletion, high cell density, the Krebs cycle, DNA replication, DNA damage, and the chromosome partitioning machinery all modulate activation of Spo0A. Mutations in *spo0A* (*rvtA11*) that bypass the need for Spo0F and Spo0B (67, 76) can at least partially bypass the DNA synthesis and damage (52, 53), Krebs cycle (56), and the chromosome partitioning controls on sporulation (54), indicating that these signals inhibit the phosphorelay and limit production of Spo0A~P. Regulation by these signals appears to serve as a developmental checkpoint, ensuring that sporulation does not begin unless it seems likely that it can be completed. However, little is known about the nature of the signals and the signal transduction pathways that control the activity of the phosphorelay.

Nutrient depletion causes a drop in the intracellular GTP level (84). Inhibiting GTP biosynthesis induces sporulation (28, 85, 96). The *rvtA11* mutation in *spo0A* does not bypass the need for nutrient depletion. However, expression of a constitutively active Spo0A (Spo0A^{sad}) is sufficient to bypass the requirement for nutrient depletion (55). These results suggest that the target of nutrient depletion signal is probably the sensor kinases (Kin). Mutations in *kinA* and *kinB* cause different phenotypes depending on the starvation condition, indicating that there might be multiple ways of sensing nutrient depletion (55). However, it is not clear how sensor kinases are activated. In addition to contributing to activation of the sensor kinases, nutrient depletion also induces several genes required for sporulation, independent of the phosphorelay, including citrate synthase genes (*cit*) (57), *ald*, encoding alanine dehydrogenase (128), and *spo0J*, a chromosome partitioning gene (54). Factors controlling expression of these genes have not been characterized.

Production of Spo0A^{sad} also bypasses the need for high cell density in sporulation, indicating the cell density signal regulates the Spo0A~P level (55). However, high cell density also induces the mutually exclusive process of competence development. Competence refers to the ability of cells to take up exogenous DNA. According to a simple

model (34), a low concentration of Spo0A~P in vegetative cells inhibits both the competence and the sporulation pathways. During the transition state (late exponential growth phase to early stationary phase), an intermediate level of Spo0A~P is optimal for competence development. A further increase of Spo0A~P crosses a threshold and induces sporulation. One of the mechanisms that cells use to respond to changes in cell density is sensing of extracellular peptide factors that accumulate in the culture medium (quorum sensing) (29). Three such peptide factors have been identified. The ComX pheromone mainly stimulates competence development (131). PhrA mainly stimulates sporulation (75, 107). CSF (competence and sporulation factor) has at least three distinct functions: stimulating competence gene expression at low concentrations, and inhibiting competence gene expression and stimulating sporulation at high concentrations (130). CSF appears to have three different targets in cells corresponding to its three functions (75). Both PhrA and CSF appear to be pentapeptides that are produced by secretion and processing of precursor molecules (75, 107, 130). They are transported back into the cell by the oligopeptide permease encoded by *spo0K*. One target of CSF is RapC, which it negative regulates (130), whereas PhrA negatively regulates the activity of RapA (107). RapA and RapC are members of the response-regulator aspartate phosphatase family that also includes RapB (105). RapA and RapB negatively regulate the phosphorelay by specifically dephosphorylating the response regulator Spo0F~P (Fig. 1.2) and thus reducing the level of Spo0A~P in cells (105). Interestingly, PhrA is derived from the product of a small gene downstream of *rapA* (107) and CSF is derived from the product of a small gene downstream of *rapC* (130). CSF and PhrA appear to represent an emerging class of cell-cell signaling molecules that are actively imported and function intracellularly.

Spo0E is another phosphatase that regulates the flow of the phosphorelay. It specifically dephosphorylates Spo0A~P (98) (Figure 3). Transcription of *spo0E* is repressed by a DNA-binding protein, AbrB, and is derepressed due to Spo0A~P repression

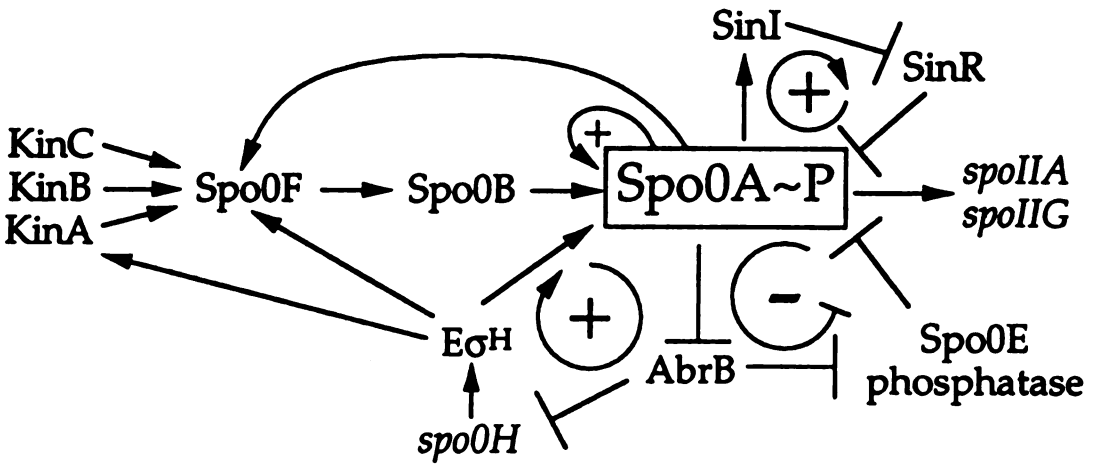


Figure 3. Positive and negative feedback loops controlling production and accumulation of Spo0A~P. Lines with arrowheads indicate positive effects on synthesis or activition. Lines with barred ends indicate negative effects on synthesis or activation. See text for details. Reprinted from ref. 34.

of *abrB* (106). This is a negative feedback loop that inhibits the accumulation of Spo0A~P (Figure 3). Spo0E may represent an independent pathway for preventing the initiation of sporulation.

Several positive feedback loops contribute to the accumulation of Spo0A~P (Figure 3). They are controlled initially by the level of Spo0A~P and can further increase the activity or expression of Spo0A. Spo0A~P directly activates transcription of *spo0A* and *spo0F*, in combination with σ^H RNAP (113, 140). In addition, Spo0A~P increases the level of σ^H by repressing *abrB* (138), which encodes a repressor of several genes involved in sporulation including *sigH* (139). This causes derepression of *sigH*. An increase in σ^H then contributes to increased transcription of *kinA* as well as *spo0F* and *spo0A* (113). SinR is a DNA-binding protein that inhibits sporulation. It represses transcription of *spo0A* by σ^H RNA polymerase (91), as well as transcription of several key stage II genes (92). Spo0A~P activates transcription of *sinI*, which encodes an inhibitor of SinR (8). Thus, inhibition of SinR by increased production of SinI probably contributes to increased transcription of *spo0A*.

In addition to stimulating the transcription of *spo0A* and *spo0F*, the accumulation of Spo0A~P activates the transcription of several key genes that govern entry into sporulation and the transition to a two-compartment sporangia. These include an unknown gene(s) that determines the switch from medial to polar division (78), the *spoIIE* gene (153) and the *spoIIA* operon (14), which are responsible for switching on gene expression in the forespore, and the *spoIIG* operon (10, 12), which is responsible for gene transcription in the mother cell. Spo0A~P activates transcription by binding to promoters of these genes or operons and stimulating the rate of initiation by modifying the RNA polymerase preinitiation complex (9, 10, 12).

Polar Division and Activation of σ^F in the Forespore. A hallmark of

sporulation is the formation of the polar septum, which partitions the sporangium into two, dissimilar-sized cellular compartments. In vegetative cells that undergo binary fission, a ring of a tubulin-like, cell division protein, FtsZ, is formed at a medial position. Later in the cell cycle, a septum forms at the site of FtsZ assembly, resulting in cytokinesis (78). In sporulating cells, FtsZ ring formation switches to sites near both poles of the sporangium, and this is under the control of Spo0A~P (78). Next, a septum is formed at one of the polar rings of FtsZ. This event is controlled by σ^H (78). Another sporulation sigma factor, σ^E, is involved in suppressing septum formation at the distal pole, because mutants defective in σ^E production undergo septation at both poles, giving rise to "disporic" sporangia (79). The genes controlled by Spo0A~P, σ^H and σ^E that are responsible for polar septation have not yet been identified.

Unlike binary fission in which chromosome segregation takes place prior to septum formation, during sporulation chromosome partitioning largely occurs only after the polar septum is formed. In *spoIIIE* mutant cells, about 30% of the chromosome corresponding to the region proximal to the replication origin becomes trapped in the forespore (151). SpoIIIE functions as a DNA translocase in wild-type cells, pumping 70% of the chromosome into the forespore compartment (151). Spo0J, which itself is located in the origin region (80, 147), is required for the orientation of the origin-proximal region of chromosomes to the pole (123).

Shortly after the formation of the polar septum, σ^F is activated and its activity is strictly confined to the forespore (42, 93). σ^F is encoded by the third gene of the *spoIIA* operon, *spoIIAC* (142). Transcription of *spoIIA* is dependent upon Spo0A~P and σ^H RNAP (9, 14, 148, 150), and begins prior to septation (31). The mechanism by which σ^F activity is confined to the forespore involves a pathway composed of proteins encoded by

the first two genes of the *spoIIA* operon, SpoIIAA and SpoIIAB, and SpoIIE (Figure 4). SpoIIAB is an anti-sigma factor that binds to σ^F and inhibits σ^F-directed gene expression (26, 95). SpoIIAA is an anti-anti-sigma factor that counteracts the inhibitory effect of SpoIIAB by binding to the SpoIIAB·σ^F complex and causing release of free and active σ^F (3, 20). SpoIIAB is also a protein kinase that phosphorylates SpoIIAA on serine residue 58 (95), thereby impairing the capacity of SpoIIAA to bind to SpoIIAB (3, 20). Central to the cell-specific activation of σ^F is SpoIIE, a specific serine phosphatase that is responsible for dephosphorylating SpoIIAA~P, thereby reactivating it for binding to SpoIIAB (Figure 4) (25). SpoIIE is an integral membrane protein that is localized initially to the two potential polar division sites before septation, disappears from the distal pole, and persists at the sporulation septum after septation (5, 11). According to current thinking, the principle determinant of σ^F activation is the cellular concentration of unphosphorylated SpoIIAA, which is governed by the opposing activities of the SpoIIE phosphatase and the SpoIIAB kinase. If SpoIIE is displayed equally on both sides of the septum, then SpoIIAA~P could be expected to be dephosphorylated at an equal rate in both compartments. But because the forespore is several-fold smaller than the mother cell, the concentration of unphosphorylated SpoIIAA would be higher in the forespore than in the mother cell. Once a critical concentration is reached, SpoIIAA would interact with SpoIIAB and discharge σ^F from the SpoIIAB·σ^F complex (Figure 4), forming the SpoIIAB·SpoIIAA complex. In this model, activation depends on the completion of septum morphogenesis. The observation that ATP stimulates the formation of the SpoIIAB·σ^F complex and ADP enhances the formation of the SpoIIAB·SpoIIAA complex (3, 20) has also led to a speculation that perhaps there is a selective decrease in the ratio of ATP to ADP in the forespore compartment. This decrease would favor the binding of SpoIIAB to SpoIIAA, rather than to σ^F. It may also hinder the phosphorylation of

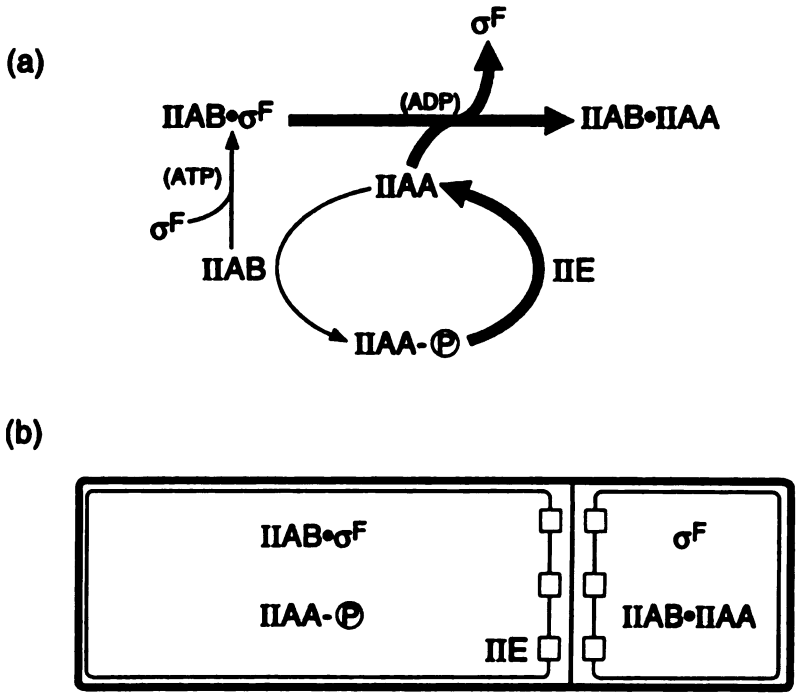


Figure 4. Model for the forespore-specific activation of σ^F. (a) Reactions that contribute to the release of free σ^F from the SpoIIAB·σ^F complex. The heavy lines indicate reactions favored in the forespore. (b) The state and cellular locations of the proteins involved in the activation of σ^F. Reprinted from ref. 137.

SpoIIAA by SpoIIAB, destabilizing the SpoIIAB·SpoIIAA complex (Figure 4).

Anti-sigma factors and anti-anti-sigma factors are also involved in regulating the activity of the *Bacillus subtilis* and *Staphylococcus aureus* stress response sigma factors. In the case of *B. subtilis* σ^B, the activity of anti-sigma factor RsbW is subject to regulation by multiple kinases and phosphatases that integrate multiple stress signals. FlgM is an anti-sigma factor that inhibits the flagellar-specific σ^{28} in *Salmonella typhimurium*. Activation of σ^{28} involves secreting FlgM outside the cell by a type III secretion apparatus that assembles functional flagella (49, 62). In this case, the integrity of the flagellar hook-basal body complex serves as a morphogenetic cue to relieve the inhibition of σ^{28} by FlgM (49, 62). Other anti-sigma factors include the T4 bacteriophage AsiA protein that binds and inhibits *E. coli* σ^{70} activity, *Myxococcus xanthus* CarR that sequesters to the inner cell membrane sigma factor CarQ involved in carotenogenesis (33), and *Pseudomonas aeruginosa* MucA that inhibits AlgT (σ^E) involved in alginate synthesis (94, 127, 152).

Signal Transduction Pathways Leading to the Activation of Compartment-specific Sigma Factors. The temporal and spatial pattern of gene expression is established, in part, by four compartment specific sigma factors: σ^F and σ^G in the forespore, σ^E and σ^K in the mother cell. Each of the sigma factors is initially inactive. Activation depends on signals generated by the previously activated sigma factor in the opposite compartment (68, 86, 137). Thus, activation of σ^F in the forespore following the formation of the polar septum triggers the activation of σ^E in the mother cell. σ^E, in turn, in conjunction with the engulfment of the forespore by the mother cell, causes the activation of σ^G in the forespore. Finally, σ^G sets in motion a chain of events that leads to the activation of σ^K.

σ^F to σ^E. σ^E is synthesized as an inactive precursor, pro-σ^E, the primary product of the promoter-distal member (*spoIIGB*) of the two-cistron *spoIIG* operon (59, 64). Pro-σ^E is converted to its active form by proteolytic removal of its pro-amino acid sequence (74), an N-terminal extension of 27 residues (97). Synthesis of pro-σ^E starts before asymmetric division due to Spo0A~P activated transcription of the *spoIIG* operon (10, 12). The first gene of the operon, *spoIIGA*, encodes a membrane protein that is sufficient for activation of pro-σ^E and is believed to be the processing enzyme (110, 135). Activation of pro-σ^E is delayed until after septation and it requires a σ^F-controlled gene *spoIIR* (48, 61, 83). Simultaneous expression of *spoIIR*, *spoIIGA*, and *spoIIGB* during exponential growth leads to efficient pro-σ^E processing, which indicates that *spoIIR* is the only σ^F-controlled gene needed for activation of pro-σ^E (83). SpoIIR contains a putative signal sequence and can be found in the supernatant fluid from a culture of *B. subtilis* cells engineered to express *spoIIR* during exponential growth (48). Thus, the simplest model is that SpoIIGA is a receptor/protease and that SpoIIR is a secreted signal protein that activates the intracellular protease domain of SpoIIGA by interacting with an extracellular receptor domain of this integral membrane protein (Figure 5a). Biochemical evidence in support of this hypothesis has come from the demonstration that partially purified SpoIIR can activate pro-σ^E processing in protoplasts and intact cells of *B. subtilis* that have been engineered to produce SpoIIGA and pro-σ^E during growth (48). Mutagenesis analysis revealed a residue near the N-terminus (D6) is required for function. This residue is in a region predicted to be exposed to the space between the mother cell and the forespore (82).

SpoIIR can also activate SpoIIGA molecules in the same cell where it is synthesized (125, 158). This raises the question of how pro-σ^E is prevented from becoming active in the forespore. Recently, it was shown that σ^E and pro-σ^E are absent in the forespore

compartment shortly after septation, suggesting that the loss of σ^E/pro-σ^E from the forespore contributes to the compartmentalization of σ^E-directed gene transcription (112). Interestingly, a null mutation in *spoIIIE*, but not a missense mutation, allows σ^E and/or pro-σ^E to persist and σ^E to become active in the forespore. The same null mutant also allows SpoIIE phosphatase to persist at the mother-cell pole after septation, causing σ^F to become active in both compartments (112). Thus, SpoIIIE may be a dual function protein. It contributes to the proper spatial regulation of both σ^E and σ^F activities, in addition to being a DNA translocase as noted above.

σ^E to σ^G. One hour after σ^F is activated, it starts to transcribe *spoIIIG*, which encodes σ^G (102). σ^G directs the transcription in the engulfed forespore of a large set of genes, including a family of small, acid-soluble proteins (SASP) that protect the spore DNA from different types of environmental insult (122). σ^G can also maintain its own synthesis by recognizing the *spoIIIG* promoter (141). σ^G-directed transcription in the forespore is controlled by mother-cell signals acting at two successive levels. First, transcription of *spoIIIG* is dependent on the presence of σ^E in the mother cell, suggesting that the mother cell generates a signal required for expression of *spoIIIG* (102). The molecular nature of this signal is unknown, but it does not appear to be related to the morphological development (137). Second, full activation of σ^G requires not only completion of engulfment, but also the eight products of the σ^E-controlled *spoIIIA* operon (134). Genetic studies suggest that like σ^F, σ^G is also held inactive by SpoIIAB (114). The *spoIIIA*-dependence of σ^G-controlled gene expression can be overcome by a mutation that impairs σ^G in its binding to SpoIIAB (63). If SpoIIAB does repress both σ^F and σ^G

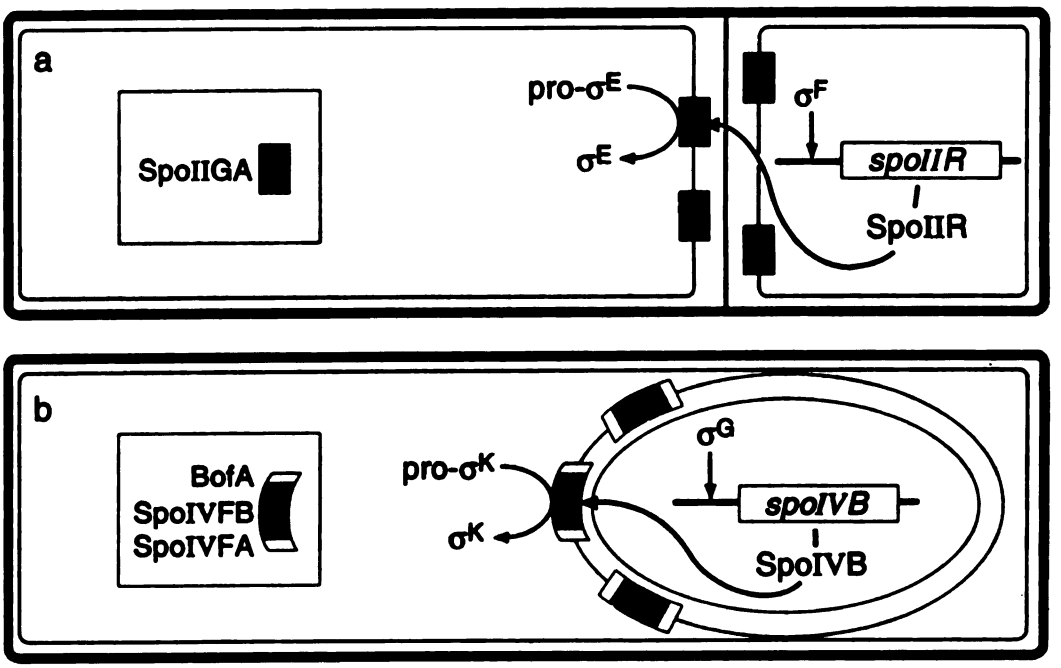


Figure 5. Models for the intercompartmental signaling pathways leading to the activation of pro-σ^E and pro-σ^K. (a) Activation of pro-σ^E. See text for details. (b) Activation of pro-σ^K. See text for details. Reprinted from ref. 137.

activity, then its repression of these sigma factors must be relieved by different mechanisms, because σ^F and σ^G are activated sequentially. There is some evidence that SpoIIAB might be preferentially degraded in the forespore in a *spoIIIA*-dependent fashion (66).

σ^G to σ^K. σ^K, like σ^E, is first made as an inactive precursor. Pro-σ^K has 20 amino acid residues at its N-terminus that are not present in σ^K (69, 136). Pro-σ^K is first made at 3 hours into sporulation, while active σ^K appears an hour later (89). Processing of pro-σ^K not only depends on several mother-cell-specific genes, but also on several forespore-specific genes, including *spoIIIG*, the gene that encodes the forespore-specific sigma factor, σ^G (18, 89). A σ^G-controlled gene, *spoIVB*, is involved in signalling the processing of pro-σ^K (17, 89, 145). Evidence shows that SpoIVB is the only protein produced under σ^G control that is needed to trigger pro-σ^K processing (32). SpoIVB may play a direct role in activating processing of pro-σ^K, possibly by interacting with the processing enzyme. Alternatively, SpoIVB may play a structural or enzymatic role in serving as part of the processing signal from the forespore. Genetic studies have suggested that SpoIVFB, the product of the promoter distal gene of the *spoIVF* operon, may encode the pro-σ^K processing enzyme or, alternatively, a regulator of the processing event (18). SpoIVFA, the product of the promoter proximal gene of the *spoIVF* operon, is suggested to play dual roles in regulating the activity of SpoIVFB (19). In its positive-acting role, SpoIVFA is required to stabilize SpoIVFB, which is suggested to be thermolabile. In its negative-acting role, SpoIVFA inhibits the activity of SpoIVFB until a signal(s) from the forespore is received. BofA, the product of the σ^E-dependent *bofA* gene, also plays a negative role in pro-σ^K processing until a signal from the forespore is

received (116). Both *spoIVF* and *bofA* are transcribed by σ^E RNAP and thereby their expression is confined to the mother cell. The proteins encoded by these genes have potential membrane spanning domains. Indeed, SpoIVFA and SpoIVFB have been localized to the membrane surrounding the forespore (115). Based on these observations and the results from the genetic studies, it was proposed these proteins form an oligomeric complex in the outer membrane of the forespore and that from this position sense either a *spoIVB*-dependent morphological change in the membrane and/or a *spoIVB*-dependent signal from the forespore (Figure 5b).

It is of interest to compare the processing machinery of pro-σ^K to that of pro-σ^E. SpoIVFB seems to be active in its default state, requiring the SpoIVB signal protein to overcome the inhibitory effects of SpoIVFA and BofA, whereas its counterpart, SpoIIGA, appears to be inactive in its default state, requiring the SpoIIR signal protein to become active (Figure 5). Consistent with SpoIVFB being an active protease, coexpression of *spoIVFB* and *sigK* genes in growing *B. subtilis* or *E. coli* enhanced pro-σ^K processing in the absence of other sporulation-specific gene products (88). Sequence analysis revealed that SpoIVFB contains a potential aspartyl protease motif. Mutation of a key amino acid residue in the motif inactivated SpoIVFB (155).

Pro-σ^K may also be subject to processing by a SpoIVFB-independent pathway. This was first suggested by an experiment in which overproducing pro-σ^K in *spoIVF* null mutant cells resulted in partial restoration of σ^K-dependent gene expression and sporulation by allowing accumulation of a small amount of σ^K (90). *sop* (suppressor of processing defect) mutants have now been isolated that are able to partially rescue σ^K-dependent gene expression and sporulation in the absence of *spoIVF* gene products (4).

In cells in which the need for pro-σ^K processing has been uncoupled from

dependence on a forespore signal(s), transcription of σ^K-dependent genes is advanced by 1 hour. This decreases the sporulation efficiency by 10-1,000 fold and the spores produced are germination defective (18, 32). In this case, coupling of σ^K activation in the mother cell to forespore morphogenesis is obviously of great biological importance. This is the only example of a cell-cell communication pathway during sporulation for which the physiological consequences of bypassing it are known.

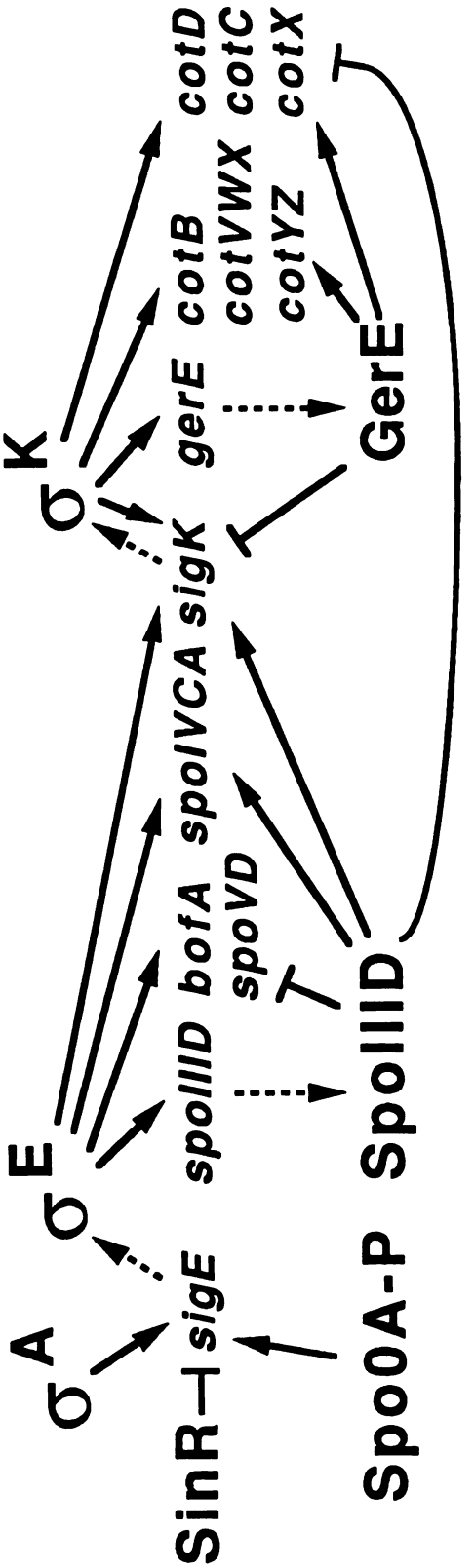
Like that of σ^E and σ^K, the activity of several eukaryotic transcription factors is regulated by proteolysis. NF-κB is a transcription factor that affects the expression of genes involved in immune function, inflammation, and cellular growth. Activation of NF-κB involves the proteasome-mediated destruction of an inhibitory protein, IκB, which masks the nuclear localization sequence of NF-κB (100). The sterol regulatory element-binding proteins (SREBPs) are derived from integral membrane proteins that are held inactive by virtue of being sequestered at the nuclear envelope and the endoplasmic reticulum. In sterol-depleted cells, the membrane-bound precursor is subject to two sequential cleavages, one within a transmembrane domain, to release a soluble fragment that translocates to the nucleus and activates transcription (117, 146). Finally, the *Drosophila* regulatory protein Ci (Cubitus interruptus) is initially inactive because it is tethered in the cytoplasm. It is cleaved to generate a form that lacks the tethering domain and migrates to the nucleus through a pathway that is governed by the Hedgehog signalling protein (6).

Hierarchical Regulatory Cascade of the Mother-cell Line of Gene Expression. Regulation of mother cell gene expression is governed by a hierarchical regulatory cascade consisting of four key regulatory proteins, σ^E, SpoIIID, σ^K, and GerE, in which each regulatory protein is responsible for the production of the next one (Figure 6). Shortly after cells commit to sporulation and before the septum forms, an increased

Spo0A-P level stimulates the transcription of *spoIIG* (*sigE*), which encodes SpoIIGA and pro-σ^E, by σ^A-RNAP (10, 12). Transcription of *sigE* is repressed by SinR, a stationary phase regulator (129). SinR inhibits sporulation by repressing transcription of *spo0A, sigE, spoIIE* and *spoIIA* (8, 91, 92).

The activation of pro-σ^E in the mother cell after the completion of septation sets in motion σ^E-dependent gene transcription, including transcription of the regulatory gene *spoIIID* (70, 132). SpoIIID is a 10.8 kDa DNA-binding protein that activates or represses certain genes in the σ^E and σ^K regulon (40, 69, 156), regulating the timing and/or level of transcription of these genes (Figure 6). Among the σ^E-dependent genes activated by SpoIIID are genes involved in the appearance of σ^K, including *spoIVCA* and *sigK* (39, 40, 69). The appearance of σ^K is subject to multiple levels of regulation (18, 89, 99, 136). First, σ^E acting in conjunction with SpoIIID turns on *spoIVCA* (39, 136), which encodes a site-specific recombinase (119). SpoIVCA recombinase catalyzes a chromosomal rearrangement event joining two truncated genes, *spoIVCB* (encoding the N-terminal half of σ^K) and *spoIIIC* (encoding the C-terminal half of σ^K) (71, 136), to form the composite *sigK* gene. Since σ^E and SpoIIID are produced exclusively in the mother cell, the chromosomal rearrangement does not occur in the forespore. A second level of regulation is the transcription of *sigK*, which requires initially the concerted action of σ^E RNAP and SpoIIID, and then σ^K and SpoIIID (40, 69, 72). The appearance of GerE, the last regulatory protein in the cascade (discussed below), negatively regulates the transcription of *sigK* by σ^K RNAP (50, 159), possibly contributing to the maintenance of a proper level of σ^K. A third level of regulation is the activation of σ^K by the removal of the pro-amino acid sequence (69, 89). As we have seen, pro-σ^K processing is coordinated with and

Figure 6. Diagram of gene regulation in the mother-cell cascade. Dashed lines with arrowheads represent gene-to-product relationships. Arrows and lines with barred ends indicate positive and negative effects, respectively, on expression. Spo0A~P activates the transcription of *sigE* by σ^A RNAP. SinR inhibits *sigE* expression. σ^E RNAP transcribes *spoIIID*. SpoIIID regulates genes in both the σ^E and σ^K regulons. Among them, it activates *sigK* transcription and represses transcription of certain *cot* genes. Transcription of *sigK* is directed first by σ^E and then by its own gene product, σ^K. The *cot D, C,* and *X* genes are transcribed by σ^K RNAP. In one feedback loop, σ^K negatively regulates the *spoIIID* mRNA level (not shown). A diminished SpoIIID level allows the previously repressed *cot* genes to be transcribed. GerE is a gene product of the σ^K regulon. It represses transcription of *sigK*, forming another feedback loop in the mother-cell cascade of gene expression. GerE also activates transcription of the *cot D, C,* and *X* genes, reinforcing the switch in the mother-cell pattern of gene expression initiated by the decrease in the level of SpoIIID.


σA
σE
σK
SinR⊣sigE
spoIIID botA spoIVCA sigK gerE cotB cotVWX cotC cotYZ cotX
spoVD
Spo0A-P
SpoIIID
GerE

controlled by events in the forespore.

SpoIIID represses several late σ^K-dependent genes, notably some of the spore coat genes (Figure 6). A decrease in the SpoIIID level late during sporulation is postulated to be critical for switching on these genes. The SpoIIID level decreases rapidly after reaching its maximum during sporulation (38). This decrease was found to depend on σ^K (38). Thus, the appearance of σ^K negatively regulates the level of SpoIIID (38). Chapters 2 and 3 of this dissertation discuss the continued investigation into the mechanism by which σ^K negatively regulates the level of SpoIIID. In addition, degradation of SpoIIID may involve the conversion of SpoIIID to an unstable 9 kDa form, apparently by removing 7 amino acid residues from its C-terminus (37). The conversion is a developmentally regulated event that is independent of σ^K (37).

GerE is an 8.5 kDa protein that contains a putative helix-turn-helix DNA-binding motif. As we have seen, GerE represses transcription of *sigK*. It activates the expression of many spore coat genes transcribed by σ^K RNAP, some of which are repressed by SpoIIID (Figure 6). Thus, the appearance of GerE reinforces the switch in the mother cell pattern of gene expression initiated by the decrease in the level of SpoIIID. Interestingly, the SpoVT DNA-binding protein encoded by a σ^G-dependent gene seems to play a similar role in the regulation of late gene expression in the forespore (7, 126). SpoVT is believed to bind to some regulatory DNA sequences and allow expression of the latest class of forespore-specific genes. Analogous to GerE repression of *sigK* transcription, SpoVT inhibits *spoIIIG* transcription, perhaps contributing to a progressive shut-off of σ^G activity (7).

Hierarchical regulatory cascades are also found in other systems. An excellent example is flagellum biosynthesis in *E. coli* and *S. typhimurium*. The flagellar genes are

grouped into 13 operons. These operons have been divided into three classes. Class I genes are required for the expression of class II genes and class II genes are required for the expression of class III genes (1, 73, 149). Environmental cues trigger the expression of Class I genes, which encode the two master regulatory proteins FlhC and FlhD. These proteins are required for the activation of σ^A RNAP for transcription of Class II genes, which encode the basal body-hook complex. Completion of this structure acts as the assembly checkpoint to regulate transcription of late σ^{28}-dependent Class III genes, by allowing anti-σ^{28} protein FlgM to be secreted outside the cell (49, 62). In *C. crescentus*, the hierarchy of flagellar assembly involves four classes of genes and two assembly checkpoints (149).

Summary. Gene expression is regulated temporally and spatially during development of *B. subtilis* through synthesis and activation of different sigma factors. Synthesis of late sigma factors depends on the activity of earlier ones in the same compartment. Activation of sigma factors involves signalling between the two compartments and in some cases appears to be coupled to the establishment of a morphological structure. Studies on the model system of *B. subtilis* sporulation are likely to continue to provide insight into the fundamental question of how genes are regulated temporally and spatially during development.

Chapter II

A Feedback Loop Regulates the Switch from One Sigma Factor to the Next in the Cascade Controlling *Bacillus subtilis* Mother-cell Gene Expression

Reprinted from the Journal of Bacteriology

JOURNAL OF BACTERIOLOGY, Oct. 1997, p. 6138–6144
0021-9193/97/$04.00+0

Vol. 179, No. 19

A Feedback Loop Regulates the Switch from One Sigma Factor to the Next in the Cascade Controlling *Bacillus subtilis* Mother Cell Gene Expression

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Received 15 May 1997/Accepted 15 July 1997

Regulation of gene expression in the mother cell compartment of sporulating *Bacillus subtilis* involves sequential activation and inactivation of several transcription factors. Among them are two sigma factors, σ^E and σ^K, and a DNA-binding protein, SpoIIID. A decrease in the level of SpoIIID is thought to relieve its repressive effect on transcription by σ^K RNA polymerase of certain spore coat genes. Previous studies showed that σ^K negatively regulates the level of *spoIIID* mRNA. Here, it is shown that σ^K does not affect the stability of *spoIIID* mRNA. Rather, σ^K appears to negatively regulate the synthesis of *spoIIID* mRNA by accelerating the disappearance of σ^E RNA polymerase, which transcribes *spoIIID*. As σ^K begins to accumulate by 4 h into sporulation, the σ^E level drops rapidly in wild-type cells but remains twofold to fivefold higher in *sigK* mutant cells during the subsequent 4 h. In a strain engineered to produce σ^K 1 h earlier than normal, twofold less σ^E than that in wild-type cells accumulates. σ^K did not detectably alter the stability of σ^E in pulse-chase experiments. However, β-galactosidase expression from a *sigE-lacZ* transcriptional fusion showed a pattern similar to the level of σ^E protein in *sigK* mutant cells and cells prematurely expressing σ^K. These results suggest that the appearance of σ^K initiates a negative feedback loop controlling not only transcription of *spoIIID*, but the entire σ^E regulon, by directly or indirectly inhibiting the transcription of *sigE*.

Sporulation of the gram-positive bacterium *Bacillus subtilis* is a model system for studying developmental gene regulation (8). In response to starvation, *B. subtilis* undergoes a series of morphological changes that culminate in the formation of an endospore. Early during sporulation, an asymmetrically positioned septum partitions the developing cell into two unequal compartments, the mother cell and the forespore, each of which carries a copy of the chromosome. The two compartments follow different programs of gene expression that drive further morphological changes, including migration of the septum to engulf the forespore, deposition of cell wall-like material called cortex between the two membranes surrounding the forespore, formation of a tough protein coat that encases the forespore, and lysis of the mother cell to release the endospore. Temporal and spatial gene regulation during sporulation is established by compartment-specific activation of a cascade of sigma factors, namely, σ^F, σ^E, σ^G, and σ^K, in order of their appearance (26, 34). The forespore-specific program of gene expression is controlled by σ^F and σ^G, while the mother cell program is controlled by σ^E and σ^K. Each sigma factor is initially inactive. σ^F is the first to become active, and this occurs only in the forespore (13, 32, 39). Activation of subsequent sigma factors in the cascade is triggered by signal transduction between the two compartments (12, 34). The inactive forms of the mother cell-specific sigma factors are precursor proteins called pro-σ^E and pro-σ^K. Each is synthesized about 1 h before it is activated by proteolysis (6, 30, 35).

Temporal gene regulation in the mother cell is established primarily by the ordered appearance of σ^E and then σ^K. Also involved is a transcription factor, SpoIIID, whose mRNA is synthesized by σ^E RNA polymerase (28, 49, 52). SpoIIID is a sequence-specific DNA-binding protein that activates or represses many different genes transcribed by σ^E and/or σ^K RNA polymerase (10, 27, 55). One of the genes activated by SpoIIID is *sigK*, which encodes pro-σ^K. The *sigK* gene is constructed during sporulation by a DNA rearrangement that joins *spoIVCB* (encoding the N-terminal part) and *spoIIIC* (encoding the C-terminal part) (51), and SpoIIID also activates transcription of *spoIVCA* (10, 45), the site-specific recombinase that catalyzes the rearrangement (29, 43, 46). Hence, SpoIIID plays a key role in progression from the early σ^E-directed pattern of gene expression to the late σ^K-directed pattern. Somewhat paradoxically, SpoIIID represses certain late genes in the σ^K regulon, apparently fine-tuning their timing and/or level of expression (10, 27, 56). How is the repressive effect of SpoIIID on late gene expression relieved? We showed previously that the SpoIIID protein level decreases abruptly when σ^K appears during sporulation (9). Also, in mutants that fail to make active σ^K, both SpoIIID and its mRNA persist at a higher level until later during sporulation compared to wild-type cells. This suggests that σ^K negatively regulates the synthesis and/or stability of *spoIIID* mRNA. As the existing SpoIIID is degraded, the σ^K-dependent genes that were repressed by SpoIIID would begin to be transcribed.

Here, we describe our continued investigation of the negative feedback loop connecting the production of σ^K to the SpoIIID decrease during sporulation. We demonstrate that σ^K does not affect the stability of *spoIIID* mRNA; therefore, it must exert its negative effect on *spoIIID* transcription. Indeed, a *spoIIID-lacZ* fusion is overexpressed in *sigK* mutant cells (28). Transcription of *spoIIID* is carried out by σ^E RNA polymerase (28, 49, 52). We show here that σ^K also negatively regulates the σ^E level, providing a simple explanation for the negative effect of σ^K on *spoIIID* transcription. σ^K directly or indirectly inhibits the transcription of *sigE* (encoding σ^E), based on the levels of expression from a *sigE-lacZ* transcriptional fusion in wild-type and different mutant strains. Thus,

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σ^K initiates a negative feedback loop that controls not only *spoIIID* expression but expression of the entire σ^E regulon.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* BK556 (*spoIVCB23*) (28), VO48 (*spoIVCBΔ19 cat*) (6), and SC776 (*bofB8 cat*) (6), which are isogenic with the wild-type Spo$^+$ strain PY79 (54), were provided by R. Losick. Strain BZ536 (P_{spoIIG}-P_{sigK}-*sigKΔ19 spc*) was constructed by first replacing the *cat* allele of VO536 (P_{spoIIG}-P_{sigK}-*sigKΔ19 cat*) (40) with a spectinomycin (*spc*) allele by using plasmid pCm::Sp (48) and then by using the chromosomal DNA of the resulting strain to transform competent PY79 cells and to select for a spectinomycin-resistant transformant.

General methods. Preparation of competent cells for transformation with plasmid DNA or chromosomal DNA was described previously (14). Sporulation was induced by resuspending growing cells in SM medium as described previously (14). The onset of sporulation (T_0) was defined as the time of resuspension. Use of the specialized transducing phage SPβ::*sigE-lacZ* (also called *spoIIG-lacZ*) has been described elsewhere (24). β-Galactosidase activity was assessed qualitatively by placing cells on DSM agar (14) containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (20 μg/ml) and was determined quantitatively with toluene to permeabilize cells and *o*-nitrophenol-β-D-galactopyranoside as the substrate (14). One unit of enzyme hydrolyzes 1 μmol of substrate per min per A_{600} of initial cell density.

Measurement of the stability of *spoIIID* mRNA. At the fourth hour of sporulation, rifampin (75 μg/ml) was added to cultures to stop transcription initiation. Samples (9 ml) were taken before and immediately after the addition of rifampin and were centrifuged at 12,000 × *g* for 1.5 min. Cell pellets were frozen in a dry ice-ethanol bath. Samples were also taken at 5 and 12 min after the addition of rifampin. The process of sample collection took about 3 min to complete; therefore, the first time point immediately after rifampin treatment was designated as 3 min after the stoppage of new transcription in cells. Likewise, the ensuing time points were designated as 8 and 15 min after the stoppage of transcription. RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform method (3) with the following modifications. Cell pellets were resuspended in 1.5 ml of denaturing buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) and 1.5 ml of acid phenol-chloroform (5:1) (Ambion) and then mixed vigorously with 1-ml glass beads (500 μm, acid washed; Sigma) to break cells. The mixture was centrifuged at 10,000 × *g* for 20 min at 4°C. After centrifugation, the aqueous phase was re-extracted with acid phenol-chloroform. RNA was precipitated by ethanol. Residual DNA was removed by digesting with RNase-free DNase. RNA (20 μg) was fractionated on a 1.2% (wt/vol) agarose gel containing 1.11% (vol/vol) formaldehyde, transferred to a nylon membrane, and hybridized to a random primed 1.1-kb DNA fragment containing the *spoIIID* coding sequence purified from pBK39 (28) digested with *Pst*I. The radioactive signals were quantified with a PhosphorImager (Molecular Dynamics).

Western blot analysis. Preparation of whole-cell proteins, electrophoresis, and electroblotting were described previously (9, 35). The membrane was probed with monoclonal anti-σ^E antibody (30) diluted 1:600 or polyclonal anti-SpoIIID antiserum (9) diluted 1:10,000. Chemiluminescence detection was performed according to the manufacturer's instructions (ECL; Amersham). When necessary, the membrane was then stripped of the bound antibodies and reprobed with polyclonal anti-pro-σ^K antiserum (35) diluted 1:10,000. Signals were quantified with a computing densitometer (Molecular Dynamics). Exposure times that gave maximum signal intensities within the linear response range of the X-ray film, as determined by control experiments, were used.

Pulse-chase and immunoprecipitation. At the third hour after the onset of sporulation, cells were pulse-labeled by adding [^{35}S]methionine (ICN) (35 μCi/ml) to the culture and incubating for 5 min. Excess (1,000-fold) unlabeled methionine and cysteine were then added, and the incubation was continued at 37°C. Cells (1 ml) were collected by centrifugation immediately following the pulse-chase and at 30-min intervals thereafter until the fifth hour after the onset of sporulation. The cells were frozen in a dry ice-ethanol bath and stored at −70°C.

Cell pellets were resuspended in 50 μl of lysis buffer (10 mM Tris-Cl [pH 8.4], 1 mM EDTA, 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.5-mg/ml lysozyme, 0.1-mg/ml DNase I) and incubated for 10 min at 37°C. Sodium dodecyl sulfate (SDS) was added to a concentration of 1%, and samples were boiled for 3 min. Lysates were centrifuged at 12,000 × *g* for 10 min. Lysates of different samples contained approximately equal amounts of radioactivity as judged by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. After centrifugation, the supernatant was diluted 10-fold in immunoprecipitation buffer (50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.2% deoxycholate, 1 mM phenylmethylsulfonyl fluoride). Monoclonal anti-σ^E antibody (80 μl), which was sufficient to quantitatively precipitate pro-σ^E and σ^E from a 1-ml culture in a control experiment, was added, and the mixture was incubated at 0°C for 2 h. A slurry (20 μl) of 1:1 (vol/vol) protein A-Sepharose CL-4B (Pharmacia)–immunoprecipitation buffer was then added, and the incubation was continued with gentle mixing on a rotary shaker at 4°C for 1.5 h. Samples were centrifuged briefly. The pellets were washed three times with 1 ml of immunoprecipitation buffer supplemented with 0.1% SDS and then resuspended in 30 μl of SDS sample buffer (31), boiled for 5 min, and centrifuged again to remove Sepharose beads. Immunoprecipitates were analyzed by SDS-PAGE. Pro-σ^E and σ^E bands were visualized by fluorography with ENTENSIFY (Dupont) as enhancing fluors and quantified by a PhosphorImager (Molecular Dynamics), with the background of each lane subtracted from the band intensity.

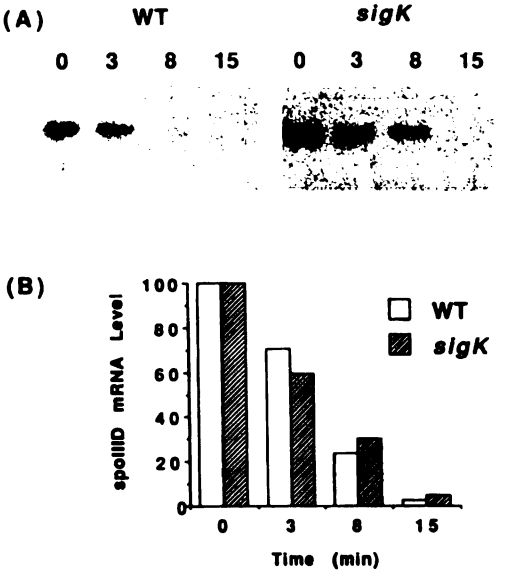


FIG. 1. The production of σ^K does not change the stability of *spoIIID* mRNA. (A) Wild-type (PY79) and *sigK* mutant (BK556) strains were induced to sporulate by resuspension in SM medium. Rifampin (75 μg/ml) was added to the medium at the fourth hour after the onset of sporulation. RNA was prepared from cells collected before and at the indicated number of minutes after the addition of rifampin, and equal amounts (20 μg) were analyzed by Northern blot analysis. (B) The level of *spoIIID* mRNA, as quantified by a PhosphorImager, is plotted as a percentage relative to the level before rifampin treatment. WT, wild type.

RESULTS

Stability of *spoIIID* mRNA in wild-type and *sigK* mutant cells. We showed previously that the *spoIIID* mRNA level reaches a higher maximum and remains higher late during sporulation of *sigK* (*spoIIIC94*; *spoIIIC* encodes the C-terminal part of σ^K) (51) mutant cells compared to that of wild-type cells (9). Similar results were obtained when cells containing another *sigK* mutation, *spoIVCB23* (*spoIVCB* encodes the N-terminal part of σ^K) (51) (both *spoIIIC94* and *spoIVCB23* cells fail to make σ^K) (35), were analyzed (data not shown). This *sigK* mutant was used in the studies reported here.

The higher level of *spoIIID* mRNA in the *sigK* mutants must be due to increased synthesis and/or stability of *spoIIID* mRNA. To measure the stability of *spoIIID* mRNA, sporulating wild-type and *sigK* mutant cells were treated with rifampin at T_4 (i.e., 4 h after starvation initiated sporulation) to stop transcription initiation. Total cellular RNA was isolated from cells collected before and at different times after the rifampin treatment. Northern blot analysis was performed to detect *spoIIID* mRNA. At T_4, there was already more *spoIIID* mRNA in *sigK* mutant cells than in wild-type cells (Fig. 1A), and a considerable amount of σ^K was present in the wild-type cells (data not shown). The amount of *spoIIID* mRNA remaining at different times after the rifampin treatment is shown in Fig. 1A and was quantified with a PhosphorImager. The half-life of *spoIIID* mRNA at T_4 is about 3.5 min in both wild-type and

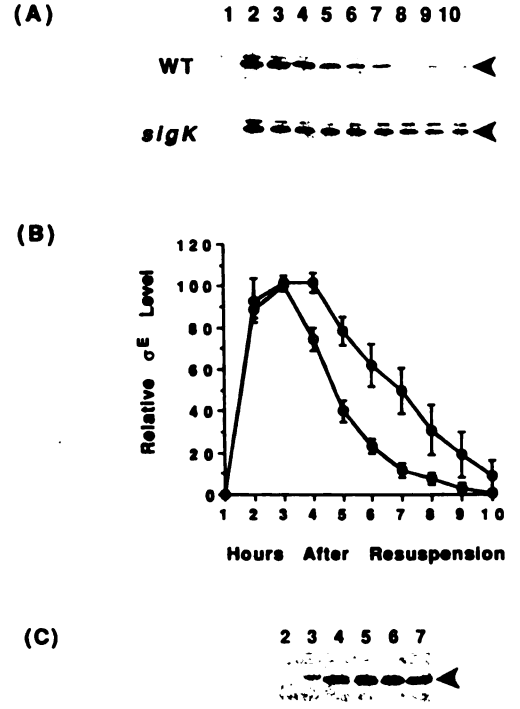


FIG. 2. σ^E persists at a higher level during sporulation of cells defective in σ^K production. Whole-cell extracts were prepared from wild-type (PY79) and *sigK* mutant (BK556) cells collected at the indicated numbers of hours after the onset of sporulation in SM medium. Proteins (5 μg) were fractionated on an SDS–12% polyacrylamide gel and subjected to Western blot analysis with either monoclonal anti-σ^E or polyclonal anti-pro-σ^K antibodies. (A) Levels of σ^E in wild-type (WT) and *sigK* mutant cells. Arrowheads, σ^E signal (the faint signal of lesser mobility most apparent at T_2 is pro-σ^E). (B) Relative amounts of σ^E in wild-type (○) and *sigK* mutant (●) cells during sporulation. The σ^E signals in three experiments with both the wild-type strain and the *sigK* mutant and two experiments with just the wild-type strain were quantified with a computing densitometer. For each experiment, the signal intensities were normalized to the maximum signal in wild-type cells. Points on the graph are averages of the normalized values, and error bars show one standard deviation of the data. (C) Levels of σ^K in wild-type cells. Arrowhead, σ^K signal (the faint signal of lesser mobility first appearing at T_3 is pro-σ^K).

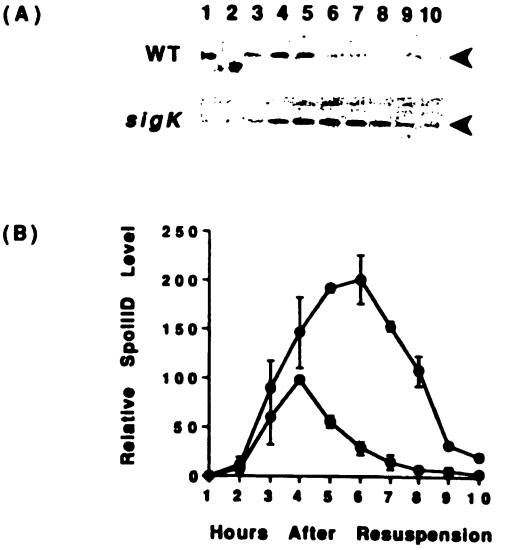


FIG. 3. SpoIIID persists at a higher level during sporulation of cells defective in σ^K production. (A) Proteins (1.7 μg) in the same samples shown in Fig. 2A were fractionated on an SDS–18% polyacrylamide gel and subjected to Western blot analysis with anti-SpoIIID antiserum. Arrowheads, SpoIIID signal. WT, wild type. (B) Relative amounts of SpoIIID in wild-type (PY79 [○]) and *sigK* mutant (BK556 [●]) cells during sporulation. The SpoIIID signals in three experiments were quantified, normalized, and plotted as described in the legend to Fig. 2.

sigK mutant cells (Fig. 1B). Since no substantial difference in the stability of *spoIIID* mRNA was detected, the higher level of *spoIIID* mRNA in sporulating *sigK* mutant cells must be due to increased synthesis of *spoIIID* mRNA. In support of this idea and in agreement with the results of Kunkel et al. (28), we found that a *spoIIID-lacZ* transcriptional fusion is overexpressed by approximately 1.7-fold in *sigK* mutant cells compared to wild-type cells (data not shown).

σ^E level in wild-type and *sigK* mutant cells. Since *spoIIID* is transcribed by σ^E RNA polymerase (28, 49, 52), we reasoned that increased *spoIIID* transcription in *sigK* mutant cells might result from an elevated level of σ^E. We measured the level of σ^E in extracts of wild-type and *sigK* mutant cells using anti-σ^E antibody (30) in Western blot analysis. To facilitate the comparison, the two strains were induced to sporulate in parallel cultures and equal amounts of protein in whole-cell extracts were electrophoresed in the same SDS-polyacrylamide gel. Figure 2A shows that in wild-type cells, σ^E was first detected at T_2 and reached a maximum level by T_3, and the level decreased rapidly thereafter. In *sigK* mutant cells, the σ^E level remained high at T_4, and thereafter its level decreased less rapidly than that in wild-type cells. The experiment was repeated several times, and the Western blot signals were quantitated. Figure 2B shows that, after T_3, σ^E reproducibly persisted at a level in *sigK* mutant cells higher than that in wild-type cells. Between T_5 and T_8, the σ^E level was twofold to fivefold higher in the *sigK* mutant than that in the wild type. Similar results were obtained when other mutants that fail to make σ^K (i.e., cells containing a *spoIIIC94* or a *spoIVCA133* mutation) (35) were tested (data not shown). We also tested mutants (i.e., those containing *spoIIIGΔ1* and *spoIVFΔAB::cat*) that produce pro-σ^K but fail to process it to active σ^K (35, 36). Again, similar results were observed (data not shown), indicating the pro-σ^K must be processed to active σ^K in order to accelerate the disappearance of σ^E from sporulating cells. Moreover, as shown in Fig. 2C, processing in wild-type cells causes σ^K to begin accumulating by T_4, which is the earliest time that the σ^E level is lower in wild-type cells than that in *sigK* mutant cells (Fig. 2B). We conclude that the appearance of active σ^K accelerates the disappearance of σ^E during sporulation.

We note that σ^K is not essential for the level of σ^E to decrease, since the σ^E level eventually declines in mutants that fail to make σ^K (Fig. 2B and data not shown). Cell lysis is not the explanation for the decrease in σ^E in the mutants or for the more rapid decrease in σ^E in wild-type cells. Although a small amount of cell lysis began to occur after T_7 in both the wild-type and the mutant cultures, the ability to recover protein from sedimented cells never varied by more than 10% during the course of our experiments.

We also measured SpoIIID levels in most of the samples used in the experiments summarized in Fig. 2B. Figure 3A shows the results for the same samples used in the experiment shown in Fig. 2A. Figure 3B shows quantitation of several experiments. SpoIIID accumulated by T_5 to a level in *sigK*

mutant cells that was twofold higher than that in wild-type cells. The level of SpoIIID in the wild-type strain decreased threefold by T_6, while in the *sigK* mutant the SpoIIID level remained high until T_6 and then declined thereafter. Thus, the levels of both SpoIIID and σ^E are significantly higher in *sigK* mutant cells than in wild-type cells between T_4 and T_8 of sporulation. The absence of σ^K has a larger effect on the SpoIIID level than on the σ^E level (compare Fig. 2B and 3B). This difference might be explained by the fact that σ^E RNA polymerase acts enzymatically to increase *spoIIID* transcription and/or by the observation that SpoIIID positively autoregulates *spoIIID* transcription (23, 28, 49, 52). Thus, a relatively small effect on σ^E could lead to a larger effect on SpoIIID. Clearly, σ^K negatively regulates the σ^E level during sporulation, providing a simple explanation for the negative effect of σ^K on the SpoIIID level.

σ^E level in cells that produce σ^K earlier. It was shown previously that earlier production of σ^K during sporulation resulted in less accumulation of SpoIIID and earlier disappearance of SpoIIID (9). To examine whether these effects might also be explained by a negative effect of σ^K on σ^E, we monitored the level of σ^E in *spoIVCBΔ19* mutant cells. In these cells, codons 2 through 20 of *sigK*, which encode the N-terminal prosequence of pro-σ^K, are missing, resulting in production of active σ^K 1 to 2 h earlier than normal (Fig. 4A) (6, 9). As documented in Fig. 4B and C, the maximum level of σ^E in *spoIVCBΔ19* mutant cells reached only about 50% of the wild-type maximum. These results support the idea that the appearance of σ^K negatively regulates the σ^E level during sporulation.

Turnover of pro-σ^E and σ^E in wild-type and *sigK* mutant cells. σ^K might negatively regulate the σ^E level by destabilizing σ^E, possibly by directly competing with σ^E for core RNA polymerase. It has been suggested that σ^E is unstable in cells when it is not bound to core RNA polymerase (21). A complication in measuring the stability of σ^E is that it is generated from pro-σ^E by proteolytic processing (30). However, since processing of pro-σ^E occurs normally in *sigK* mutant cells (Fig. 2A), we reasoned that a comparison of the total amounts of pro-σ^E and σ^E remaining at different times after pulse-labeling of *sigK* mutant cells and wild-type cells should reveal a difference in σ^E stability, if it exists. Sporulating wild-type and *sigK* mutant cells were pulse-labeled at T_3 with [^{35}S]methionine and chased with an excess amount of unlabeled methionine. We chose T_3 to perform the labeling because during the subsequent hours of sporulation large differences in the levels of σ^E between wild-type and *sigK* mutant cells were observed (Fig. 2B). Samples were collected every half hour after the pulse-labeling, and pro-σ^E and σ^E in crude cell extracts were immunoprecipitated with monoclonal anti-σ^E antibody. The pro-σ^E and σ^E signals were revealed by SDS-PAGE and fluorography (Fig. 5A). The [^{35}S]methionine was first incorporated into pro-σ^E through protein synthesis and then appeared as σ^E upon proteolytic cleavage of the N-terminal sequence from pro-σ^E. A small portion of the pro-σ^E had already been processed into σ^E at the end of the 5 min of pulse-labeling (labeled 0 min in Fig. 5A). Upon incubation, the ^{35}S label was chased into σ^E, and eventually σ^E was degraded. Figure 5B shows that the decay rate of pro-σ^E plus σ^E was similar in wild-type and *sigK* mutant cells, as judged by the quantification of the combined signal intensities of pro-σ^E and σ^E. When the experiment was repeated and samples were collected at 45-min intervals after pulse-labeling, again no substantial difference between wild-type and *sigK* mutant cells was observed (data not shown). Therefore, destabilization of σ^E upon the appearance of σ^K cannot explain the level of σ^E in wild-type cells being lower than that in *sigK* mutant cells at T_4 to T_5 of sporulation (Fig. 2).

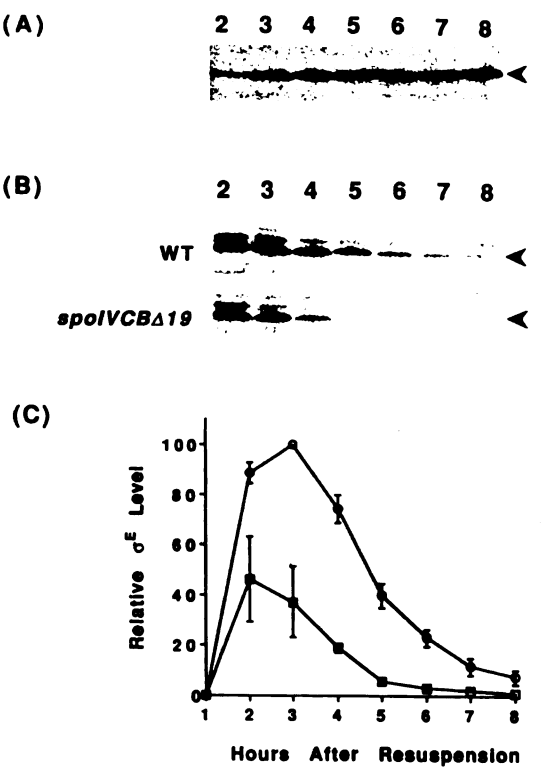


FIG. 4. σ^E disappears earlier during sporulation of cells that produce σ^K earlier than normal. Whole-cell extracts were prepared from wild-type (PY79) and *spoIVCBΔ19* mutant (VO48) cells collected at the indicated numbers of hours after the onset of sporulation in SM medium. Proteins (5 μg) were fractionated on an SDS–12% polyacrylamide gel and subjected to Western blot analysis with either monoclonal anti-σ^E or polyclonal anti-pro-σ^K antibodies. (A) The level of σ^K in the *spoIVCBΔ19* mutant. Arrowhead, σ^K signal. (B) Levels of σ^E in wild-type (WT) and *spoIVCBΔ19* mutant cells. Arrowheads, σ^E signal (the faint signal of lesser mobility most apparent at T_2 is pro-σ^E). (C) Relative amounts of σ^E in wild-type (○) and *spoIVCBΔ19* (□) cells during sporulation. For the wild-type strain, the data shown in Fig. 2B are also shown here. In three of the experiments with wild-type cells, the *spoIVCBΔ19* mutant was induced to sporulate in a parallel culture. The σ^E signals were quantified, normalized, and plotted as described in the legend to Fig. 2.

Expression of a *sigE-lacZ* transcriptional fusion in wild-type cells, *sigK* mutant cells, and cells producing σ^K earlier. Since σ^K did not appear to affect the stability of σ^E, we tested the possibility that σ^K may affect the transcription of the *sigE* gene that encodes pro-σ^E. *sigE* (also called *spoIIGB*) is the second gene in the *spoIIG* operon (22, 24). The first gene of the operon, *spoIIGA*, encodes a putative protease that processes pro-σ^E to σ^E (16, 41, 50). First, we tried to directly compare the levels of *sigE* mRNA in sporulating wild-type and *sigK* mutant cells by Northern blot analysis. In agreement with a previous report (24), we found that *sigE* mRNA was unstable and subject to processing or breakdown. Despite the difficulty in detecting *sigE* mRNA, we noticed that slightly more *sigE* mRNA appeared to be present in *sigK* mutant cells than that in wild-type cells at T_3 and later times during development (data not shown). We then examined expression of a *sigE-lacZ* transcriptional fusion as a simple, albeit indirect, measure of *sigE* transcription. We introduced a *sigE-lacZ* transcriptional fusion

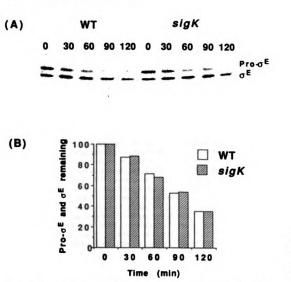


FIG. 5. The production of σ^K does not alter the stability of pro-σ^E and σ^E. (A) Wild-type (PY79) and *sigK* mutant (BKS56) cells were labeled at the third hour after the onset of sporulation in SM medium with [^{35}S]methionine for 5 min and chased with excess amounts of unlabeled methionine and cysteine. Cells were collected immediately and at the indicated numbers of minutes following pulse-labeling. Whole-cell extracts were prepared, and pro-σ^E and σ^E were immunoprecipitated with monoclonal anti-σ^E antibody. Immunoprecipitates from 300 μl of the sporulating cell culture were separated by SDS-PAGE and detected by fluorography. (B) Pro-σ^E and σ^E were quantified with a PhosphorImager and plotted as percentages relative to the levels immediately after pulse-labeling. WT, wild type.

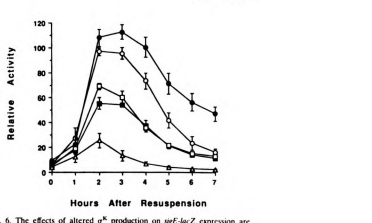


FIG. 6. The effects of altered σ^K production on *sigE-lacZ* expression are similar to the effects on the σ^K level. Wild-type (PY79 [○]) cells, *sigK* mutant (BKS56 [●]) cells, and *spoIVCBΔ19* (VO48 [■]), *bofB8* (SC776 [□]), and P_{spac}-P_{spac}-*sigKΔ19* (BZ536 [△]) cells that produce σ^K earlier than normal were lysogenized with phage SPβ::*sigE-lacZ*, and the resulting strains were induced to sporulate in SM medium. Samples were collected at the indicated times following the initiation of sporulation and assayed for β-galactosidase activity. Experiments were performed at least twice for each strain. For each experiment, the specific activities were normalized to the maximum specific activity in wild-type cells (typically 70 U). Points on the graph are averages of the normalized values, and error bars show one standard deviation of the data.

carried on an SPβ phage (24), via specialized transduction, into the chromosomes of wild-type cells, *sigK* (*spoIVCB23*) mutant cells, and mutants (carrying *spoIVCBΔ19*, *bofB8*, or P_{spac}-P_{spac}-*sigKΔ19*) that produce active σ^K earlier than normal. The *bofB8* mutant, like the *spoIVCBΔ19* mutant, produces active σ^K about 1 h earlier than normal because processing of pro-σ^K is uncoupled from its normal dependence on a signal from the forespore compartment (6). The P_{spac}-P_{spac}-*sigKΔ19* mutant contains in its chromosome the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, *spac*, fused to a copy of the *sigK* gene (*sigKΔ19*) that permits production of active σ^K without the need for the site-specific recombination event that normally joins the two parts (i.e., *spoIVCB* and *spoIIIC*) of the *sigK* gene and without the need for processing (40). Thus, P_{spac}-P_{spac}-*sigKΔ19* cells produce σ^K upon the addition of IPTG (11, 40). *sigE-lacZ* expression was highest in *sigK* mutant cells, lower in wild-type cells, even lower in *spoIVCBΔ19* and *bofB8* mutant cells, and lowest in P_{spac}-P_{spac}-*sigKΔ19* cells, as judged by the intensity of blue of colonies sporulating on DSM agar containing X-Gal and IPTG (data not shown). In agreement with these qualitative results were the results of quantitative β-galactosidase assays of cells sporulating in SM liquid. Figure 6 shows that in wild-type cells, *sigE*-directed β-galactosidase activity increased at T_1, reached its peak level at T_2 or T_3, and decreased thereafter. In *sigK* mutant cells, β-galactosidase activity rose to a slightly higher level and remained higher late in sporulation. β-Galactosidase activity was reduced in *spoIVCBΔ19* and *bofB8* mutant cells that produce σ^K about 1 h earlier than normal. The effects on *sigE-lacZ* expression in the *sigK* mutant and the *spoIVCBΔ19* mutant were similar to the effects on the σ^E level (Fig. 2 and 4). When production of σ^K was induced 30 min before cells were resuspended to initiate sporulation, *sigE-lacZ* expression was even lower (Fig. 6). Taken together, these results suggest that σ^K negatively regulates the σ^E level by affecting the transcription of *sigE*.

DISCUSSION

We have demonstrated that σ^K negatively regulates the level of σ^E during sporulation. In wild-type cells, the level of σ^E begins to decrease when active σ^K begins to accumulate (Fig. 2). In mutants defective in σ^K production, σ^E persists at an elevated level for several hours (Fig. 2 and data not shown). In cells engineered to produce σ^K earlier than normal, twofold less σ^E than that in wild-type cells accumulates (Fig. 4). A similar pattern of effects in *sigK* mutant cells and cells prematurely expressing σ^K is observed for expression of a *sigE-lacZ* fusion (Fig. 6), suggesting that σ^K exerts its negative effect at the level of *sigE* transcription.

The finding that σ^K negatively regulates σ^E provides a simple explanation for the previous observation that σ^K negatively regulates SpoIIID (9). As depicted in Fig. 7, σ^K RNA polymerase transcribes the *spoIIID* gene (28, 49, 52). A decrease in the σ^E level brought about by a negative effect of σ^K on *sigE* transcription (Fig. 7) would reduce the synthesis of *spoIIID* mRNA, assuming that σ^E RNA polymerase becomes limiting for *spoIIID* transcription. It seems likely that *spoIIID* transcription is limited by the availability of σ^E, because earlier production of σ^K reduces the σ^E level (Fig. 4), and the level of SpoIIID is likewise reduced (9). Conversely, the failure to make σ^K results in an elevated σ^E level beginning at T_4 of sporulation (Fig. 2), and the level of SpoIIID is also elevated (Fig. 3). We found no evidence that σ^K affects *spoIIID* expression at the level of mRNA stability (Fig. 1). Also, there is no evidence that σ^K regulates the SpoIIID level via a posttranscriptional mechanism. The difference in the SpoIIID protein level between wild-type and *sigK* mutant cells (Fig. 3) is similar

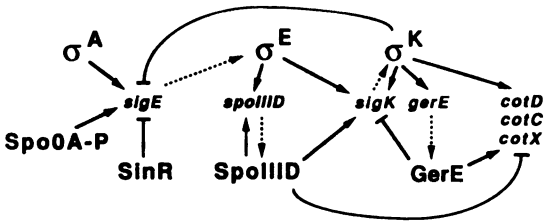


FIG. 7. Model for gene regulation in the mother cell cascade. Dashed lines with arrowheads, gene-to-product relationships; arrows and lines with barred ends, positive and negative effects, respectively, on expression. The hallmark of initiation of sporulation is an increase in the level of Spo0A-P. Spo0A-P activates transcription of *sigE* by σ^A RNA polymerase. SinR directly or indirectly inhibits *sigE* expression. σ^E RNA polymerase transcribes *spoIIID*. SpoIIID regulates genes in both the σ^E and σ^K regulons. Among them, it activates *sigK* transcription and represses transcription of certain *cot* genes. Transcription of *sigK* is directed first by σ^E and then by its own gene product, σ^K. The *cotD*, *-C*, and *-X* genes are transcribed by σ^K RNA polymerase. σ^K negatively regulates *spoIIID* and the entire σ^E regulon by negatively regulating *sigE* transcription. A diminished SpoIIID level allows the previously repressed *cot* genes to be transcribed. GerE is a gene product of the σ^K regulon. It represses transcription of *sigK*, forming another feedback loop in the mother cell cascade of gene expression. GerE also activates transcription of the *cotD*, *-C*, and *-X* genes, reinforcing the switch of the mother cell gene expression pattern initiated by the decrease in the level of SpoIIID.

to the difference in the *spoIIID* mRNA level (9) (data not shown). In addition, the pattern of overaccumulation of β-galactosidase activity from a *spoIIID-lacZ* transcriptional fusion in sporulating *sigK* mutant cells (28) (data not shown) was similar to the pattern of overaccumulation of SpoIIID (Fig. 3). Therefore, we propose that σ^K directly or indirectly inhibits *sigE* transcription, reducing synthesis of σ^E, which in turn reduces transcription of *spoIIID*, and, as the level of SpoIIID declines, its repressive effect on σ^K-dependent genes such as *cotD*, *cotX*, and *cotC* is relieved (9, 10, 19, 56, 58) (Fig. 7).

Transcription of *sigE* is carried out by σ^A RNA polymerase and requires Spo0A phosphate (25, 47) (Fig. 7). σ^A is the major sigma factor present in growing cells, in which it directs transcription of most genes (12). Spo0A is also present in growing cells, and an increase in the level of phosphorylated Spo0A (Spo0A-P) initiates sporulation gene expression, including directly activating *sigE* transcription by σ^A RNA polymerase, in response to nutritional, extracellular, and cell cycle signals (1, 20). Transcription of *sigE* is also subject to negative control by SinR (37, 38) (Fig. 7). We are currently trying to determine whether σ^K exerts its negative effect on *sigE* transcription by affecting σ^A, Spo0A-P, or SinR.

The negative effect of σ^K on *sigE* transcription may explain why a *sigE-lacZ* fusion is overexpressed in *sigE* mutant cells (24). Since the *sigE* mutant fails to make σ^K, the negative feedback on *sigE* transcription would not occur, resulting in *sigE* overexpression. Similarly, the elevated level of σ^E found in *sigK* mutant cells might cause overexpression of other σ^E-dependent genes in addition to *spoIIID*. The promoter of *spoIID* is a well-known example of a σ^E-dependent promoter that is independent of SpoIIID for transcription (4, 44). We found that *spoIID-lacZ* is overexpressed in *sigK* mutant cells (data not shown).

The negative effect of σ^K on σ^E and SpoIIID is not the only example of a feedback loop in the cascade of transcription factors controlling mother cell gene expression. As illustrated in Fig. 7, σ^K RNA polymerase transcribes the *gerE* gene and GerE limits the σ^K level by repressing *sigK* transcription (19, 58). It was attractive to think that in addition to repressing *sigK* transcription, GerE might repress the transcription of *sigE* and/or *spoIIID*. However, expression of *sigE-lacZ* and *spoIIID-lacZ* transcriptional fusions is indistinguishable in wild-type and *gerE* mutant cells (data not shown).

The finding that σ^K negatively regulates *sigE* transcription provides an alternative to the model that each subsequent σ in a cascade competes more effectively for a limiting amount of core RNA polymerase (33). In vitro studies with phage σ factors involved in cascade regulation support the direct σ competition model in some cases (2, 18), but not in others (53). Recently, Hicks and Grossman (15) presented in vivo experiments that suggest that σ^A competes with σ^H for binding to core RNA polymerase. If σ^K could outcompete σ^E for core binding, it seemed likely that the appearance of σ^K in cells would destabilize σ^E, since it had been suggested that free σ^E is unstable (21). However, σ^K did not affect the stability of σ^E (Fig. 5).

In the σ cascade controlling *B. subtilis* sporulation gene expression, each σ is either made as an inactive precursor or is initially held inactive by an anti-σ factor (12, 34). This ensures that later-acting σ factors accumulate sufficiently before negatively regulating earlier-acting σ factors that control their synthesis. Regulation of σ factor activity also appears to couple the program of gene expression in the mother cell and forespore during *B. subtilis* sporulation (26, 34). For example, proteolytic processing of inactive pro-σ^K to active σ^K in the mother cell is governed by a signal transduction pathway that emanates from the forespore and may depend on a morphological feature of the developing sporangium (5–7, 35). In this case, the primary event responding to morphological and/or cell-cell signals is pro-σ^K processing. Loss of σ^E and SpoIIID is a secondary event brought about by the negative effect of σ^K on *sigE* transcription. In contrast, loss of a transcription factor from cells due to secretion is the primary event regulated by morphological cues or cell-cell interactions in a few examples that have emerged recently (17, 42, 57). These examples highlight the importance of considering the disappearance of existing transcription factors, as well as the appearance of new ones, during adaptive processes.

ACKNOWLEDGMENTS

We thank V. Oke and R. Losick for providing bacterial strains, C. Buckner and C. Moran for providing SPβ::*sigE-lacZ*, and W. Haldenwang for providing monoclonal anti-σ^E antibody. We thank A. Hofmeister and W. Haldenwang for helpful comments on the manuscript.

This research was supported by the Michigan Agricultural Experiment Station and grant GM43585 from the National Institutes of Health.

REFERENCES

1. **Burbulys, D., K. A. Trach, and J. A. Hoch.** 1991. Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. Cell **64**:545–552.
2. **Chelm, B. K., J. J. Duffy, and E. P. Geiduschek.** 1982. Interaction of *Bacillus subtilis* RNA polymerase core with two specificity-determining subunits. J. Biol. Chem. **257**:6501–6508.
3. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
4. **Clarke, S., I. Lopez-Diaz, and J. Mandelstam.** 1986. Use of *lacZ* fusions to determine the dependence pattern of the sporulation gene *spoIID* in *spo* mutants of *Bacillus subtilis*. J. Gen. Microbiol. **132**:2987–2994.
5. **Cutting, S., A. Driks, R. Schmidt, B. Kunkel, and R. Losick.** 1991. Forespore-specific transcription of a gene in the signal transduction pathway that governs pro-σ^K processing in *Bacillus subtilis*. Genes Dev. **5**:456–466.
6. **Cutting, S., V. Oke, A. Driks, R. Losick, S. Lu, and L. Kroos.** 1990. A forespore checkpoint for mother-cell gene expression during development in *Bacillus subtilis*. Cell **62**:239–250.
7. **Cutting, S., S. Roels, and R. Losick.** 1991. Sporulation operon *spoIVF* and the characterization of mutations that uncouple mother-cell from forespore

gene expression in *Bacillus subtilis*. J. Mol. Biol. **221**:1237–1256.
8. **Errington, J.** 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. **57**:1–33.
9. **Halberg, R., and L. Kroos.** 1992. Fate of the SpoIIID switch protein during *Bacillus subtilis* sporulation depends on the mother-cell sigma factor, σ^K. J. Mol. Biol. **228**:840–849.
10. **Halberg, R., and L. Kroos.** 1994. Sporulation regulatory protein SpoIIID from *Bacillus subtilis* activates and represses transcription by both mother-cell-specific forms of RNA polymerase. J. Mol. Biol. **243**:425–436.
11. **Halberg, R., V. Oke, and L. Kroos.** 1995. Effects of *Bacillus subtilis* sporulation regulatory protein SpoIIID on transcription by σ^K RNA polymerase in vivo and in vitro. J. Bacteriol. **177**:1888–1891.
12. **Haldenwang, W. G.** 1995. The sigma factors of *Bacillus subtilis*. Microbiol. Rev. **59**:1–30.
13. **Harry, E. J., K. Pogliano, and R. Losick.** 1995. Use of immunofluorescence to visualize cell-specific gene expression during sporulation in *Bacillus subtilis*. J. Bacteriol. **177**:3386–3393.
14. **Harwood, C. R., and S. M. Cutting.** 1990. Molecular biological methods for *Bacillus*. John Wiley & Sons, Chichester, England.
15. **Hicks, K. A., and A. D. Grossman.** 1996. Altering the level and regulation of the major sigma subunit of RNA polymerase affects gene expression and development in *Bacillus subtilis*. Mol. Microbiol. **20**:201–212.
16. **Hofmeister, A., A. Londono-Vallejo, E. Harry, P. Stragier, and R. Losick.** 1995. Extracellular signal protein triggering the proteolytic activation of a developmental transcription factor in *B. subtilis*. Cell **83**:219–226.
17. **Hughes, K. T., K. L. Gillen, M. J. Semon, and J. E. Karlinsey.** 1993. Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator. Science **262**:1277–1280.
18. **Hyde, E. I., M. D. Hilton, and H. R. Whiteley.** 1986. Interactions of *Bacillus subtilis* RNA polymerase with subunits determining the specificity of initiation. J. Biol. Chem. **261**:16565–16570.
19. **Ichikawa, H., and L. Kroos.** Unpublished data.
20. **Ireton, K., D. Z. Rudner, K. J. Siranosian, and A. D. Grossman.** 1993. Integration of multiple developmental signals in *Bacillus subtilis* through the *spo0A* transcription factor. Genes Dev. **7**:283–294.
21. **Jonas, R. M., H. K. Peters, and W. G. Haldenwang.** 1990. Phenotypes of *Bacillus subtilis* mutants altered in the precursor-specific region of σ^E. J. Bacteriol. **172**:4178–4186.
22. **Jonas, R. M., E. A. Weaver, T. J. Kenney, C. P. Moran, and W. G. Haldenwang.** 1988. The *Bacillus subtilis spoIIG* operon encodes both σ^E and a gene necessary for σ^E activation. J. Bacteriol. **170**:507–511.
23. **Jones, C. H., and C. P. Moran.** 1992. Mutant σ factor blocks transition between promoter binding and initiation of transcription. Proc. Natl. Acad. Sci. USA **89**:1958–1962.
24. **Kenney, T. J., and C. P. Moran.** 1987. Organization and regulation of an operon that encodes a sporulation-essential sigma factor in *Bacillus subtilis*. J. Bacteriol. **169**:3329–3339.
25. **Kenney, T. J., K. York, P. Youngman, and C. P. Moran.** 1989. Genetic evidence that RNA polymerase associated with σ^A uses a sporulation-specific promoter in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **86**:9109–9113.
26. **Kroos, L., and S. Cutting.** 1994. Intercellular and intercompartmental communication during *Bacillus subtilis* sporulation, p. 155–180. *In* P. J. Piggot, C. P. Moran, and P. Youngman (ed.), Regulation of bacterial differentiation. American Society for Microbiology, Washington, D.C.
27. **Kroos, L., B. Kunkel, and R. Losick.** 1989. Switch protein alters specificity of RNA polymerase containing a compartment-specific sigma factor. Science **243**:526–529.
28. **Kunkel, B., L. Kroos, H. Poth, P. Youngman, and R. Losick.** 1989. Temporal and spatial control of the mother-cell regulatory gene *spoIIID* of *Bacillus subtilis*. Genes Dev. **3**:1735–1744.
29. **Kunkel, B., R. Losick, and P. Stragier.** 1990. The *Bacillus subtilis* gene for the developmental transcription factor σ^K is generated by excision of a dispensable DNA element containing a sporulation recombinase gene. Genes Dev. **4**:525–535.
30. **LaBell, T. L., J. E. Trempy, and W. G. Haldenwang.** 1987. Sporulation-specific sigma factor, σ^{29}, of *Bacillus subtilis* is synthesized from a precursor protein, P^{31}. Proc. Natl. Acad. Sci. USA **84**:1784–1788.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680–685.
32. **Lewis, P. J., C. E. Nwoguh, M. R. Rarer, C. J. Harwood, and J. Errington.** 1994. Use of digitized video microscopy with a fluorogenic enzyme substrate to demonstrate cell- and compartment-specific gene expression in *Salmonella enteritidis* and *Bacillus subtilis*. Mol. Microbiol. **13**:3849–3853.
33. **Losick, R., and J. Pero.** 1981. Cascades of sigma factors. Cell **25**:582–584.
34. **Losick, R., and P. Stragier.** 1992. Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*. Nature **355**:601–604.
35. **Lu, S., R. Halberg, and L. Kroos.** 1990. Processing of the mother-cell σ factor, σ^K, may depend on events occurring in the forespore during *Bacillus subtilis* development. Proc. Natl. Acad. Sci. USA **87**:9722–9726.
36. **Lu, S., and L. Kroos.** 1994. Overproducing the *Bacillus subtilis* mother-cell sigma factor precursor, pro-σ^K, uncouples σ^K-dependent gene expression from dependence on intercompartmental communication. J. Bacteriol. **176**:3936–3943.
37. **Mandic-Mulec, I., L. Doukhan, and I. Smith.** 1995. The *Bacillus subtilis* SinR protein is a repressor of the key sporulation gene *spo0A*. J. Bacteriol. **177**:4619–4627.
38. **Mandic-Mulec, I., N. Gaur, U. Bai, and I. Smith.** 1992. Sin, a stage-specific repressor of cellular differentiation. J. Bacteriol. **174**:3561–3569.
39. **Margolis, P., A. Driks, and R. Losick.** 1991. Establishment of cell type by compartmentalized activation of a transcription factor. Science **254**:562–565.
40. **Oke, V., and R. Losick.** 1993. Multilevel regulation of the sporulation transcription factor σ^K in *Bacillus subtilis*. J. Bacteriol. **175**:7341–7347.
41. **Peters, H. K., and W. G. Haldenwang.** 1994. Isolation of a *Bacillus subtilis spoIIGA* allele that suppresses processing-negative mutations in the pro-σ^E gene (*sigE*). J. Bacteriol. **176**:7763–7766.
42. **Pettersson, J., R. Nordfelth, E. Dubinina, T. Bergman, M. Gustafsson, K. E. Magnusson, and H. Walf-Watz.** 1996. Modulation of virulence factor expression by pathogen target cell contact. Science **273**:1231–1233.
43. **Popham, D. L., and P. Stragier.** 1992. Binding of the *Bacillus subtilis spoIVCA* product to the recombination sites of the element interrupting the σ^K-encoding gene. Proc. Natl. Acad. Sci. USA **89**:5991–5995.
44. **Rong, S., M. S. Rosenkrantz, and A. L. Sonenshein.** 1986. Transcriptional control of the *Bacillus subtilis spoIID* gene. J. Bacteriol. **165**:771–779.
45. **Sato, T., K. Harada, Y. Ohta, and Y. Kobayashi.** 1994. Expression of the *Bacillus subtilis spoIVCA* gene, which encodes a site-specific recombinase, depends on the *spoIIGB* product. J. Bacteriol. **176**:935–937.
46. **Sato, T., Y. Samori, and Y. Kobayashi.** 1990. The *cisA* cistron of *Bacillus subtilis* sporulation gene *spoIVC* encodes a protein homologous to a site-specific recombinase. J. Bacteriol. **172**:1092–1098.
47. **Satola, S. W., J. M. Baldus, and C. P. Moran.** 1992. Binding of Spo0A stimulates *spoIIG* promoter activity in *Bacillus subtilis*. J. Bacteriol. **174**:1448–1453.
48. **Steinmetz, M., and R. Richter.** 1994. Plasmids designed to alter the antibiotic resistance expression by insertion mutations in *Bacillus subtilis*, through *in vivo* recombination. Gene **142**:79–83.
49. **Stevens, C. M., and J. Errington.** 1990. Differential gene expression during sporulation in *Bacillus subtilis*: structure and regulation of the *spoIIID* gene. Mol. Microbiol. **4**:543–552.
50. **Stragier, P., C. Bonamy, and C. Karmazyn-Campelli.** 1988. Processing of a sporulation sigma factor in *Bacillus subtilis*: how morphological structure could control gene expression. Cell **52**:697–704.
51. **Stragier, P., B. Kunkel, L. Kroos, and R. Losick.** 1989. Chromosomal rearrangement generating a composite gene for a developmental transcription factor. Science **243**:507–512.
52. **Tatti, K. M., C. H. Jones, and C. P. Moran.** 1991. Genetic evidence for interaction of σ^E with the *spoIIID* promoter in *Bacillus subtilis*. J. Bacteriol. **173**:7828–7833.
53. **Williams, K. P., G. A. Kassavetis, and E. P. Geiduschek.** 1987. Interaction of the bacteriophage T4 gene 55 product with *Escherichia coli* RNA polymerase. J. Biol. Chem. **262**:12365–12371.
54. **Youngman, P., J. B. Perkins, and R. Losick.** 1984. Construction of a cloning site near one end of Tn*917* into which foreign DNA may be inserted without affecting transposition in *Bacillus subtilis* or expression of the transposon-borne *erm* gene. Plasmid **12**:1–9.
55. **Zhang, B., R. Daniel, J. Errington, and L. Kroos.** 1997. *Bacillus subtilis* SpoIIID protein binds to two sites in the *spoVD* promoter and represses transcription by σ^E RNA polymerase. J. Bacteriol. **179**:972–975.
56. **Zhang, J., H. Ichikawa, R. Halberg, L. Kroos, and A. I. Aronson.** 1994. Regulation of the transcription of a cluster of *Bacillus subtilis* spore coat genes. J. Mol. Biol. **240**:405–415.
57. **Zhang, J. P., and S. Normark.** 1996. Induction of gene expression in *E. coli* after pilus-mediated adherence. Science **273**:1234–1236.
58. **Zheng, L., R. Halberg, S. Roels, H. Ichikawa, L. Kroos, and R. Losick.** 1992. Sporulation regulatory protein GerE from *Bacillus subtilis* binds to and can activate or repress transcription from promoters for mother-cell-specific genes. J. Mol. Biol. **226**:1037–1050.

Chapter III

Characterization of the Mechanism by which σ^K Negatively Regulates *sigE* Transcription during Sporulation of *Bacillus subtilis*

ABSTRACT

Temporal and spatial gene regulation during *B. subtilis* sporulation involves the activation and inactivation of multiple sigma subunits of RNA polymerase in a cascade. It was shown previously that transcription of the *sigE* gene encoding the earlier-acting sigma factor σ^E is negatively regulated by the later-acting sigma factor σ^K in the mother cell compartment of sporulating cells. Here, it is shown that σ^K does not do so by increasing the level or activity of SinR, which is an inhibitor of *sigE* transcription. A null mutation in *sinR* did not change the expression of *sigE-lacZ* during sporulation in otherwise wild-type cells, in *sigK* mutant cells, or in cells engineered to produce σ^K earlier than normal. A mutation in the *spo0A* gene that bypasses the phosphorelay leading to the phosphorylation of Spo0A partially relieved the negative effect of σ^K on *sigE* transcription. This suggests that σ^K affects the phosphorylation of Spo0A, an activator of *sigE* transcription. σ^K also affected expression of the Spo0A-independent alanine dehydrogenase promoter late in sporulation. This promoter, like the *sigE* promoter, is thought to be recognized by σ^A RNA polymerase, suggesting that σ^K inhibits σ^A activity. In contrast, σ^K did not inhibit σ^H-dependent gene expression as strongly. We propose that the product(s) of a σ^K-dependent gene(s) lowers the level of phosphorylated Spo0A and that σ^K competes with σ^A for binding to core RNAP. By negatively regulating both positive factors required for *sigE* transcription, the appearance of σ^K would facilitate the switch from early σ^E-directed gene expression to late σ^K-directed gene expression in the mother cell compartment of sporulating *B. subtilis*.

INTRODUCTION

In response to nutrient depletion, *Bacillus subtilis* undergoes a developmental process that culminates with the formation of a dormant spore (137). Two compartments, the mother cell and the forespore, are formed early during the sporulation process due to the formation of an asymmetric septum. The forespore is later engulfed within the mother cell, being completely surrounded by the two membranes of the septum. The mother cell contributes to the synthesis of many components necessary for forespore maturation, including a thick layer of peptidoglycan called cortex and a tough proteinaceous spore coat, and is discarded by lysis at the end of sporulation, releasing the mature spore.

Sporulation involves highly ordered programs of gene expression in the two compartments that are regulated primarily by the ordered appearance of two series of alternate sigma factors (68, 137). Upon starvation, multiple signals impinge on a phosphorelay system composed of kinases, phosphotransferases, and phosphatases (16, 34, 104). The result is an elevated level of phosphorylated Spo0A (Spo0A~P), a transcription factor that activates σ^A RNA polymerase (RNAP) and σ^H RNAP to transcribe the genes encoding σ^E and σ^F, respectively (10, 12). After formation of the asymmetric septum, σ^F becomes active in the forespore and directs transcription of the gene encoding σ^G (42, 72, 93). Similarly, σ^E becomes active in the mother cell and directs transcription of the gene encoding σ^K (23, 42, 103, 141).

Communication between the mother cell and the forespore compartments regulates sigma factor activity. All the compartment-specific sigma factors are initially inactive. In the forespore, σ^F and perhaps σ^G are held inactive by an anti-sigma factor, SpoIIAB (26, 66, 95). In the mother cell, σ^E and σ^K are first synthesized as inactive precursor proteins, pro-σ^E and pro-σ^K (18, 74, 89). Compartmentalized activation of these sigma factors,

except for σ^F, depends on intercompartmental signal transduction (137). In this way, the programs of gene expression in the two compartments are coupled. In addition to controlling the synthesis and activation of subsequent sigma factors in the cascade, each σ directs core RNAP to transcribe different genes whose products drive morphogenesis (137).

Although the synthesis and activation of sigma factors during *B. subtilis* sporulation has been relatively well-studied, little is known about how later sigma factors replace the earlier ones. We showed previously that in the mother cell compartment, the appearance of σ^K accelerates the disappearance of σ^E and facilitates the switch of gene expression from the σ^E-dependent pattern to the σ^K-dependent pattern (157). In mutants that fail to produce σ^K, a *sigE-lacZ* transcriptional fusion is overexpressed late in sporulation. In mutants that produce σ^K earlier than normal, *sigE-lacZ* expression is reduced. These results suggest that σ^K negatively regulates *sigE* transcription. Transcription of *sigE* is carried out by σ^A RNAP, and is activated by Spo0A~P (12, 65, 120) and repressed by SinR (91, 92). We show here that σ^K does not affect the activity of SinR. σ^K, or, more likely, the product of a gene(s) under its control, does appear to inhibit the phosphorylation of Spo0A. σ^K also appears to inhibit σ^A activity, perhaps by competing directly for binding to core RNAP.

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* strains used in this study are listed in Table 1. To introduce gene fusions and mutations into the wild-type strain PY79 and its derivatives BK556, VO48 and BZ536, chromosomal DNA was prepared from a strain containing the desired fusion or mutation, and used to transform competent cells of the recipient strain (43). Transformants were selected on LB plates containing appropriate antibiotics. Chloramphenicol was used at 5 μg/ml and spectinomycin was used at 100 μg/ml. Resistance to macrolide-lincosamide-streptogramin B (MLS) antibiotics, encoded by Tn*917*, was selected by using a combination of erythromycin (1 μg/ml) and lincomycin (25 μg/ml). Colonies of cells containing the *sinR* null mutation displayed a characteristic "rough" phenotype (30). The *rvtA11* mutation in AG919 is 80-90% linked by co-transformation to a downstream chloramphenicol resistance gene marker (35). To verify the presence of the *rvtA11* mutation in a chloramphenicol-resistant transformant, chromosomal DNA was used to transform competent AG1431 cells. DNA from isolates containing the *rvtA11* mutation rescued the Spo^- and Pig^- AG1431 cells to Spo^+ and Pig^+ at a frequency of 80-90%. Specialized transduction was used to move *lacZ* fusions carried on SPβ phages into various strains (43).

Cell growth and sporulation. Sporulation was induced by resuspending growing cells in SM medium as described previously (43). The onset of sporulation (T_0) is defined as the time of resuspension. The sporulation efficiency was measured as described (43). For experiments involving the induction of P_{spac}-P_{sigK}-*sigKΔ19* in strain BZ536 during vegetative growth, cells were first grown overnight (12-14 h) in LB medium at 37 °C. The overnight culture was used to inoculate fresh LB medium to an optical density (OD) at 600 nm of 0.05. The culture was divided into aliquots of equal volume

Table 1. *B. subtilis* strains used.

Strain	Relevant Genotype	Sources
PY79	wild type	(154)
BK556[a]	*spoIVCB23*	(70)
VO48[a]	*spoIVCBΔ19 cat*	(18)
BZ536[a]	P_{spac}-P_{sigK}-*sigKΔ19 spc*	(157)
AG919[b]	*rvtA11 cat*	(35)
KI220[b]	*ald::*Tn917*lac MLS*	(128)
KI1261[b]	*amyE::spo0A-lacZ cat*	(52)
AG1431[b]	*spo0FΔ, spo0BΔ*	(35)
IS432[c]	*sinR cat*	(30)
ZB307[b]	SPβ::*spoVG42-lacZ*	(160)
DZR67[b]	*amyE::spo0K-lacZ cat*	A. Grossman
KH566[b]	*spo0H-lacZ cat*	A. Grossman
KI1202[b]	SPβ::*spoIIA-lacZ*	A. Grossman, (150)
KY9[b]	SPβ::*spoIIE-lacZ*	(36)

a, derived from PY79.
b, derived from JH642.
c, derived from IS75.
Abbreviations: *cat,* chloramphenicol resistance; *spc*, spectinomycin resistance; MLS, resistance to macrolide-lincosamide-streptogramin B antibiotics.

when it reached an OD_{600} of 0.3-0.5. Different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) were then added to the aliquots. Incubation was continued and samples were taken every half hour.

β-galactosidase assays. ß-galactosidase activity was assessed qualitatively by placing cells on DSM agar (43) containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (20 μg/ml) and was determined quantitatively using toluene to permeabilize cells and *o*-nitrophenol-*β*-D-galactopyranoside as the substrate (43). One unit of enzyme hydrolyses 1 μmol of substrate per minute per A_{600} of initial cell density.

RESULTS AND DISCUSSION

σ^K does not inhibit *sigE* transcription by increasing the level or activity of SinR. The gene that encodes pro-σ^E, *sigE* (also called *spoIIGB*), is the second gene in the *spoIIG* operon (59, 64). The first gene of the operon, *spoIIGA*, encodes a putative protease that processes pro-σ^E to σ^E (48, 110, 135). Using a transcriptional fusion between the *spoIIG* promoter and *lacZ* (64), which we referred to as *sigE-lacZ* since it provided an indirect measure of *sigE* transcription, we showed previously that σ^K appears to negatively regulate transcription of *sigE* (157). In *spoIVCB23* (*spoIVCB* encodes the N-terminal part of σ^K) mutant cells that fail to produce σ^K, *sigE-lacZ* was overexpressed late in sporulation. In *spoIVCBΔ19* cells that make active σ^K 1 h earlier than normal due to a deletion in the pro-sequence of pro-σ^K, *sigE-lacZ* expression was reduced. To further explore the mechanism by which σ^K inhibits *sigE* transcription, we introduced additional mutations into the mutants with altered σ^K production and measured *sigE-lacZ* expression.

The first mutation we tested in this way was a null mutation in *sinR* (30). SinR is a transcription factor that inhibits the transcription of some early sporulation genes, including *sigE* (91, 92). A simple mechanism by which σ^K might decrease the transcription of *sigE* is by increasing the level or activity of SinR. To test this hypothesis, we introduced a *sinR* null mutation into wild type cells, and *spoIVCB23* and *spoIVCBΔ19* mutant cells. We then introduced the *sigE-lacZ* transcriptional fusion carried on an SPβ phage into the chromosome of each strain via specialized transduction. Expression of *sigE-lacZ* was monitored in these strains by measuring the β-galactosidase activity of samples collected at hourly intervals during sporulation.

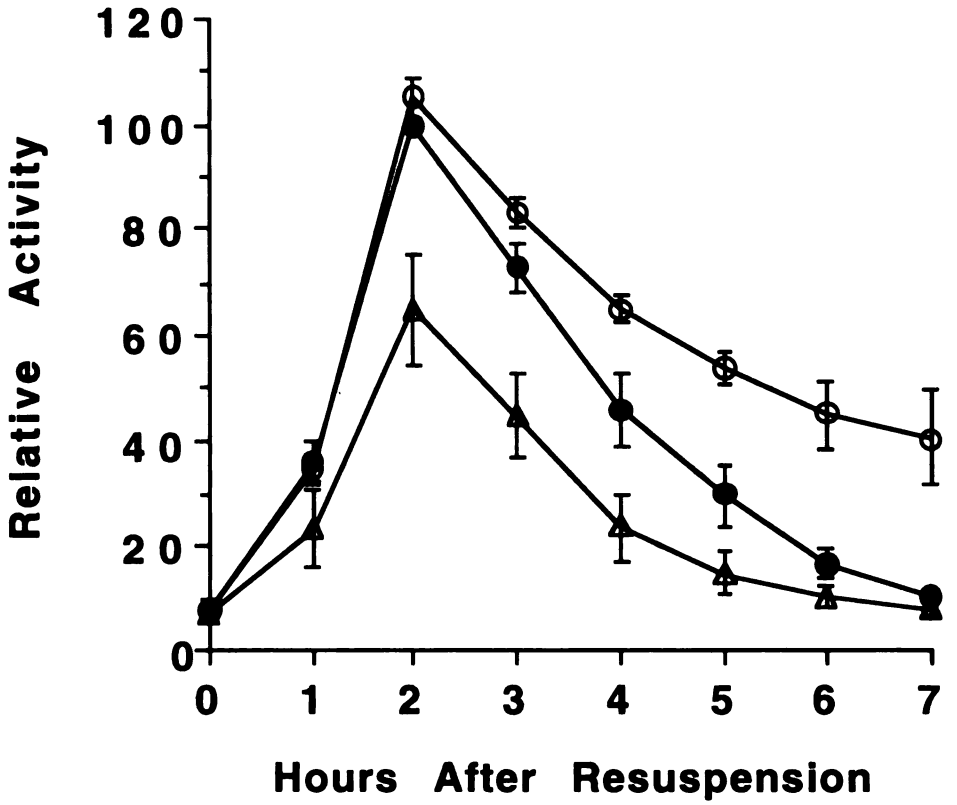


Figure 1. The effect of a *sinR* null mutation on *sigE-lacZ* expression. The *sinR* mutation was introduced into wild-type (PY79; ●) cells, *sigK* (*spoIVCB23*) mutant cells (BK556, ○), and *spoIVCBΔ19* (VO48, Δ) cells that produce σ^K earlier than normal. The resulting strains were lysogenized with phage SP*ß::sigE-lacZ* and induced to sporulate in SM media. Samples were collected at the indicated times following the initiation of sporulation and assayed for ß-galactosidase activity. Experiments were performed at least twice for each strain. For each experiment, the specific activities were normalized to the maximum specific activity in cells containing the *rvtA11* mutation in the wild-type PY79 background (typically 130 units). Points on the graph are averages of the normalized values and error bars show one standard deviation of the data.

As shown in Figure 1, the pattern of *sigE-lacZ* expression was preserved in these strains relative to the parental strains without the *sinR* mutation (157). In cells containing only the *sinR* mutation, *sigE-lacZ* expression increased and decreased with similar timing as in wild-type cells, but reached a 2-fold higher maximum level [130 units versus 70 units (157)], consistent with the finding reported previously that SinR inhibits *sigE* expression (91, 92). In *sinR spoIVCB23* mutant cells that fail to make σ^K, *sigE-lacZ* expression was higher late in sporulation (i.e. at T_3 - T_7) than in cells containing only the *sinR* mutation. In *sinR spoIVCBΔ19* cells that make σ^K earlier than normal, *sigE-lacZ* expression was reduced at T_2 - T_5 compared to that in *sinR* mutant cells. Thus, *sinR* is not required for the negative effect of σ^K on *sigE* transcription. We conclude that σ^K does not affect the transcription of *sigE* by increasing the level or activity of SinR.

Bypassing the phosphorelay leading to the activation of Spo0A partially relieves the negative effect of σ^K on *sigE* transcription. The second mutation we tested for an effect on the σ^K-dependent inhibition of *sigE* transcription was a missense mutation in *spo0A* called *rvtA11* (124). At the onset of sporulation, multiple signals activate a milticomponent phosphorelay system to phosphorylate Spo0A (34, 46, 104). Only after it is phosphorylated can Spo0A activate the transcription of *sigE* and other early sporulation genes (10, 12). The *rvtA11* mutation bypasses the need for the phosphorelay and renders Spo0A able to be phosphorylated by an alternate kinase (76). If σ^K inhibits *sigE* transcription by affecting a component of the phosphorelay so as to lower the level of Spo0A~P, then the *rvtA11* mutation might bypass this effect and relieve the *rvtA11* mutation was introduced into wild-type (PY79; ●) cells, *sigK* (*spoIVCB23*) mutant cells (BK556, ○), and *spoIVCBΔ19* (VO48, Δ) cells that produce σ^K earlier than normal. The resulting strains were lysogenized with phage SP*β*::*sigE-lacZ* and induced to sporulate

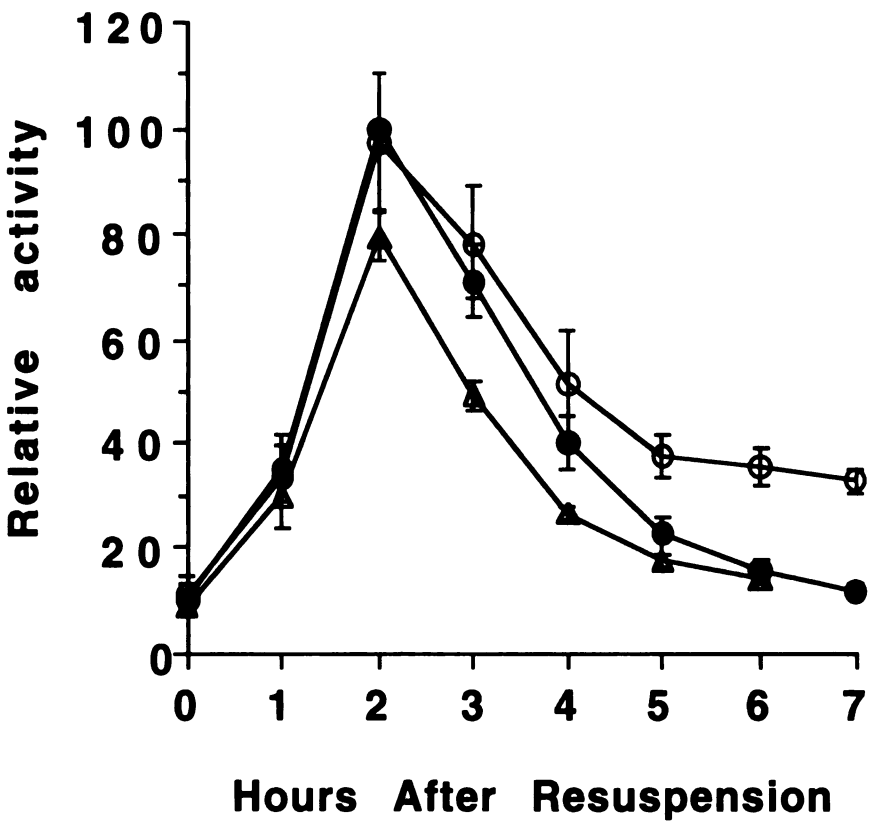


Figure 2. The effect of bypassing the phosphorelay on *sigE-lacZ* expression. The in SM media. Samples were collected at the indicated times following the initiation of sporulation and assayed for ß-galactosidase activity. Experiments were performed at least twice for each strain. For each experiment, the specific activities were normalized to the maximum specific activity in cells containing the *rvtA11* mutation in the wild-type PY79 background (typically 70 units). Points on the graph are averages of the normalized values and error bars show one standard deviation of the data.

inhibition of *sigE* transcription by σ^K.

We introduced the *rvtA11* mutation into wild-type cells, and *spoIVCB23* and *spoIVCBΔ19* mutant cells. The *sigE-lacZ* transcriptional fusion, carried on phage SPβ, was then integrated into the chromosomes of these strains. *sigE-lacZ* expression was first examined by allowing the strains to sporulate on DSM agar containing X-gal. The pattern of expression was preserved to some extent as judged by the intensity of blue color of colonies (data not shown). That is, expression appeared to be highest in *rvtA11 spoIVCB23* cells, lower in cells containing only the *rvtA11* mutation, and lowest in *rvtA11 spoIVCBΔ19* cells. However, the differences did not appear to be as great as when the corresponding strains without the *rvtA11* mutation were examined on DSM agar containing X-gal (data not shown). To test these qualitative observations more carefully, the *rvtA11* mutant strains were induced to sporulate in SM liquid and the β-galactosidase activity was quantitatively assayed. In agreement with the qualitative observations, Figure 2 shows that the pattern of *sigE-lacZ* expression was preserved in these strains, but the differences were not as great as for the parental strains without the *rvtA11* mutation (157) or the strains with the *sinR* mutation (Figure 1). For example, *sigE-lacZ* expression in *rvtA11 spoIVCBΔ19* cells reached 80% of the maximum level observed (at T_2) in cells containing only the *rvtA11* mutation, whereas *sigE-lacZ* expression in *spoIVCBΔ19* cells reached only 55% of the maximum level observed in wild-type cells (157), and expression in *sinR spoIVCBΔ19* cells reached only 65% of the maximum observed in cells containing only the *sinR* mutation. Thus, the negative effect of σ^K on *sigE* expression was partially relieved by bypassing the phosphorelay, suggesting that σ^K negatively regulates the activity of Spo0A by interfering with the phosphorelay.

How might σ^K affect the phosphorylation of Spo0A? The phosphorelay is a complicated variation of the two-component signal transduction system (34, 46). Two

independent histidine kinases, KinA and KinB, phosphorylate a response regulator, Spo0F. The phosphate group of Spo0F~P is then transferred to another response regulator, Spo0A, by the phosphotransferase Spo0B. Spo0F~P can also be dephosphorylated by a family of phosphatases (104). The phosphorelay provides many regulatory sites to integrate a large variety of intracellular and extracellular signals that regulate sporulation initiation. The *rvtA11* mutation in *spo0A* bypasses the need for the phosphorelay (124). The observation that the negative effect of σ^K on *sigE* transcription is partially relieved in the presence of the *rvtA11* mutation (Figure 2) suggests that σ^K targets a component(s) of the phosphorelay and/or a Spo0F~P phosphatase(s). It seems likely that this is an indirect effect of σ^K due to the transcription of one or more σ^K-dependent genes.

Clearly though, the *rvtA11* mutation did not completely relieve the inhibition of *sigE* transcription by σ^K. Compared to cells containing only the *rvtA11* mutation, cells that in addition contained a *spoIVCB23* mutation and therefore failed to make σ^K overexpressed *sigE-lacZ* at T_5 - T_7, whereas *rvtA11 spoIVCBΔ19* cells that make σ^K earlier than normal exhibited slightly reduced *sigE-lacZ* expression at T_2 - T_4 (Figure 2). This implies that σ^K can also affect Spo0A~P activity in a way that is not bypassed by the *rvtA11* mutation and/or that σ^K affects σ^A RNAP activity, since σ^A RNAP transcribes the *sigE* gene (12, 65).

Expression of a Spo0A-independent gene remains high late during sporulation in *sigK* mutant cells. We next examined whether σ^K negatively regulates expression of a Spo0A-independent gene. For this purpose, we chose a *lacZ* fusion created by insertion of Tn*917lac* into the *ald* gene (128), which encodes alanine dehydrogenase. Like expression of *sigE-lacZ*, expression of *ald*::Tn*917lac* increases at the onset of sporulation and transcription is thought to be directed by σ^A RNAP (128).

Induction of *ald* transcription has been postulated to involve an unidentified regulatory factor(s), but does not require Spo0A~P (128).

The *ald* locus of wild-type cells and *spoIVCB23* and *spoIVCBΔ19* mutant cells was replaced with *ald*::Tn*917lac* by transformation with chromosomal DNA from KI220 (128). The strain containing *ald*::Tn*917lac* in an otherwise wild-type background sporulated poorly in DS medium, consistent with the previous report (128). However, the sporulation efficiency of this strain was comparable to that of the wild-type strain in SM resuspension medium (data not shown). Apparently, the *ald* locus is dispensable for sporulation in SM medium. Therefore, the strains containing *ald*::Tn*917lac* were sporulated in SM medium and expression of *ald-lacZ* was measured by determining the β-galactosidase activity of samples collected at hourly intervals. As shown in Figure 3, there was no difference in the level of *ald-lacZ* expression in the strains early during sporulation, but expression remained high late during sporulation of *spoIVCB23* mutant cells that fail to make σ^K. Thus, σ^K inhibits expression of the Spo0A-independent *ald-lacZ* fusion in wild-type cells late during sporulation. σ^K also inhibits *sigE-lacZ* expression in late sporulating cells (157), and this effect does not require SinR (Figure 1) and is not completely bypassed by the *rvtA11* mutation in *spo0A* (Figure 2). Since *ald* is thought to be transcribed by σ^A RNAP and *sigE* is known to be, we propose that σ^K inhibits the expression of both genes late during sporulation by inhibiting the activity of σ^A RNAP. Perhaps σ^K competes directly with σ^K for binding to core RNAP.

The finding that expression of the Spo0A-independent *ald-lacZ* fusion was not reduced in *spoIVCBΔ19* cells compared to wild-type cells (Figure 3) is in striking contrast to the result with the Spo0A-dependent *sigE-lacZ* fusion (157) (Figure 1). Taken together, these results suggest that the inhibition of *sigE-lacZ* expression observed when σ^K is produced earlier than normal (157) (Figure 1) is due to an effect on Spo0A~P activity.

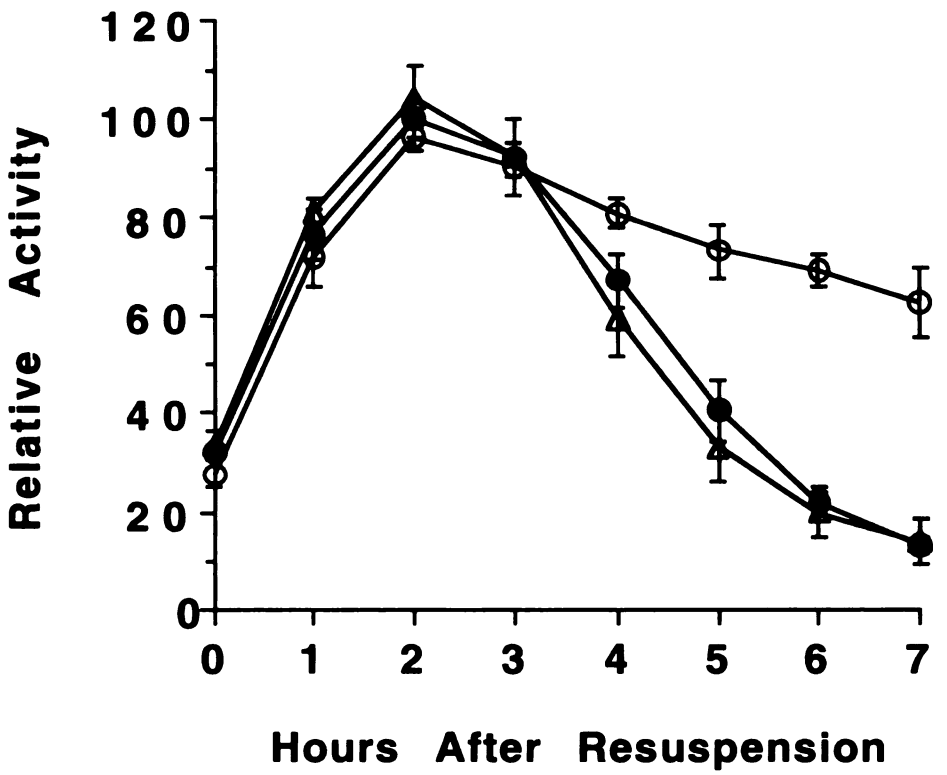
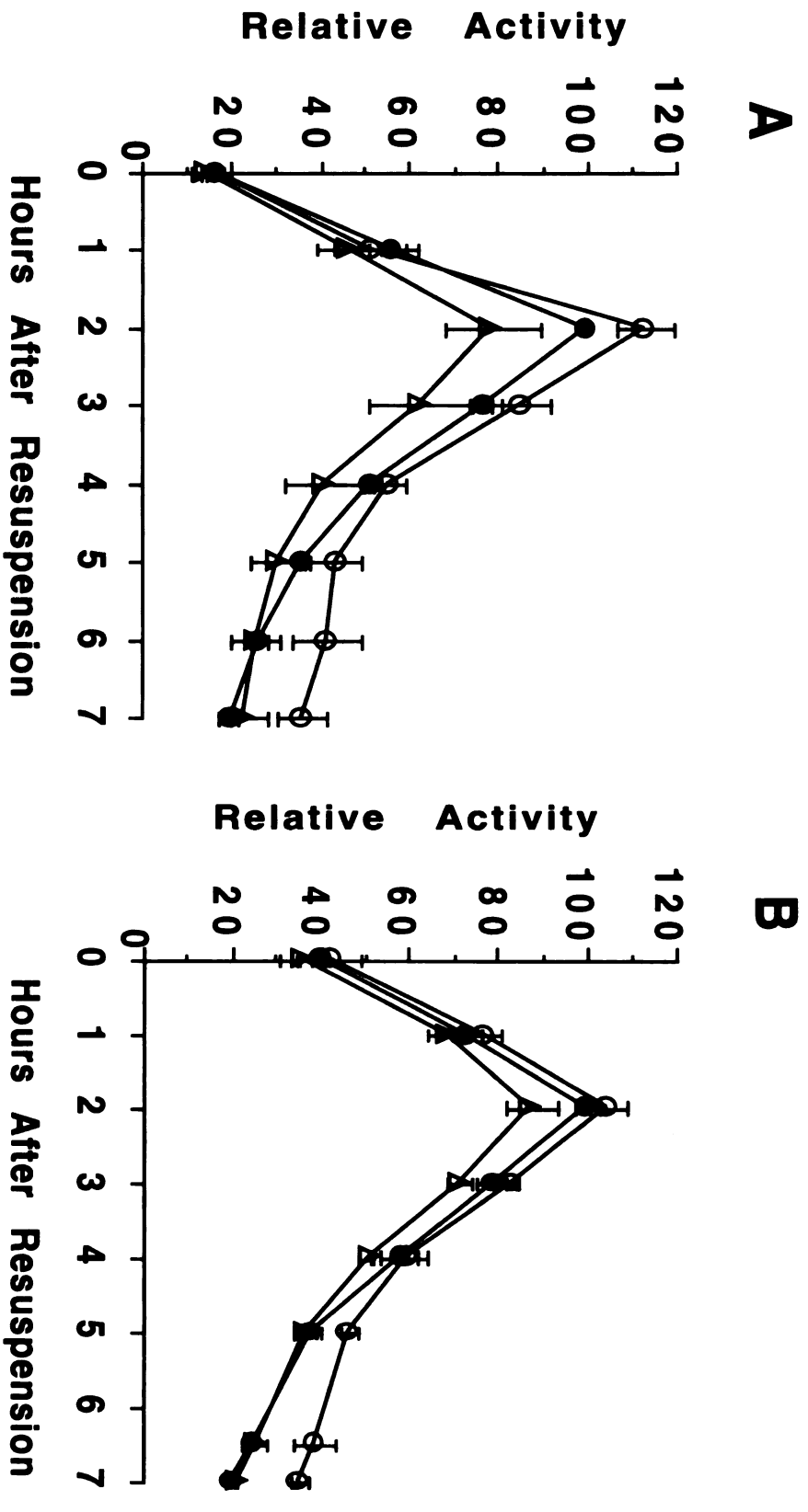


Figure 3. The effects of altered σ^K production on *ald-lacZ* expression. The *ald* locus of wild-type (PY79; ●) cells, *sigK* (*spoIVCB23*) mutant cells (BK556, ○), and *spoIVCBΔ19* (VO48, Δ) cells that produce σ^K earlier than normal was replaced with *ald*::Tn*917lac*. The resulting strains were induced to sporulate in SM media. Samples were collected at the indicated times following the initiation of sporulation and assayed for ß-galactosidase activity. Experiments were performed at least twice for each strain. For each experiment, the specific activities were normalized to the maximum specific activity in wild-type cells (typically 300 units). Points on the graph are averages of the normalized values and error bars show one standard deviation of the data.

This hypothesis is also supported by the finding that the *rvtA11* mutation in *spo0A* diminished the effect of earlier σ^K production (Figure 2).

σ^K has little effect on expression of σ^H-dependent genes. Does σ^K also exert a negative effect on σ^H-dependent gene expression? σ^H is an alternate sigma factor active during growth and with increased activity during sporulation (44). At the onset of sporulation, σ^H transcribes the *spoIIA* operon , which encodes σ^F, the early-acting, forespore-specific sigma factor (23, 42, 148, 150). Since σ^F does not become active in the mother cell, it may not be important for σ^K to negatively regulate the transcription of the *spoIIA* operon. On the other hand, some degree of inhibition was expected because expression of *spoIIA,* like expression of *sigE*, is positively regulated by Spo0A-P (15, 108, 150). For comparison, we also measured expression of the *spoVG42* promoter. This promoter, like that of the *spoIIA* operon, is σ^H-dependent, but the *spoVG42* mutation renders the promoter independent of the activity of the AbrB repressor (160), and therefore independent of Spo0A~P activity (Spo0A~P is a repressor of *abrB* transcription). Figure 4A shows that expression of a *spoIIA-lacZ* fusion was slightly higher in the *spoIVCB23* mutant that fails to make σ^K, and slightly lower in the *spoIVCBΔ19* mutant that makes σ^K earlier, compared to expression in wild-type cells. The differences in expression of *spoIIA-lacZ* in mutants with altered σ^K production were smaller than the differences in expression of *sigE-lacZ* (157) (Figure 1). These small differences may be due to the negative effect of σ^K on the phosphorelay that activates Spo0A, and/or on activity of σ^H RNAP. As shown in Figure 4B, the Spo0A-independent *spoVG42-lacZ* fusion exhibited very small difference in expression in mutants with altered σ^K production. We conclude that σ^K has less of a negative effect on expression of σ^H-dependent genes during

Figure 4. The effects of altered σ^K production on *spoIIA-lacZ* and *spoVG42-lacZ* expression. Wild-type (PY79; ●) cells, *sigK* (*spoIVCB23*) mutant cells (BK556, ○), and *spoIVCBΔ19* (VO48, Δ) cells that produce σ^K earlier than normal, were lysogenized with phage SP*β::spoIIA-lacZ* and SP*β::spoVG42-lacZ*, respectively. The resulting strains were induced to sporulate in SM media. Samples were collected at the indicated times following the initiation of sporulation and assayed for ß-galactosidase activity. Experiments were performed at least twice for each strain. Specific activities for SP*β::spoIIA-lacZ* (A) or SP*β::spoVG42-lacZ* (B) were normalized to the maximum specific activity in wild-type cells (typically 200 units for the *spoIIA-lacZ* fusion and 350 units for the *spoVG42-lacZ* fusion. Points on the graph are averages of the normalized values and error bars show one standard deviation of the data.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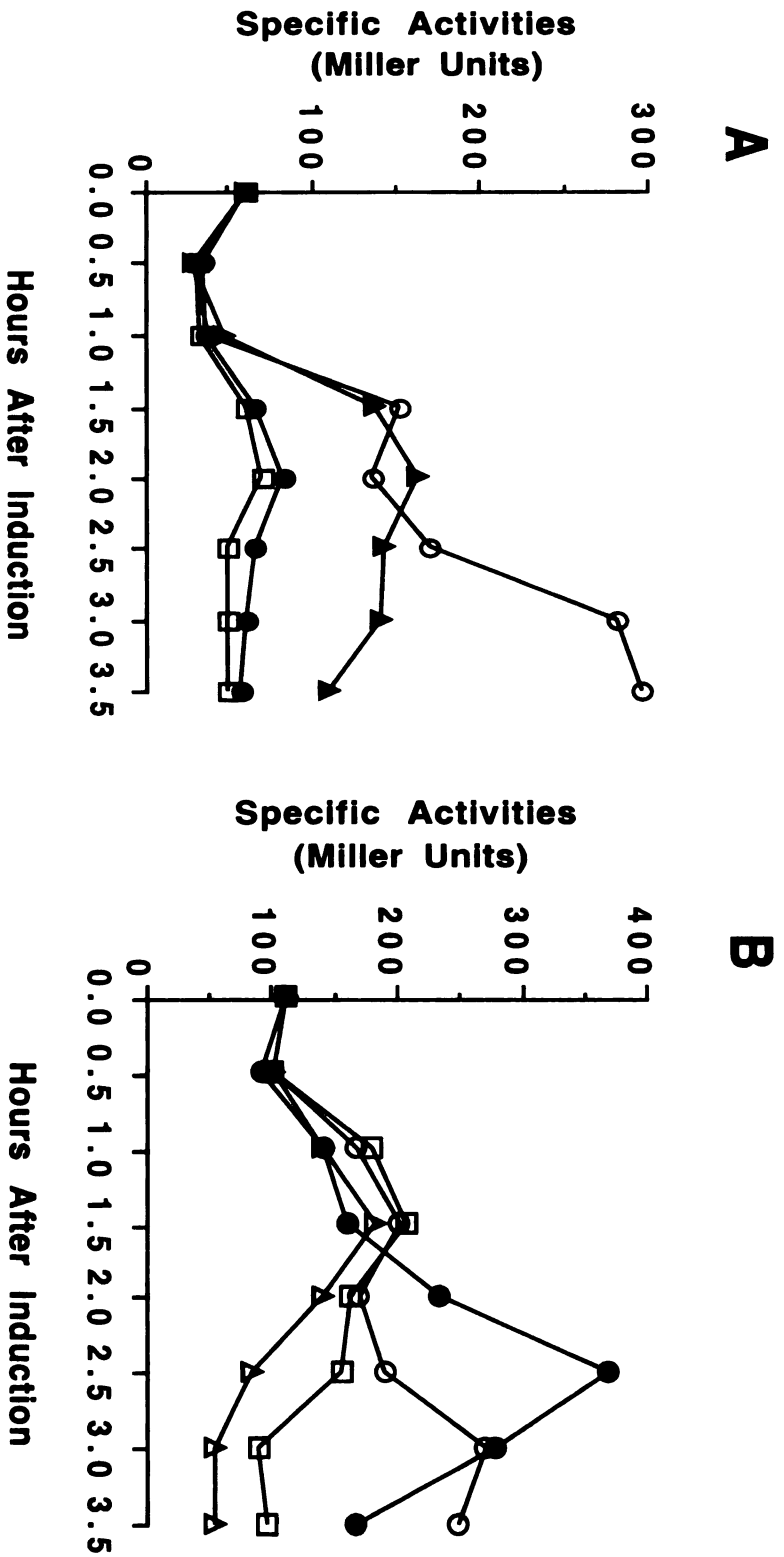
sporulation than it has on expression of the σ^A-dependent *sigE* gene (157) (Figure 1), or the *ald* gene (Figure 3), which is thought to be σ^A-dependent.

Effect of inducing σ^K during vegetative growth on expression of *ald* and *spoVG42*. Expression of the *spoVG42-lacZ* fusion was relatively insensitive to the appearance of σ^K in late sporulating cells (Figure 4B), compared with expression of *ald-lacZ* (Figure 3). Since both these fusions are expressed during vegetative growth, we examined the effect on expression of making different amounts of σ^K using P_{spac}-P_{sigK}-*sigKΔ19* mutant cells (99, 157). The P_{spac}-P_{sigK}-*sigKΔ19* mutant contains in its chromosome the IPTG-inducible promoter, *spac*, fused to a copy of the *sigK* gene (*sigKΔ19*) that permits production of active σ^K without the need for the site-specific recombination event that normally joins the two parts (i.e., *spoIVCB* and *spoIIIC*) of the *sigK* gene and without the need for processing (99). Thus, P_{spac}-P_{sigK}-*sigKΔ19* cells produce σ^K upon the addition of IPTG (40, 99).

We induced different levels of σ^K production in P_{spac}-P_{sigK}-*sigKΔ19* cells during vegetative growth by adding to cultures different concentrations of IPTG, and monitored the expression of *ald-lacZ* (*ald*::Tn917*lac*) and *spoVG42-lacZ* (carried on an SPβ phage) fusions. At a low concentration of IPTG (25 μM), *ald-lacZ* expression was reduced after 3 h of induction (Fig. 5A), compared to the parallel culture without IPTG addition. At higher concentrations of IPTG (50 or 100 μM), the *ald-lacZ* expression was reduced after 1.5 h of induction (Figure 5A). As noted above, the *ald* gene is thought to be transcribed by σ^A RNAP. Therefore, we examined the expression of several genes that are known to be transcribed by σ^A RNAP during vegetative growth, including *spo0K, spo0A,* and

Figure 5. The effect of making σ^K during vegetative growth on *ald-lacZ* and *spoVG42-lacZ* expression. The *ald*::Tn*917* and SP*β*::*spoVG42-lacZ* fusions were introduced into P_{spac}-P_{sigK}-*sigKΔ19* (BZ536) cells by transformation and transduction, respectively. Cells were grown in LB medium to early exponential phase. The cultures were divided into aliquots of equal volume, and IPTG was added to some of the aliquots to induce σ^K production. Incubation was continued and samples were taken every half hour for *β*-galactosidase assay. (A) Expression of *ald-lacZ*. IPTG concentrations were: 0 μM (○), 25 μM (▲), 50 μM (●), and 100 μM (❑). (B) Expression of *spoVG42-lacZ*. IPTG concentrations were: 0 μM (○), 50 μM (●), 100 μM (❑), and 200 μM (Δ). Experiments were performed at least twice for each strain, and data from a representative experiment are shown.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(Miller Units)
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Hours After Induction
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Hours After Induction

spo0H. Expression of *lacZ* fusions to these genes was reduced within 1.5 h after adding 1 mM IPTG to P_{spac}-P_{sigK}-*sigKΔ19* cells, compared with the corresponding strains without IPTG addition (data not shown). Thus, σ^K production in vegetative cells appears to exert a general negative effect on σ^A-dependent gene expression.

Expression of the σ^H-dependent *spoVG42-lacZ* fusion was also reduced when σ^K production was induced in P_{spac}-P_{sigK}-*sigKΔ19* cells; however, a higher concentration of IPTG (100 μM IPTG) and a longer induction time (approximately 3 h) were required (Figure 4B). Thus, it appears that a higher level of σ^K is required to inhibit transcription of the *spoVG42* promoter than the *ald* promoter. Perhaps the level of σ^K produced late in sporulation is sufficient to inhibit *ald* expression (Figure 3) but insufficient to inhibit *spoVG42* expression (Figure 4B). It is possible that the inhibition of *spoVG42* expression observed in vegetative cells (Figure 4B) is due to the negative effect of σ^K on σ^A-dependent gene expression, because the gene encoding σ^H, *spo0H,* is σ^A-dependent, and its expression was reduced after σ^K production was induced (data not shown).

We propose that σ^K inhibits σ^A-dependent gene expression in growing and sporulating *B. subtilis* cells by competing with σ^A for binding to core RNAP. This hypothesis assumes that the amount of core RNAP available for binding to sigma factors is limiting. Core RNAP does appear to be limiting in growing cells because competition has been demonstrated between σ^H and σ^A (45). In sporulating cells, the level of σ^A remains high, but its ability to associate with core RNAP is diminished early during sporulation (81, 143), (data not shown). Perhaps the mother-cell-specific sigma factors, σ^E and σ^K, have a higher affinity than σ^A for binding to core RNAP. This can be tested by

competition experiments in vitro. Alternatively, the products of genes under σ^E and σ^K control might prevent σ^A from associating with core RNAP.

In summary, we have presented evidence that σ^K directly or indirectly inhibits the activity of both positive regulators of *sigE* transcription, Spo0A~P and σ^A RNAP. Hence, the appearance of σ^K both turns on late mother cell gene expression and turns off early σ^E-directed gene expression.

ACKNOWLEDGMENTS

We are very grateful to A. Grossman, I. Smith, P. Zuber, P. Youngman, for providing *B. subtilis* strains. This research was supported by the Michigan Agricultural Experiment Station, by grant GM43585 from the National Institutes of Health.

Chapter IV

The Pro-sequence of Pro-σ^K Promotes Membrane Association and Inhibits RNA Polymerase Core Binding

ABSTRACT

Pro-σ^K is the inactive precursor of σ^K, a mother-cell-specific sigma factor responsible for the transcription of late sporulation genes of *Bacillus subtilis*. Upon subcellular fractionation, the majority of the pro-σ^K was present in the membrane fraction. The rest of the pro-σ^K was in a large complex that did not contain RNA polymerase core subunits. In contrast, the majority of the σ^K was associated with core RNA polymerase. Virtually identical fractionation properties were observed when pro-σ^E was analyzed. Pro-σ^K was completely solubilized from the membrane fraction and the large complex by Triton X-100, and was partially solubilized from the membrane fraction by NaCl and KSCN. The membrane-association of pro-σ^K did not require *spoIVF* gene products, which appear to be located in the mother cell membrane that surrounds the forespore, and govern pro-σ^K processing in the mother cell. Furthermore, pro-σ^K associated with the membrane when overproduced in vegetative cells. Overproduction of pro-σ^K in sporulating cells resulted in more pro-σ^K in the membrane fraction. In agreement with the cell fractionation studies, immunofluorescence microscopy showed that pro-σ^K was localized to the mother cell membranes that surround the mother cell and the forespore in sporulating wild-type cells and mutant cells that do not process pro-σ^K. Treatment of extracts with 0.6 M KCl appeared to free most of the pro-σ^K and σ^K from other cell constituents. After salt removal, σ^K, but not pro-σ^K, reassociated with exogenous core RNA polymerase to form holoenzyme. These results suggest that the pro-sequence inhibits RNA polymerase core binding and targets pro-σ^K to the membrane, where it may interact with the processing machinery.

INTRODUCTION

Endospore formation in the Gram-positive bacterium *Bacillus subtilis* involves the formation of two cellular compartments of unequal size. The two compartments, namely, the mother cell and the forespore, are generated by the asymmetric positioning of a septum. The smaller forespore compartment is later engulfed inside the mother cell through a phagocytic-like process. The mother cell nurtures the forespore during sporulation and is discarded by lysis upon maturation of the endospore. Gene expression in the two compartments is driven by a cascade of σ factors, namely, σ^F, σ^E, σ^G, and σ^K, in order of their activity (27, 41, 68, 86). The forespore-specific program of gene expression is controlled by σ^F and σ^G, while the mother-cell program is controlled by σ^E and σ^K. Each sigma factor is initially inactive. σ^F is the first to become active and this occurs only in the forespore. Activation of subsequent sigma factors in the cascade is triggered by signal transduction between the two compartments. The inactive forms of the mother-cell-specific sigma factors are precursor proteins called pro-σ^E and pro-σ^K. Each is synthesized about one hour before it is activated by proteolysis (18, 74, 89).

The processing of mother-cell-specific σ factors is controlled by signals from the forespore. The putative processing enzyme for the conversion of pro-σ^E to σ^E is SpoIIGA (59, 135), which receives a signal from a protein, SpoIIR, generated in the forespore under the control of σ^F (48, 61, 83). Conversion of pro-σ^K to σ^K requires SpoIVFB (18, 19, 89), which is either the processing enzyme or its regulator, and is negatively regulated by SpoIVFA and BofA (18, 19, 51, 116). SpoIVFA, SpoIVFB, and BofA appear to be integral membrane proteins (19, 115, 116), and SpoIVFA and SpoIVFB have been shown to be localized at the boundary between the mother cell and the forespore (115). Activation of SpoIVFB for pro-σ^K processing requires the production of SpoIVB under the control of

σ^G in the forespore (17, 32). SpoIVB is inferred to be a secreted protein and is presumed to overcome the inhibitory effect of SpoIVFA and BofA (17, 145).

Pro-σ^K has 20 amino acid residues at its N-terminus which must be removed to generate active σ^K (69, 89, 136). Two lines of evidence indicate that pro-σ^K is transcriptionally inactive (89). First, expression of σ^K-dependent gene fusions does not begin until processing occurs. Second, when added to core RNA polymerase (RNAP), pro-σ^K fails to direct transcription from σ^K-dependent promoters in vitro. The role of the pro-sequence in preventing transcription is not clear. One function of the pro-sequence may be to mask the DNA-binding activity of σ^K , since the affinity binding constant of purified pro-σ^K for promoter DNA is one order of magnitude lower than that of σ^K (21). The results presented here suggest additional functions of the pro-sequence. We show that the majority of pro-σ^K is membrane-associated in cell extracts, and is not associated with the core subunits of RNAP. In agreement with this observation, we find that pro-σ^K immunolocalizes to the mother cell membranes that surround the mother cell and the forespore in sporulating cells. Moreover, pro-σ^K fails to bind to core RNAP in vitro under conditions that permit σ^K binding. These results suggest that two more functions of the pro-sequence of pro-σ^K are to inhibit RNAP core binding and to promote association with the membrane, where processing may occur.

MATERIALS AND METHODS

General methods. Sporulation was induced by resuspending growing cells in SM medium as described previously (43). The onset of sporulation (T_0) is defined as the time of resuspension. The *B. subtilis* strains used in this study are: PY79 (wild-type) (154), PY79/pSL1 (89), BSL51 (*spoIVFΔAB::cat*) (90), BK183 (*spoIVA67 trpC2*) (118), RL831 (*spoIIIGΔ::neo*) (121), RL87 (*spoIVFB152*) (18) and RL136 (*spoIVFB152 spoIVCBΔ19*) (18).

Western blot analysis. Samples of different fractions equivalent to the same original volume of culture, or containing the same amount of protein, as determined by the Bradford method (13), were separated on SDS-12% Prosieve polyacrylamide gels (FMC) using Tris/Tricine electrode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS) and electroblotted to Immobilon-P membrane (Millipore). The membrane was probed with either polyclonal anti-pro-σ^K antiserum (89), anti-FtsH antiserum (a gift from S. Cutting and T. Ogura), anti-*E.coli* core RNAP antiserum (a gift from M. Chamberlin and C. Kane), or monoclonal anti-σ^E antibody (a gift from W. Haldenwang). In some experiments, the membrane was stripped and reprobed with a different antibody. Horseradish peroxidase conjugated secondary antibody was either goat-anti-rabbit IgG or goat-anti-mouse IgG (Bio-Rad). Chemiluminescence detection was performed following the manufacturer's instructions (Amersham, ECL).

Column chromatography. Mini-columns (5.5x70 mm) were made from pasteur pipettes with the narrow end cut off and sealed with glass fiber and beads. Three types of gel filtration media were used: Sephacryl S-300, Sephadex G-200, and Sephadex G-100 (Pharmacia). The flow rate was controlled by gravity and ranged from 50 to 80 µl/min. The void volume and fractionation range were determined by passing various combinations of Dextran Blue, alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic

anhydrase (29 kDa) and cytochrome C (12.4 kDa) through the columns. Usually a 100 µl sample was loaded, eluted with the same buffer, and 120 µl fractions were collected. Salt or detergent treated fractions were eluted with buffer adjusted to contain the same concentration of salt or detergent. If necessary, the column fractions were concentrated by 10% trichloroacetic acid (TCA) precipitation.

Subcellular fractionation. Figure 1 is a diagram showing the fractionation scheme used in our experiments. Cells were collected by centrifugation (5,000g), washed with 1 M NaCl, and stored at -80°C. The cell pellet was resuspended in 7.5% of the original volume in lysis buffer [25 mM Hepes-KOH (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 10% glycerol, 1 mg/ml lysozyme, 0.1 mg/ml DNase I, 20 µg/ml RNase A, 1 mM PMSF] and incubated for 10 min at 37°C. Cells were then chilled and lysed by passage through a French Pressure cell twice at 1,800 psi. The crude lysate was incubated at 30°C for 10 min. Cell debris was removed by centrifugation at 12,000g for 10 min. No nucleic acids were detected when the supernatant was analyzed by 2% agarose gel electrophoresis. The supernatant was then subjected to high speed centrifugation (200,000g) for 1.5 hour at 4°C. The pellet was homogenized in 1/5 of the lysate volume in sucrose gradient buffer [25 mM Hepes-KOH (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM PMSF] plus 5% sucrose and loaded on top of a sucrose density gradient made with 2 ml of 55% (wt/vol) and 2 ml of 25% (wt/vol) sucrose in buffer in a 5-ml ultracentrifuge tube. After centrifugation at 200,000g for 4 hr at 4°C, the membrane fraction was recovered at the interface between 25% and 55% sucrose. The supernatant (cytoplasmic fraction) after the initial high speed centrifugation (100 µl) was loaded onto a gel filtration column and eluted with lysis buffer omitting the lysozyme, DNase I and RNase A. Fractions of 120 µl were collected and analyzed by Western blotting.

In the experiments testing the effect of salt and detergent, the supernatant after low speed centrifugation was divided into six aliquots. Salt or detergent was added to different

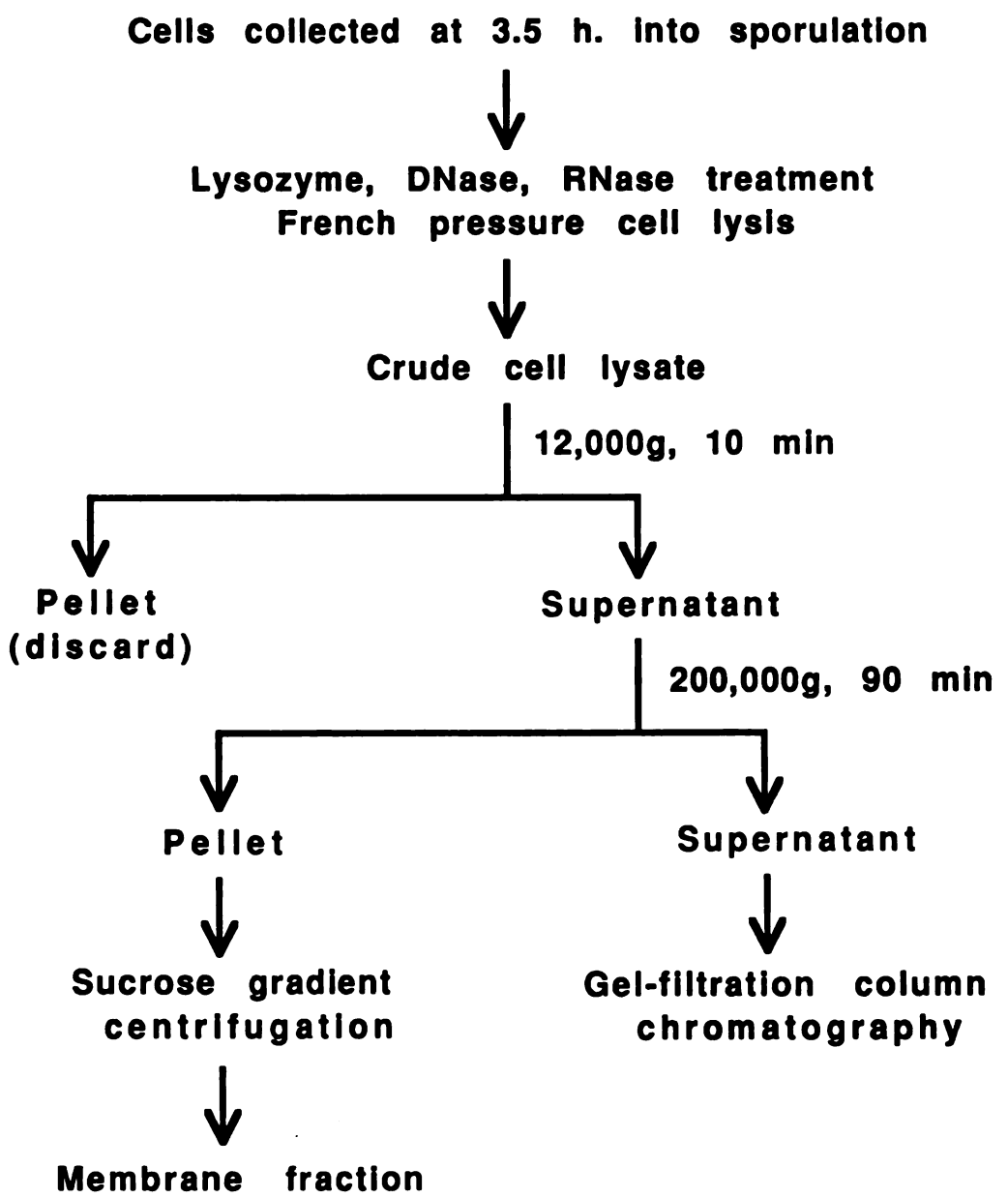


Figure 1. Diagram of subcellular fractionation of sporulating *B. subtilis* cells. See Materials and Methods for details.

final concentrations and one fraction was left untreated. All aliquots were kept for 20 min at 4°C and then subjected to high speed centrifugation as noted above. The supernatant and pellet fractions were analyzed by Western blotting.

Immunofluorescence microscopy and image processing. The affinity purified rabbit polyclonal anti-σ^K antibodies (115) were a gift of O. Resnekov, and were used at a 1:500 dilution. The secondary antibodies (Jackson Immunolabs) were affinity-purified donkey anti-rabbit antibodies conjugated to fluorescein isothiocyanite (FITC), and were used at a 1:100 dilution. DNA was stained with propidium iodide (PI; Molecular Probes) at a final concentration of 10 μg/ml. Cells were harvested 2.5 and 3.5 h after the onset of sporulation. Immunofluorescence experiments were performed as described by Pogliano et al. (112). Fluorescence micrographs were recorded using a cooled CCD camera (Princeton Instruments) and a PC with the MetaMorph imaging system (version 3.0; Universal Imaging Corp.). PI images were assigned to the red channel and FITC images to the green channel. Adobe Photoshop (version 3.0.5) was used to overlay FITC images on PI images.

In vitro reconstitution of RNA polymerase holoenzyme. *B. subtilis* core RNAP was partially purified as described previously (69). To isolate pro-σ^K and σ^K, PY79/pSL1 cells were collected at 4.5 hours into sporulation without IPTG induction. Cells from 3 ml of culture were pelleted by centrifugation at 5,000g for 5 min, resuspended in 100 μl lysis buffer substituting KCl for NaCl, and incubated at 37°C for 10 min. The KCl concentration was adjusted to 0.6 M and the lysate sonicated. After 10 min at 30°C, the lysate was cleared of unlysed cell debris by centrifugation for 10 min in a microfuge. The supernatant (120 μl) was loaded onto a Sephadex G-100 column and eluted with lysis buffer containing 0.6 M KCl without enzymes. Fractions in the molecular weight range of monomeric pro-σ^K were pooled and dialyzed against lysis buffer to remove the salt. The

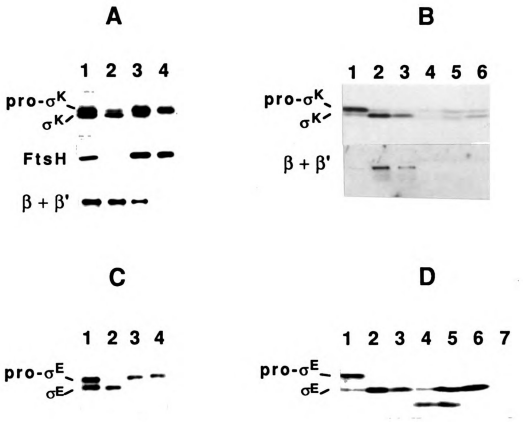
dialyzed sample was divided into two 100 µl aliquots, each containing approximately 5 pmole of pro-σ^K and 1 pmole of σ^K, as determined by Western blotting. Ten microliters of core RNAP in storage buffer, containing approximately 15 pmole of core subunits, as determined by SDS-PAGE and Coomassie blue staining, was added to one aliquot, and 10 µl of storage buffer was added to the second aliquot. The two aliquots were incubated on ice for 1 hr. Each aliquot was then fractionated on the same Sephadex G-100 column. Column fractions were precipitated with TCA and analyzed by Western blotting.

RESULTS

The majority of pro-σ^K is membrane-associated. To investigate whether pro-σ^K is associated with core RNAP, we fractionated crude lysates of sporulating wild-type *B. subtilis*. To facilitate the comparison of pro-σ^K and σ^K, cells were collected at 3.5 h after the onset of sporulation ($T_{3.5}$), a time at which approximately equal amounts of pro-σ^K and σ^K are present in cells. Cells were treated with lysozyme and lysed by passage through a French Pressure cell. The crude lysate was cleared of cell debris by low speed centrifugation (12,000g) and the supernatant was then subjected to high speed centrifugation (200,000g). The resulting pellet was further fractionated on a sucrose density gradient. Samples of different fractions were analyzed by Western blotting using anti-pro-σ^K antibodies (89). As shown in Figure 2A, the majority of pro-σ^K was detected in the high-speed pellet (lane 3), while σ^K was predominantly present in the high-speed supernatant (cytoplasmic fraction) (lane 2). After further fractionation of the high speed pellet on a sucrose density gradient, pro-σ^K remained in the membrane fraction, whereas the small amount of σ^K in the sample formed a pellet at the bottom of the sucrose gradient tube (data not shown), suggesting that it was present in residual cell debris or in a large aggregate of proteins. The cytoplasmic fraction was apparently depleted of membrane vesicles as FstH, an integral membrane protein, was not detected (lane 2). All the FtsH was found in the initial high speed pellet (lane 3) and was recovered in the purified membrane fraction (lane 4). The purified membrane fraction was essentially free of core RNAP, as little ß and ß′ subunits were detected (lane 4). These results show that the majority of the pro-σ^K in the crude lysate, unlike σ^K, is not associated with core RNAP, but is membrane-associated.

To ask whether pro-σ^K in the cytoplasmic fraction was associated with core RNAP,

Figure 2. Subcellular fractionation of extracts of sporulating wild-type cells. Cell extracts were fractionated as diagramed in Figure 1. Proteins in different fractions were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting. (A) Samples of different fractions equivalent to the same original volume of wild-type $T_{3.5}$ culture were analyzed for pro-σ^K and σ^K, as well as FtsH and the ß and ß′ subunits of RNAP by Western blotting. Lane 1, supernatant after 12,000g centrifugation. Lane 2, supernatant after 200,000g centrifugation. Lane 3, pellet after 200,000g centrifugation. Lane 4, membrane fraction purified by sucrose density gradient. (B) The supernatant after 200,000g centrifugation was subjected to size-fractionation by passage through a Sephacryl S-300 column. Equal volumes of the column fractions were analyzed for pro-σ^K and σ^K and the RNAP ß and ß′ subunits. Fraction numbers are indicated. (C) Samples of different fractions equivalent to the same original volume of wild-type $T_{1.7}$ culture were analyzed for pro-σ^E and σ^E. Lane contents are the same as for panel A. (D) The supernatant after 200,000g centrifugation was subjected to size-fractionation by passage through a Sephacryl S-300 column. Equal volumes of the column fractions were analyzed for pro-σ^E and σ^E.


A
1 2 3 4
pro-σK
σK
FtsH
β + β'
B
1 2 3 4 5 6
pro-σK
σK
β + β'
C
1 2 3 4
pro-σE
σE
D
1 2 3 4 5 6 7
pro-σE
σE

the supernatant after high speed centrifugation was size-fractionated by passage through a Sephacryl S-300 column, which has a fractionation range of 10 kDa to 1,500 kDa. The molecular weight of σ^K RNAP holoenzyme is about 370 kDa, which should render it readily separated from very high molecular weight complexes and from free pro-σ^K (29 kDa) in this column. The column fractions were analyzed by Western blotting using anti-pro-σ^K antibodies or antibodies against *E. coli* core RNAP. As shown in Fig. 2B, most of the pro-σ^K was eluted at or near the void volume of the column (lane 1), suggesting that it is part of a very large complex (>1,500 kDa). σ^K was eluted later than pro-σ^K and was co-eluted with the ß and ß′ subunits of RNAP (lanes 2 and 3), indicating that σ^K was present in the holoenzyme form. Taken together, these results show that pro-σ^K is not associated with core RNAP in the crude extract of sporulating *B. subtilis*; rather, most of it is associated with membrane and the rest is present in a large complex of unknown composition.

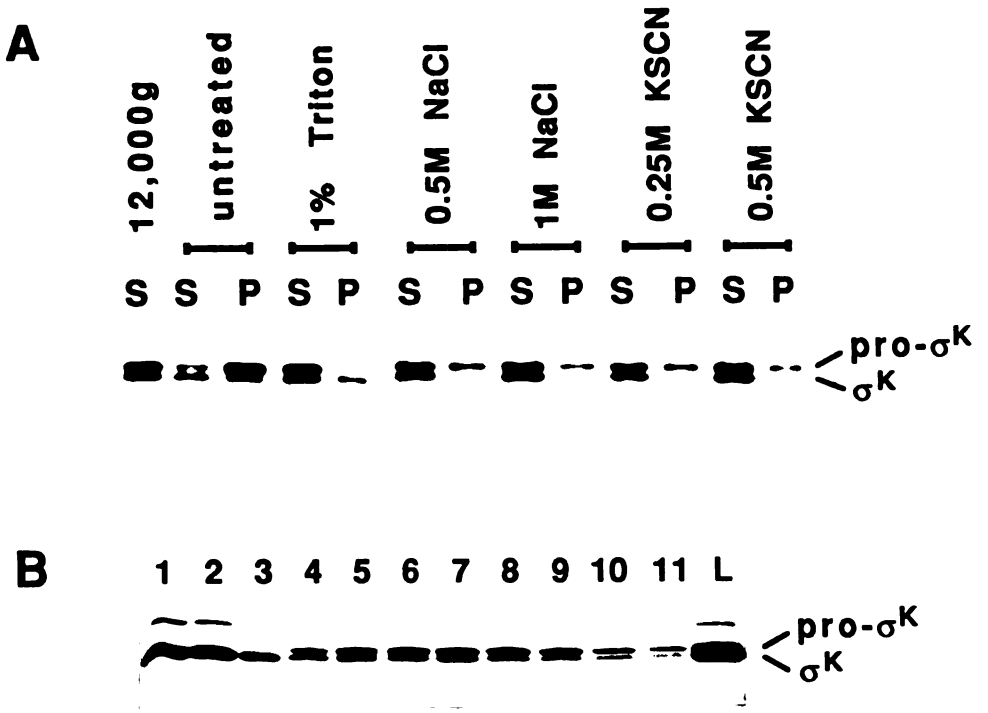
We next asked whether pro-σ^E fractionates in the same way as pro-σ^K. Pro-σ^E is the inactive precursor of σ^E. Since pro-σ^E is synthesized earlier than pro-σ^K, wild-type cells were collected at 1 h and 40 minutes after the onset of sporulation, a time at which approximately equal amounts of pro-σ^E and σ^E are present. Cell extracts were prepared and fractionated as described above. Pro-σ^E and σ^E were analyzed by Western blotting using monoclonal anti-σ^E antibody. As shown in Figure 2C, pro-σ^E fractionated in a pattern similar to that of pro-σ^K. The majority of pro-σ^E was detected in the high speed pellet (lane 3) and was recovered in the purified membrane fraction (lane 4). The small amount of pro-σ^E in the cytoplasmic fraction (lane 2) was eluted in the void volume of a Sephacryl S-300 column (Figure 2D, lane 1), suggesting it is part of a very large complex

(>1,500 kDa) of unknown composition. σ^E was found almost exclusively in the cytoplasmic fraction (lane 2), and co-eluted with core RNAP (Fig. 2D, lanes 2 and 3) or as free σ^E (Figure 2D, lanes 5 and 6) from the sizing column.

Effect of detergent and salt treatment on the membrane association of pro-σ^K. After the lysate of wild-type cells collected at $T_{3.5}$ was cleared of cell debris by low speed centrifugation (12,000g), the supernatant was treated with detergent or salt, then subjected to high speed centrifugation (200,000g). The resulting supernatant and pellet fractions were analyzed by Western blotting to further characterize the membrane-association of pro-σ^K. As expected for a protein interacting with membranes, pro-σ^K was solubilized by 1% Triton X-100 treatment (Figure 3A). In contrast, the small amount of σ^K found in the high speed pellet remained in the pellet upon detergent treatment (Figure 3A). This result is consistent with the finding that σ^K in the pellet did not fractionate with membrane in a sucrose gradient and supports the idea that σ^K is not membrane-associated. Instead, we speculate that it may be associated with residual cell debris or a large aggregate of proteins.

To determine the size of pro-σ^K and σ^K in the Triton X-100-treated supernatant, instead of subjecting it to high speed centrifugation it was size-fractionated by passage through a Sephadex G-200 column, which has a fractionation range of 5 kDa to 600 kDa. Figure 3B shows that the majority of σ^K was eluted near the void volume, suggesting that most of the σ^K was not dissociated from core RNAP by 1% Triton X-100. Pro-σ^K was eluted in the included volume, indicating that pro-σ^K was dissociated from membranes. In addition, the large complex that had remained in the supernatant of extracts not treated with detergent (Figure 2B, lane 1) appeared to be dissociated by Triton X-100, suggesting that

Figure 3. Effects of detergent and salt treatment on fractionation of pro-σ^K and σ^K. (A) Crude cell extract was cleared of cell debris by 12,000 g centrifugation. The supernatant (S 12,000g) was divided into six aliquots and treated with either 1% Triton X-100, 0.5 M NaCl, 1 M NaCl, 0.25 M KSCN, 0.5 M KSCN, or left untreated. These aliquots were then subjected to high speed centrifugation (90 min, 200,000g). Samples of the supernatant (S) and pellet (P) fractions equivalent to the same original volume of wild-type $T_{3.5}$ culture were analyzed by Western blotting using anti-pro-σ^K antibodies. (B) The supernatant after 12,000g centrifugation was treated with 1% Triton X-100 (lane L) and size-fractionated by passage through a Sephadex G-200 column (lanes 1 through 11). Equal volumes of the column fractions were analyzed for pro-σ^K and σ^K by Western blotting. Numbers indicate the column fractions. Fractions 1 and 2 contained materials eluted in the void volume of the column.


A
12,000g
untreated
1% Triton
0.5M NaCl
1M NaCl
0.25M KSCN
0.5M KSCN
S S P S P S P S P S P S P
pro-σK
σK
B
1 2 3 4 5 6 7 8 9 10 11 L
pro-σK
σK

the interactions of pro-σ^K in the large complex are primarily hydrophobic in nature.

Both a nonchaotropic salt (NaCl) and a chaotropic salt (KCNS) partially solubilized pro-σ^K from the membrane (Figure 3A), suggesting that both ionic and hydrophobic interactions are likely to be involved in the binding of pro-σ^K to the membrane. The pro-σ^K remaining in the pellet after salt treatment may be present inside vesicles and therefore incapable of release by salt. In contrast, both 0.5 M NaCl and 0.25 M KCNS completely solubilized the residual σ^K from the pellet.

Membrane association of pro-σ^K does not depend upon sporulation-specific gene products. The products of the mother cell-expressed *spoIVF* operon are thought to be intimately involved in the processing of pro-σ^K. SpoIVFB is either the processing enzyme or a regulator of the processing enzyme (18, 19, 88, 89). SpoIVFA negatively regulates the activity of SpoIVFB and these proteins are thought to form a complex in the mother cell membrane that surrounds the forespore (18, 19, 115). To investigate whether *spoIVF* gene products are required for the membrane association of pro-σ^K, a lysate from *spoIVF* null mutant cells was fractionated and analyzed by Western blotting. As shown in Figure 4A (lanes 1-3), the majority of pro-σ^K was present in the pellet after high speed centrifugation, just as in wild-type cells (Figure 2A, lanes 1-3). Since *spoIVF* null mutant cells are processing deficient, only pro-σ^K is present. We conclude that the membrane association of pro-σ^K does not depend upon *spoIVF* gene products. Another mother cell-specific protein, SpoIVA, is located at the forespore surface and controls the assembly of the spore cortex and coat (24). Pro-σ^K processing is impaired in *spoIVA* mutant cells (89). We found that pro-σ^K associates with the membrane in *spoIVA* mutant (*spoIVA67*) cells (data not shown), suggesting that the *spoIVA* mutation

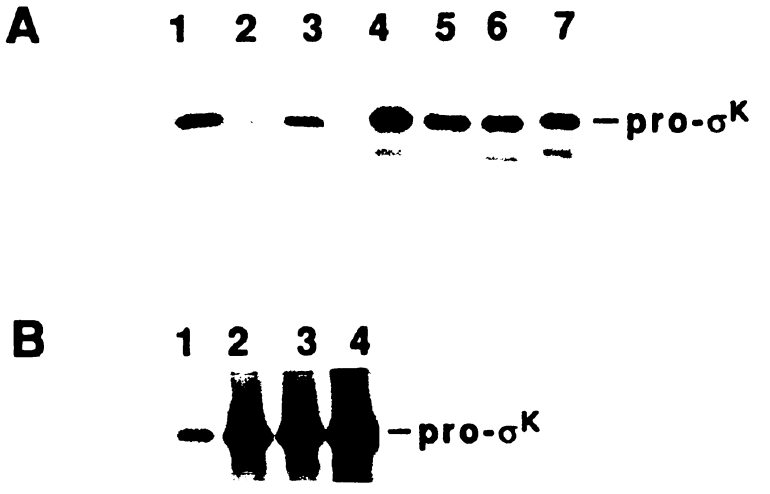


Figure 4. Specificity of the membrane-association of pro-σ^K. (A) Sporulating ($T_{3.5}$) *spoIVF* null mutant (BSL51) cells and vegetative wild-type cells expressing pro-σ^K from a plasmid (pSL1) were fractionated. Proteins equivalent to the same original volume of cells were analyzed by Western blotting. Lanes 1-3, 12,000 g supernatant, 200,000 g supernatant and 200,000 g pellet, respectively, of sporulating BSL51 cells. Lanes 4-7, 12,000 g supernatant, 200,000 g supernatant, 200,000 g pellet and gradient-purified membrane, respectively, of vegetative PY79/pSL1 cells. (B) Western blot analysis of 2 μg of protein from sucrose gradient-purified membrane of sporulating ($T_{3.5}$) wild-type (PY79) cells (lane 1) and sporulating ($T_{3.5}$) wild-type cells containing plasmid pSL1 after being induced to make pro-σ^K for 10 min (lane 2), 30 min (lane 3) or 3.5 h (lanes 4).

does not impair the processing of pro-σ^K by interfering with its membrane association.

To ask whether membrane association of pro-σ^K occurs in the absence of any sporulation-specific gene products, we induced production of pro-σ^K during vegetative growth from a multicopy plasmid, pSL1, which has the intact *sigK* gene fused to an IPTG-inducible promoter (P_{spac}) (89). A lysate was prepared from IPTG-induced cells containing pSL1, fractionated, and analyzed by Western blotting. About half of the pro-σ^K was pelleted by high speed centrifugation and it remained in the membrane fraction after sucrose gradient purification (Figure 4A, lanes 4-7). Hence, membrane association of pro-σ^K does not require expression of any sporulation-specific genes. In this experiment, about half of the pro-σ^K remained in the supernatant after high speed centrifugation (Figure 4A, lane 5), whereas in sporulating cells only a small amount of pro-σ^K was found in the cytoplasmic fraction (Figure 2A, lane 2). The difference may be due to overproduction of pro-σ^K in IPTG-induced vegetative cells containing pSL1. Like the pro-σ^K in the cytoplasmic fraction of sporulating cells (Figure 2B, lane 1), the pro-σ^K in the cytoplasmic fraction of the vegetative cells appeared to be present in a large complex (>1,500 kDa) of unknown composition (data not shown).

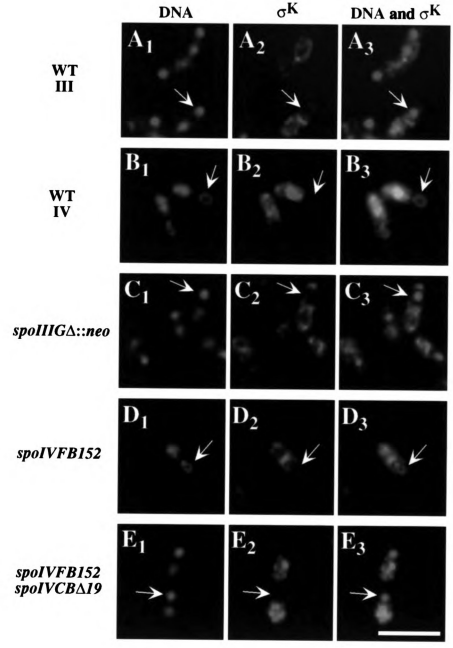
Pro-σ^K binding sites are not saturated on the membranes of sporulating cells. To test whether membranes in sporulating cells have the ability to bind more pro-σ^K, we induced the production of pro-σ^K from pSL1 during sporulation. Wild-type cells bearing pSL1 were induced with IPTG for 10 min, 30 min, or 3.5 h before being harvested at $T_{3.5}$. Membrane fractions from these cells were purified by sucrose gradients. Two micrograms of protein from each membrane preparation was analyzed by Western blotting. As shown in Figure 4B, more pro-σ^K was detected in membranes

prepared from cells overproducing pro-σ^K, as compared to membranes prepared from wild-type cells. These results indicate that pro-σ^K binding sites are not saturated on the membrane of sporulating cells.

Pro-σ^K localizes to the mother cell membranes that surround the forespore and the mother cell of the postengulfment sporangium. Pro-σ^K and σ^K were immunolocalized in sporulating cells using affinity purified anti-σ^K antibodies (115), secondary antibodies coupled to FITC, and fluorescence microscopy. The rabbit polyclonal anti-σ^K antibodies bind pro-σ^K as well as σ^K. Therefore, we were able to distinguish both forms of the transcription factor only by co-staining the nucleoids to determine the stage of sporulation and by analyzing mutants that are either deficient in pro-σ^K processing or are known to synthesize mature σ^K in the absence of processing. Postengulfment sporangia at stages III and IV in sporulation can be readily identified by their DNA staining pattern. Whereas the forespore chromosome of stage III sporangia (Figure $5A_1$, red, arrow) more closely resembles the mother cell chromosome, albeit slightly more condensed, the forespore nucleoid of stage IV sporangia assumes a characteristic toroidal structure (Figure $5B_1$, red, arrow) upon association with the α/ß-type SASP (111).

Wild-type postengulfment sporangia, which could be assigned to stage III in sporulation by virtue of their DNA staining pattern (Figure $5A_1$, red), displayed pro-σ^K/σ^K immunostaining (Figure $5A_2$, green) in the periphery of most of the mother cell and on one side of the engulfed forespore. The peripheral forespore staining was often evident as a crescent at the interface between the forespore and the larger volume of the mother cell. There was very little overlap between the green immunostaining of pro-σ^K/σ^K and the red

Figure 5. Immunolocalization of pro-σ^K and σ^K in sporulating cells. The sporangia were harvested at $T_{2.5}$ in (A) and at $T_{3.5}$ in (B) through (E) and prepared for immunofluorescence microscopy as described in Materials and Methods. Arrows point to the engulfed forespore compartment and are oriented perpendicularly to the long axis of the sporangia. DNA was stained with PI (red) (A_1, B_1, C_1, D_1, E_1). Immunostaining of pro-σ^K and σ^K is shown in green (A_2, B_2, C_2, D_2, E_2). Where the red and green fluophores overlap, as in the doubly exposed images shown in B_3 and E_3, a yellow to orange color is visible. (A) Wild-type sporangia with almost equally as condensed mother cell and forespore nucleoids, which is characteristic of cells at stage III in sporulation before pro-σ^K is processed to σ^K. (B) Wild-type sporangia at stage IV in sporulation, when the forespore nucleoid has assumed its toroidal shape and pro-σ^K has been processed to σ^K in the mother cell. (C) Pro-σ^K processing deficient *spoIIIGΔ::neo* mutant sporangia of strain RL831. (D) Processing deficient *spoIVFB152* mutant sporangia of strain RL87. (E) *spoIVFB152 spoIVCBΔ19* doubly mutant sporangia of strain RL136, which synthesize mature σ^K in the absence of a functional protease for pro-σ^K processing.


DNA
σK
DNA and σK
WT
III
WT
IV
spoIIIGΔ::neo
spoIVFB152
spoIVFB152
spoIVCBΔ19
A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3
E1
E2
E3

nucleoid staining (Figure $5A_3$), indicating that pro-σ^K/σ^K is associated with the mother cell membranes that surround the mother cell and the forespore. After sporulation proceeded to stage IV, as indicated by the toroidal forespore nucleoid (Figure $5B_1$, red, arrow), when pro-σ^K is known to be converted to σ^K in the mother cell (89), the pattern of pro-σ^K/σ^K immunostaining changed to include the cytoplasm of the mother cell (Figure $5B_2$, green). This staining pattern was consistent with the previously reported even distribution of σ^K throughout the mother cell (115).

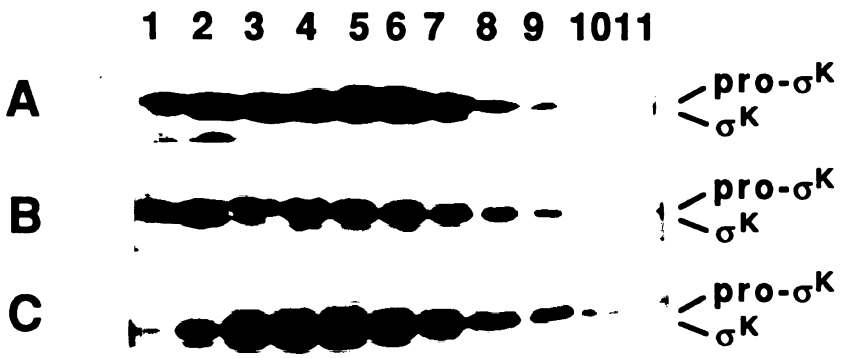
In *spoIIIG* and *spoIVFB* mutant sporangia, which are deficient in pro-σ^K processing and do not proceed in development beyond stages III and IV, respectively, pro-σ^K immunostaining was detected in the periphery of the mother cell and forespore (Figure 5C and 5D, green). Because this staining pattern was similar to the one observed in wild-type stage III sporangia (Figure 5A, green), we conclude that pro-σ^K is associated with the mother cell membranes that surround the mother cell and the forespore. In sporangia of a *spoIVFB* mutant that produces mature σ^K without processing due to a deletion (*spoIVCBΔ19*) in the pro-sequence-encoding portion of *sigK*, immunostaining of σ^K was detected throughout the mother cell (Figure 5E, green). As this staining pattern is reminiscent of the one observed in wild-type stage IV sporangia (Figure 5B, green), we infer that after proteolytic activation σ^K is released from the membrane and becomes soluble in the mother cell cytoplasm. Therefore, the change in pro-σ^K/σ^K immunostaining from stage III to stage IV in sporulation resulted from the conversion of membrane associated pro-σ^K to soluble σ^K, consistent with our subcellular fractionation results (Figure 2).

Pro-σ^K does not bind to exogenous core RNAP in vitro. Very little, if

any, of the pro-σ^K in lysates is associated with core RNAP (Figure 2). Is this because pro-σ^K is unable to bind to core RNAP (due either to intrinsic inability to bind or to associate with other cellular components like membranes) or because core RNAP is not available for binding? To address this question, our strategy was to dissociate both pro-σ^K and σ^K from other components in the cell lysates and incubate them with exogenous core RNAP. To increase the production of pro-σ^K and σ^K, we used wild-type cells containing pSL1. In the absence of IPTG induction, leaky expression from the P_{spac} promoter in pSL1 allows accumulation of pro-σ^K during sporulation so that when cells are harvested at $T_{4.5}$, a time at which more σ^K has accumulated, both pro-σ^K and σ^K are present at a higher level than in wild-type cells at $T_{3.5}$.

A crude lysate was prepared from cells harvested at $T_{4.5}$ and KCl was added to a final concentration of 0.6 M. The salt-treated lysate was then size-fractionated on a Sephadex G-100 column, which has a fractionation range of 4-150 kDa. Both pro-σ^K and σ^K in untreated crude lysate were excluded from this column (data not shown). After salt treatment, a portion of the pro-σ^K and σ^K was retained in the column (Figure 6A), indicating that σ^K was partially dissociated from core RNAP and pro-σ^K was partially dissociated from the membrane and/or the large complex that remained in the supernatant after high speed centrifugation (Figure 2B, lane 1). Fractions 5-7 containing dissociated pro-σ^K and σ^K were pooled and dialyzed to remove the salt. The dialyzed sample was incubated with either partially purified core RNAP or with the core RNAP storage buffer, and then fractionated in separate experiments on the same Sephadex G-100 column. Upon incubation with core RNAP (Figure 6B), σ^K was eluted in the void volume, suggesting

Figure 6. σ^K, but not pro-σ^K, reassociates with core RNAP after being dissociated by salt treatment. The 12,000g supernatant was prepared in the presence of 0.6 M KCl and separated by a Sephadex G-100 column (A). The void volume of this column was fractions 1-2, wherein pro-σ^K and σ^K would be eluted if not treated with salt. Fractions 5-7 containing dissociated pro-σ^K and σ^K in approximately the monomeric size range were pooled and dialyzed. The dialyzed fractions were incubated with (B) and without (C) exogenous core RNAP. Proteins were then separated by the same Sephadex G-100 column and analyzed by Western blotting with anti-pro-σ^K antibodies. Only σ^K shifted back to the void volume upon incubation with core RNAP (panel B, lanes 1 and 2), indicating formation of the holoenzyme.


1 2 3 4 5 6 7 8 9 10 11
A
B
C
pro-σK
σK
pro-σK
σK
pro-σK
σK

that it had reassociated with core RNAP. Pro-σ^K was eluted in the included volume after incubation with either core RNAP (Figure 6B) or storage buffer (Figure 6C). The same results were obtained when the experiment was repeated with a lysate made from wild-type cells harvested at $T_{3.5}$ (data not shown). These results indicate that pro-σ^K does not bind to core RNAP, even after it has been dissociated from other cellular components, whereas σ^K readily reassociates with core RNAP under these conditions.

DISCUSSION

We have demonstrated that the majority of pro-σ^K in cell lysates is membrane-associated and is not bound to core RNAP. In contrast, nearly all of the σ^K in lysates of sporulating cells is present in the cytoplasmic fraction and appears to be bound to core RNAP. In sporulating cells, pro-σ^K appears to associate with the mother cell membranes that surround the mother cell and the forespore, as visualized by immunofluorescence microscopy. Processing releases σ^K into the mother cell cytoplasm. Most of the pro-σ^K and σ^K can be dissociated from large components in the cell extract by 0.6 M KCl. After removal of the salt, σ^K, but not pro-σ^K, could bind to added core RNAP. These results indicate that the pro-sequence of pro-σ^K promotes membrane association and inhibits RNAP core binding.

The ability of pro-σ^K to associate with a membrane may facilitate its proteolytic processing to active σ^K. SpoIVFB has been proposed to be either the protease that processes pro-σ^K or a regulator of the protease (18, 19, 88, 89). Encoded in the same operon as SpoIVFB is SpoIVFA, which appears to inhibit SpoIVFB activity until a signal is received from the forespore (17-19). SpoIVFB and SpoIVFA have been shown to be localized at the boundary between the mother cell and the forespore (115). As depicted in Figure 7, these proteins presumably insert into the mother cell membrane that surrounds the forespore since the *spoIVF* operon is expressed in the mother cell (19). Likewise, *bofA* is thought to be expressed in the mother cell (116). Although BofA has not yet been shown to be localized to the mother cell membrane that surrounds the forespore, it has three putative transmembrane segments and, like SpoIVFA, it appears to inhibit SpoIVFB activity (18, 116). The signal that relieves this inhibition and leads to pro-σ^K processing is

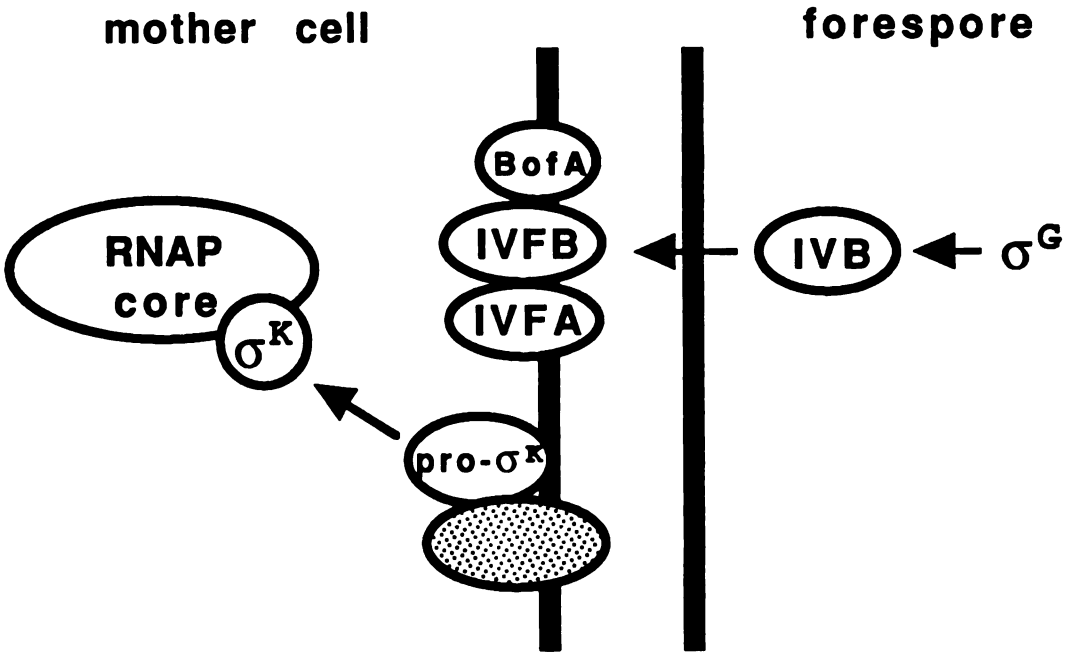


Figure 7. Model depicting association of pro-σ^K with the mother cell membrane that surrounds the forespore and signal transduction between the forespore and the mother cell leading to the processing of pro-σ^K. The shaded oval represents a possible abundant membrane protein that interacts with pro-σ^K. See the text for details.

generated in the forespore by σ^G-dependent expression of *spoIVB* (17, 32) (Figure 7). SpoIVB appears to have a signal sequence, so it may cross the forespore membrane in order to accomplish its signaling function (17, 145). If processing of pro-σ^K requires it to directly interact with SpoIVFB, then the ability of pro-σ^K to associate with the mother cell membrane that surrounds the forespore may facilitate processing by promoting this interaction.

Our immunolocalization studies showed that pro-σ^K interacts not only with the mother cell membrane that surrounds the forespore but also with the membrane that surrounds the mother cell in sporulating wild-type cells, as well as in *spoIIIG* and *spoIVFB* mutant cells (Figure 5). Does the pro-σ^K associated with the membrane that surrounds the mother cell get processed? It appears that most, if not all, of the pro-σ^K produced in sporulating cells is processed to σ^K. First, very little pro-σ^K was detected late during sporulation (89). Second, a pulse-chase experiment demonstrated that the half-life of pro-σ^K is about 30 min. The majority of the ^{35}S-label in pro-σ^K at T_3 was found in σ^K by T_4 (data not shown). Therefore, it seems likely that pro-σ^K associated with the membrane that surrounds the mother cell is either processed there or it dissociates and is processed elsewhere (e.g., at the mother cell membrane that surrounds the forespore). However, we cannot rule out the possibility that some of the pro-σ^K that associates with the membrane that surrounds the mother cell is degraded.

The pro-sequences of both mother cell specific σ factors appear to promote membrane association. We found that pro-σ^E in lysates of sporulating cells had very similar fractionation properties as pro-σ^K (Figure 2). The majority of pro-σ^E was membrane-associated and not bound to core RNAP. σ^E, like σ^K, appeared to be associated

with core RNAP in the cytoplasmic fraction. The pro-sequence of pro-σ^E has been proposed to form an amphipathic α helix with a highly charged face (109), which could presumably interact with negatively charged head groups of membrane lipids, but this would not explain the preferential localization of pro-σ^E to the sporulation septum (47, 60). Genetic suppression (110) and chemical cross-linking studies (47) suggest that pro-σ^E interacts with its putative processing enzyme, SpoIIGA. However, pro-σ^E may also interact with another protein in the septal membrane since localization of pro-σ^E (47) and a pro-σ^E::GFP fusion protein (60) to the septal membrane occurs in *spoIIGA* mutant cells.

The 20 amino acid pro-sequence of pro-σ^K is very hydrophobic, except for two charged residues at positions 13 and 14 from the N-terminus (136). The charged residues might prevent the pro-sequence from inserting into the membrane like a transmembrane domain of a typical integral membrane protein. In support of this prediction, virtually all the pro-σ^K was found in the aqueous phase of a Triton X-114 fractionation experiment (data not shown). We speculate that pro-σ^K is peripherally associated with the membrane, perhaps via binding of the pro-sequence to an abundant integral membrane protein (Figure 7), since the membranes in sporulating cells have the capacity to bind much more pro-σ^K when it is overproduced (Figure 4B). Alternatively, it is possible that the pro-sequence of pro-σ^K does not interact directly with a membrane component. Removal of the pro-sequence could induce a conformational change that prevents membrane association and/or uncovers a site that gives σ^K a higher affinity for core RNAP than for the membrane. The interaction of pro-σ^K with membranes does not require *spoIVF* gene products (Figures 4A and 5D), or the products of *spoIVA* (data not shown) or *spoIIIG* (Figure 5C). Indeed, the interaction does not require any sporulation-specific gene

products since pro-σ^K produced in vegetative cells was membrane-associated (Figure 4A).

A small portion of the pro-σ^K and pro-σ^E in cell lysates was not membrane-associated (Figures 2A and 2C). Rather, the pro-σ factors appeared to be present in large complexes (>1,500 kDa) (Figures 2B and 2D) of unknown composition. The large complexes could be aggregates of the pro-σ factors alone or in combination with other proteins. Different methods of cell breakage had little effect on the proportion of pro-σ^K that was membrane-associated versus present in a large complex. We tested sonication and osmotic shock lysis procedures (data not shown) in addition to the French pressure cell lysis method reported here.

In addition to promoting the membrane association of pro-σ^K, the pro-sequence also appears to inhibit RNAP core binding. The ß and ß′ subunits of core RNAP were barely detectable in a membrane fraction that contained abundant pro-σ^K (Figures 2A, lane 4). Also, the pro-σ^K that was not membrane-associated appeared to be present in a large complex (>1,500 kDa) containing very little ß and ß′ (Figure 2B, lane 1). Moreover, much less pro-σ^K than σ^K bound to core RNAP after both had been released from large cellular components by salt treatment and the salt was removed by dialysis (Figure 6B). We cannot rule out the possibility that pro-σ^K remained in small complexes with itself or another protein(s) upon treatment with 0.6 M KCl. However, pro-σ^K showed a similar elution profile from a sizing column as σ^K both in the presence of 0.6 M KCl (Figure 6A) and after salt removal when core RNAP was not added (Figure 6C). It seems unlikely that pro-σ^K was irreversibly denatured by 0.6 M KCl since σ^K readily associated with core RNAP upon its addition (Figure 6B). Under these conditions, the pro-sequence greatly hindered RNAP core binding. The pro-sequence may be close to the core-binding domain in the

three-dimensional structure of pro-σ^K, directly blocking core binding. Alternatively, cleavage of the pro-sequence may result in a conformational change which activates core binding. In agreement with our findings, Johnson and Dombroski (58) recently demonstrated that purified σ^K competes much more efficiently than purified pro-σ^K for binding to *E. coli* core RNAP. Removal of only 6 amino acid residues from the N-terminus of pro-σ^K restored core binding and the holoenzyme was transcriptionally active.

σ^{70} of *E. coli* does not bind to promoter DNA unless its amino-terminal region 1.1 is removed (22). Removal of the pro-sequence from pro-σ^K results in a ten-fold increase in DNA-binding activity (21). Our results suggest that in addition to modulating DNA-binding activity, the pro-sequence of pro-σ^K promotes its membrane association, perhaps facilitating processing to σ^K. Removal of the pro-sequence releases σ^K from the membrane and appears to unmask its RNAP core-binding activity, allowing the functional holoenzyme to form.

ACKNOWLEDGMENTS

We are very grateful to S. Lu, O. Resnekov, W. Haldenwang, M. Chamberlin, C. Kane, S. Cutting, and T. Ogura for providing antibodies. We thank B. Johnson and A. Dombroski for communicating their results prior to publication.

This research was supported by the Michigan Agricultural Experiment Station, by grant GM43585 from the National Institutes of Health to L. K., and by NIH grant GM18568 to R. Losick. A. Hofmeister was a postdoctoral fellow of the Alexander von Humboldt Foundation and performed the immunofluorescence microscopic studies described in this chapter.

Chapter V

Summary and Perspective

Mother-cell gene expression during *B. subtilis* sporulation is governed by the concerted action of transcription factors σ^E, SpoIIID, σ^K, and GerE. While the timely appearance of these transcription factors is critical to the temporal regulation of mother-cell gene expression, the timely disappearance of the early transcription factors is important as well. The replacement of σ^E and SpoIIID with σ^K and GerE completes a switch in the mother cell pattern of gene expression. Experiments described in this dissertation demonstrate that the appearance of σ^K initiates a negative feedback loop to facilitate the disappearance of σ^E and SpoIIID. σ^K appears to affect the activity of the two positive regulators of *sigE* transcription, σ^A and Spo0A~P. σ^K could inhibit σ^A activity by competing with σ^A for binding to core RNAP. The negative effect on Spo0A~P is likely to be mediated by a σ^K-dependent gene product(s).

The biological significance of the negative feedback regulation of σ^K on the levels of SpoIIID and σ^E needs to be addressed. The negative effect of σ^K on *sigE* and *spoIIID* transcription could be bypassed by fusing these genes to a σ^K-dependent promoter (e.g. the *gerE* promoter). For example, in an otherwise wild-type background, a single copy of P_{gerE}-$P_{spoIIID}$-*spoIIID* in the chromosome should result in a higher level of SpoIIID expression late during sporulation. This is predicted to inhibit expression of certain *cot* genes (e.g. *cotC, cotD,* and *cotX*) that are repressed by SpoIIID, based on in vitro studies (39, 40, 50, 69). It would be interesting to determine whether the resistance or structural characteristics of spores is altered when the negative feedback regulation of σ^K on SpoIIID or σ^E is bypassed.

The negative effect of σ^K on Spo0A~P may be difficult to investigate because there are many potential regulatory sites in the phosphorelay (34). It is possible to investigate the

negative effect of σ^K on σ^A activity. To test the model that σ^K has higher affinity than σ^A for core RNAP, in vitro assays could be developed to directly compare the core-binding activity of various sigma factors. If these studies support the idea that σ^K outcompetes σ^A for binding to core RNAP, it may be possible to swap the core-binding domain of σ^A with that of σ^K so as to increase the core-binding affinity of σ^A. The strain containing σ^A with the swapped core-binding domain would then be analyzed for sporulation phenotype and the production of σ^E and SpoIIID.

The appearance of σ^K not only turns on expression of genes in the σ^K regulon, but also turns off genes in the σ^E regulon. Earlier production of σ^K by deleting the part of the *sigK* gene encoding the pro-sequence results in lower expression of *sigE* and the whole σ^E regulon, including *spoIIID*. Thus, one function of making σ^K first as an inactive precursor is to avoid initiating the negative feedback loop prematurely.

Pro-σ^K appears to be kept inactive by at least two different mechanisms. First, the pro-sequence inhibits the RNAP core binding activity of σ^K. This is supported by the observation that pro-σ^K in extracts of sporulating cells is not associated with core RNAP and the observation that pro-σ^K fails to bind to core RNAP in vitro under conditions that permit σ^K binding. A simple model is that the pro-sequence interacts with and masks the core-binding domain of σ^K. If this is true, using the pro-σ^K with mutations in the pro-sequence that abolish membrane-association but retain inhibition of RNAP core-binding (discussed below), intragenic suppressor mutations in the core-binding region of σ^K could be identified that relieve the inhibitory effect of the pro-sequence. A second mechanism of inactivation involves the tethering of pro-σ^K to the membrane surrounding the mother cell

and the membrane surrounding the forespore, physically sequestering it from core RNAP in the mother cell cytoplasm. Membrane-association is apparently mediated by the pro-sequence since processing releases σ^K to the cytoplasm of mother cell where it associates with core RNAP. It is not clear how pro-σ^K interacts with the membrane. Residues in the pro-sequence critical for membrane-association and core-binding could be identified by a systematic mutagenesis analysis of the 20 amino acid residues in the pro-sequence, and/or by making a set of nested deletions of the pro-sequence. Testing these mutants for core-binding and membrane association could yield information about the mechanism by which the pro-sequence promotes membrane-association and inhibits core-binding.

Since the pro-σ^K processing machinery appears to be localized to the membrane surrounding the forespore (115), association of pro-σ^K with this membrane may facilitate processing by bringing pro-σ^K and the processing protease in proximity. During the course of subcellular fractionation studies, various fractions, including the membrane fraction, were incubated under different conditions to test for processing activity. No in vitro processing of pro-σ^K was observed. There is evidence that SpoIVFB is a labile protein, which could explain the failure to observe in vitro processing. In wild type cells, SpoIVFB is stabilized and inhibited by SpoIVFA. Relieve of the inhibitory effect of SpoIVFA depends on a forespore signal protein SpoIVB. Certain mutations in SpoIVFA (*bofB*) render SpoIVFB active independent of SpoIVB. In *spoIIID* mutant cells, σ^K is not produced but SpoIVFB overaccumulates 10-fold (115). Further attempts to develop an in vitro processing system may involve mixing the membrane fraction of cells overexpressing *spoIVFB* (e.g. *bofB spoIIID* double mutant cells) with pro-σ^K (purified or in a membrane fraction from processing deficient cells) and monitoring the production of σ^K.

APPENDIX

Appendix

Bacillus subtilis SpoIIID protein binds to two sites in the *spoVD* promoter and represses transcription by σ^E RNA polymerase

Reprinted from the Journal of Bacteriology

JOURNAL OF BACTERIOLOGY, Feb. 1997, p. 972-975
0021-9193/97/$04.00+0

Vol. 179, No. 3

Bacillus subtilis SpoIIID Protein Binds to Two Sites in the *spoVD* Promoter and Represses Transcription by σ^E RNA Polymerase

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Received 26 September 1996/Accepted 15 November 1996

The *Bacillus subtilis spoVD* gene encodes a penicillin-binding protein required for spore morphogenesis. SpoIIID is a sequence-specific DNA-binding protein that activates or represses the transcription of many different genes. We have defined the *spoVD* promoter region and demonstrated that it is recognized by σ^E RNA polymerase in vitro and that SpoIIID represses *spoVD* transcription. Two strong SpoIIID-binding sites were mapped in the *spoVD* promoter region, one overlapping the −35 region and the other encompassing the −10 region and the transcriptional start site.

In response to starvation, *Bacillus subtilis* initiates a developmental process involving a series of morphological changes driven by a program of gene expression (6). One of the early morphological changes is the formation of an asymmetric septum that divides the bacterium into two compartments, the mother cell and the forespore. Subsequent migration of the septum engulfs the forespore in a double membrane, pinching it off as a free protoplast within the mother cell. Cell wall-like material known as the cortex is then deposited between the two membranes. Finally, the mother cell synthesizes so-called coat proteins that assemble on the surface of the forespore and the mature spore is released by lysis of the mother cell. The program of gene expression driving these morphological changes involves a cascade of sigma factor activity (9, 18). In the mother cell, the products of genes transcribed by σ^E RNA polymerase ($E\sigma^E$) are primarily responsible for engulfment, cortex formation, and production of σ^K, while the products of genes transcribed by σ^K RNA polymerase ($E\sigma^K$) function mainly in formation of the spore coat, as well as mother cell lysis (6, 9). Another key regulator of mother cell gene expression is SpoIIID, a sequence-specific DNA-binding protein that activates or represses many genes in both the σ^E and σ^K regulons (8, 14). Previous genetic studies suggested that the *spoVD* gene is likely to be a member of the σ^E regulon and showed that *spoVD* is overexpressed in *spoIIID* mutant cells (3). The product of *spoVD* is a penicillin-binding protein. Penicillin-binding proteins generally carry out the final steps of the synthesis of peptidoglycan, which is the major component of the cortex. Consistent with a proposed role of SpoVD in cortex synthesis, *spoVD* mutant cells are defective in spore cortex development (1, 3). Here we show that $E\sigma^E$ can transcribe *spoVD* in vitro and that SpoIIID can repress *spoVD* transcription by binding to the promoter region.

***spoVD* is transcribed by $E\sigma^E$ in vitro, and SpoIIID represses this transcription.** Several lines of evidence suggest that *spoVD* is transcribed by $E\sigma^E$ (3). First, expression of a *spoVD-lacZ* fusion was reduced or abolished in strains defective in σ^E production. Second, *spoVD-lacZ* was expressed when transcription of the *spoIIG* operon encoding pro-σ^E and its putative processing enzyme, SpoIIGA, was induced in vegetative cells. Third, the *spoVD* promoter region shows sequence similarity to other σ^E-dependent promoters. The upstream boundary of the *spoVD* promoter region was defined by deletion analysis with exonuclease III-S1 nuclease digestion and the integrational plasmid vector pSG1301 (3). Plasmids with inserts extending from a range of positions in the vicinity of the transcriptional start site (TSS) to a unique *Hin*dIII site in the middle of the *spoVD* coding region were integrated into the chromosome of Spo$^+$ strain SG38 (7). Inserts with their upstream ends located up to and including −28 bp relative to the TSS gave a Spo$^-$ phenotype upon integration, indicating that promoter function was absent, whereas inserts extending to −42 bp or farther upstream were Spo$^+$, indicating at least partial promoter activity. The minimal sequences needed for *spoVD* expression thus lie within 42 bp of the TSS. To determine whether $E\sigma^E$ could recognize this promoter in vitro, a plasmid extending well upstream of the promoter (to −211) and to position +103 downstream (pSG1362; reference 3) was used as the template in runoff transcription assays. The plasmid was linearized by digestion with restriction enzymes that cleave at different sites downstream of the *spoVD* promoter, generating DNA templates that would produce *spoVD* runoff transcripts of different lengths. In vitro transcription reactions were performed as described previously (8). Runoff transcripts were electrophoresed in a 5% polyacrylamide gel containing 8 M urea and detected by autoradiography. Partially purified $E\sigma^E$ produced runoff transcripts of the expected sizes (Fig. 1, lanes 2 and 3). Some promoters are transcribed by both $E\sigma^E$ and $E\sigma^K$ (8, 16, 24), presumably because both forms of RNA polymerase recognize some similar sequences in promoters (5). To demonstrate that σ^E can direct transcription from the *spoVD* promoter and to test whether σ^K can too, gel-purified σ^E and σ^K were reconstituted with core RNA polymerase as described previously (14) and used to transcribe a *spoVD* template. Only $E\sigma^E$, not $E\sigma^K$, produced a runoff transcript (Fig. 1, lanes 5 and 6). A control experiment showed that the reconstituted $E\sigma^K$ was active because a strong signal was produced from a template containing the σ^K-dependent *cotD* promoter (lane 7). We concluded that *spoVD* is transcribed by $E\sigma^E$. Since $E\sigma^E$ is active only in the mother cell compartment during sporulation (4, 10, 17), expression of *spoVD* is expected to be mother cell specific. This is consistent with the results of a

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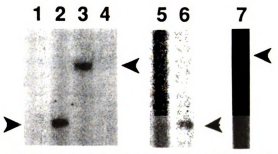

FIG. 1. In vitro transcription of *spoVD* by $E\sigma^E$ and effect of SpoIIID. pSG1362 (2 μg), containing the *spoVD* promoter (3), was digested with *Eco*RI (lanes 1 and 2, 117-base transcript) or *Xho*I (lanes 3 and 4, 150-base transcript) and transcribed in a total volume of 42 μl with $E\sigma^E$ (0.2 μg) alone (lanes 2 and 3) or with 0.1 μg of gel-purified SpoIIID added (lanes 1 and 4). pSG1362 (2 μg) was digested with *Eco*RI and transcribed with reconstituted $E\sigma^A$ (lane 5) or with reconstituted $E\sigma^E$ (lane 6). pLRK100 (2 μg) containing the *cotD* promoter (14) was digested with *Hin*dIII (225-bp transcript) and transcribed with reconstituted $E\sigma^A$ (lane 7). Arrowheads denote the positions of runoff transcripts of the expected sizes, as judged from the migration of end-labeled DNA fragments of *Msp*I-digested pBR322.

genetic experiment showing that expression of *spoVD* only in the mother cell is sufficient to allow sporulation (3).

The ability to transcribe *spoVD* in vitro with $E\sigma^E$ allowed us to test whether SpoIIID can directly affect *spoVD* transcription. The twofold overexpression of *spoVD-lacZ* in *spoIIID* mutant cells suggested that SpoIIID might repress transcription of *spoVD* (3). Addition of SpoIIID to the in vitro transcription reaction abolished the *spoVD* signal (Fig. 1, lanes 1 and 4). This was not due to general inhibition of transcription by SpoIIID, because the same preparation of SpoIIID activated transcription of *sigK* by $E\sigma^K$ (data not shown), as reported previously (8). We concluded that SpoIIID can specifically repress *spoVD* transcription by $E\sigma^E$ in vitro.

The *spoIIID* gene is transcribed by $E\sigma^E$ (13, 15, 22, 23), yet SpoIIID represses transcription of *spoVD* (Fig. 1) and some other σ^E-dependent genes. Thus, SpoIIID limits the expression of some genes in its own regulon. Other genes in the σ^E regulon that are repressed by SpoIIID include *bofA*, as demonstrated in vivo (12) and in vitro (8), and probably the *spoIIIA* operon, as implied by in vivo data (11). Another possible SpoIIID-repressed operon in the σ^E regulon is *spoIVF*, based on the observation that a *spoIVF-lacZ* fusion is overexpressed in *spoIIID* mutant cells (2) and the fact that three near-perfect matches to the SpoIIID binding site consensus sequence (8) can be found in the *spoIVF* promoter. Perhaps SpoIIID helps confine the expression of some genes in the σ^E regulon to a physiologically relevant level or time period. Consistent with this notion, in *spoIIID* mutant cells, the products of *spoIVF* overaccumulate and SpoIVFA is mislocalized (20).

SpoIIID binds to two sites in the *spoVD* promoter region. Gel retardation assays indicated that SpoIIID binds to DNA fragments containing the *spoVD* promoter region (data not shown). To localize the binding sites of SpoIIID in this promoter more precisely, DNase I protection experiments were performed as described previously (8). Radioactive DNA probes, labeled separately on the nontranscribed or tran-

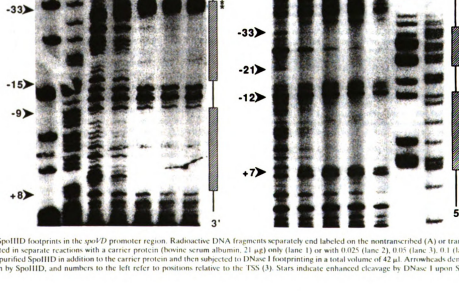

FIG. 2. SpoIIID footprints in the *spoVD* promoter region. Radioactive DNA fragments separately end labeled on the nontranscribed (A) or transcribed (B) strand were incubated in separate reactions with a carrier protein (bovine serum albumin, 21 μg) only (lane 1) or with 0.025 (lane 2), 0.05 (lane 3), 0.1 (lane 4), or 0.2 (lane 5) μg of gel-purified SpoIIID in addition to the carrier protein and then subjected to DNase I footprinting in a total volume of 42 μl. Arrowheads denote the boundaries of protection by SpoIIID, and numbers to the left refer to positions relative to the TSS (3). Stars indicate enhanced cleavage by DNase I upon SpoIIID binding.

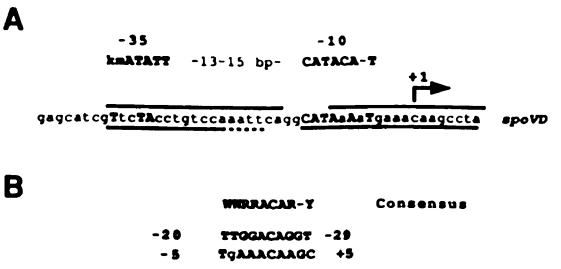

FIG. 3. Position of SpoIIID binding sites in the *spoVD* promoter region and alignment of sequences within these sites with the consensus sequence for SpoIIID binding. (A) The nucleotide sequence of the *spoVD* promoter region is aligned with respect to conserved nucleotides found in the −10 and −35 regions of promoters transcribed by $E\sigma^E$ (21), shown at the top (k means G or T, and m means A or C). Nucleotides in the *spoVD* promoter that match the consensus are shown as boldface capital letters. Overlining and underlining indicate regions on the nontranscribed and transcribed strands, respectively, protected by SpoIIID from digestion with DNase I (Fig. 2). The dashed portion of the line indicates a region of uncertain protection due to a lack of DNase I digestion in this region. (B) Nucleotide sequences within the SpoIIID-protected regions of the *spoVD* promoter are aligned with respect to the consensus sequence for SpoIIID binding (8), shown at the top (W means A or T, R means purine, and Y means pyrimidine). Numbers refer to positions relative to the TSS. Note that the sequence shown for the binding site between −20 and −29 is from the strand opposite that shown in panel A. Nucleotides that match the consensus are shown as boldface capital letters.

scribed strand, were prepared as follows. pSG1362 (3) was digested with *Hin*dIII, which cleaves in the multiple cloning site downstream of the *spoVD* promoter, and labeled either at the 3′ end by the Klenow enzyme fill-in reaction and [α-^{32}P]dATP or at the 5′ end by treatment with alkaline phosphatase followed by T4 polynucleotide kinase and [γ-^{32}P]ATP. In both cases, the labeled DNA was digested with *Sac*I, which cleaves upstream of the *spoVD* promoter, to produce a 340-bp fragment that was purified by elution from an 8% polyacrylamide gel. The purified DNA fragments were incubated with different amounts of SpoIIID and then mildly digested with DNase I. After DNase I treatment, the partially digested DNAs were electrophoresed in 6% polyacrylamide gels containing 8 M urea alongside a sequencing ladder generated by subjecting the appropriate end-labeled DNA to the chemical cleavage reactions of Maxam and Gilbert as described previously (19). Figure 2 shows that SpoIIID protected two regions of the *spoVD* promoter from DNase I digestion. Site 1 spanned positions −33 to −15 on the nontranscribed strand (Fig. 2A) and −33 to −21 on the transcribed strand (Fig. 2B), while protection in site 2 spanned positions −9 to +8 on the nontranscribed strand (Fig. 2A) and −12 to +7 on the transcribed strand (Fig. 2B). The results of DNase I protection experiments are summarized in Fig. 3A. Greater than half-maximal protection of both sites 1 and 2 was achieved at a 50 nM concentration of SpoIIID (Fig. 2A and B, lanes 2), putting the two sites into the group of strong SpoIIID binding sites along with sites 1 and 2 of *bofA*, site 1 of *spoIVCA*, sites 2 and 3 of *cotD*, and site 2 of *sigK* (8). Since we estimated from Western blot experiments that the SpoIIID concentration reaches the 1 μM range in sporulating cells (data not shown), it seems likely that SpoIIID occupies sites 1 and 2 in the *spoVD* promoter in vivo and accounts for the twofold lower expression of *spoVD-lacZ* in wild-type cells than in *spoIIID* mutant cells (3).

By aligning the nucleotide sequences of known SpoIIID binding sites, a consensus sequence, WWRRACAR-Y (where W is A or T, R is purine, and Y is pyrimidine), was found (8). SpoIIID binding site 1 in the *spoVD* promoter region contains a perfect match to the consensus sequence, while binding site 2 contains a sequence with just one mismatch (Fig. 3B). While many strong SpoIIID binding sites exhibit a second good match to the consensus sequence in inverted orientation relative to the best match (8), no such second match is found in either of the two protected regions in the *spoVD* promoter.

Of the two SpoIIID binding sites mapped in the *spoVD* promoter region, one overlaps the −35 region and the other encompasses the −10 region and the TSS. Hence, SpoIIID binding to either site is likely to interfere with promoter recognition by $E\sigma^E$, explaining the strong repressive effect of SpoIIID on *spoVD* transcription in vitro (Fig. 1). A previous study showed that SpoIIID binding in the −35 region of the *cotD* promoter is sufficient to mediate repression (8). Also, the SpoIIID-repressed *bofA* promoter has a SpoIIID-binding site encompassing the −10 region and the TSS, although it was not determined whether a second SpoIIID-binding site located at +54 to +74 is needed for repression (8). Mutational analysis of the *spoVD* promoter region should permit dissection of whether the two SpoIIID binding sites function in an additive, cooperative, or redundant fashion to repress transcription by $E\sigma^E$.

We thank C. Moran for sending partially purified $E\sigma^E$ and R. Halberg for advice on purification of σ^E, σ^K, and SpoIIID.

This work was supported by NIH grant 43585 to L.K. and by the Michigan Agricultural Experimentation Station. Work in the Errington laboratory was supported by the Biotechnology and Biological Sciences Research Council of the U.K.

REFERENCES

1. **Coote, J. G.** 1972. Sporulation in *Bacillus subtilis*. Characterization of oligosporogenous mutants and comparison of their phenotypes with those of asporogenous mutants. J. Gen. Microbiol. **71**:1–15.
2. **Cutting, S., S. Roels, and R. Losick.** 1991. Sporulation operon *spoIVF* and the characterization of mutations that uncouple mother-cell from forespore gene expression in *Bacillus subtilis*. J. Mol. Biol. **221**:1237–1256.
3. **Daniel, R. A., S. Drake, C. E. Buchanan, R. Scholle, and J. Errington.** 1994. The *Bacillus subtilis spoVD* gene encodes a mother-cell-specific penicillin-binding protein required for spore morphogenesis. J. Mol. Biol. **235**:209–220.
4. **Driks, A., and R. Losick.** 1991. Compartmentalized expression of a gene under the control of sporulation transcription factor σ^E in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **88**:9934–9938.
5. **Errington, J.** 1991. Possible intermediate steps in the evolution of a prokaryotic developmental system. Proc. R. Soc. Lond. B **244**:117–121.
6. **Errington, J.** 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. **57**:1–33.
7. **Errington, J., and J. Mandelstam.** 1986. Use of a *lacZ* gene fusion to determine the dependence pattern of sporulation operon *spoIIA* in *spo* mutants of *Bacillus subtilis*. J. Gen. Microbiol. **132**:2967–2976.
8. **Halberg, R., and L. Kroos.** 1994. Sporulation regulatory protein SpoIIID from *Bacillus subtilis* activates and represses transcription by both mother-cell-specific forms of RNA polymerase. J. Mol. Biol. **243**:425–436.
9. **Haldenwang, W. G.** 1995. The sigma factors of *Bacillus subtilis*. Microbiol. Rev. **59**:1–30.
10. **Harry, E. J., K. Pogliano, and R. Losick.** 1995. Use of immunofluorescence to visualize cell-specific gene expression during sporulation in *Bacillus subtilis*. J. Bacteriol. **177**:3386–3393.
11. **Illing, N., and J. Errington.** 1991. The *spoIIIA* operon of *Bacillus subtilis* defines a new temporal class of mother-cell-specific sporulation genes under the control of the σ^E form of RNA polymerase. Mol. Microbiol. **5**:1927–1940.
12. **Ireton, K., and A. Grossman.** 1992. Interaction among mutations that cause altered timing of gene expression during sporulation in *Bacillus subtilis*. J. Bacteriol. **174**:3185–3195.
13. **Jones, C. H., K. M. Tatti, and C. P. Moran.** 1992. Effects of amino acid substitutions in the −10 binding region of σ^E from *Bacillus subtilis*. J. Bacteriol. **174**:6815–6821.
14. **Kroos, L., B. Kunkel, and R. Losick.** 1989. Switch protein alters specificity of RNA polymerase containing a compartment-specific sigma factor. Science **243**:526–529.
15. **Kunkel, B., L. Kroos, H. Poth, P. Youngman, and R. Losick.** 1989. Temporal and spatial control of the mother-cell regulatory gene *spoIIID* of *Bacillus subtilis*. Genes Dev. **3**:1735–1744.
16. **Kunkel, B., K. Sandman, S. Panzer, P. Youngman, and R. Losick.** 1988. The promoter for a sporulation gene in the *spoIVC* locus of *Bacillus subtilis* and

its use in studies of temporal and spatial control of gene expression. J. Bacteriol. **170**:3513–3522.

17. **Lewis, P. J., C. E. Nwoguh, M. R. Rarer, C. J. Harwood, and J. Errington.** 1994. Use of digitized video microscopy with a fluorogenic enzyme substrate to demonstrate cell- and compartment-specific gene expression in *Salmonella enteritidis* and *Bacillus subtilis*. Mol. Microbiol. **13**:3849–3853.
18. **Losick, R., and P. Stragier.** 1992. Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*. Nature **355**:601–604.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Resnekov, O., S. Alper, and R. Losick.** 1996. Subcellular localization of proteins governing the proteolytic activation of a developmental transcription factor in *Bacillus subtilis*. Genes Cells **1**:529–542.
21. **Roels, S., A. Driks, and R. Losick.** 1992. Characterization of *spoIVA*, a sporulation gene involved in coat morphogenesis in *Bacillus subtilis*. J. Bacteriol. **174**:575–585.
22. **Stevens, C. M., and J. Errington.** 1990. Differential gene expression during sporulation in *Bacillus subtilis*: structure and regulation of the *spoIIID* gene. Mol. Microbiol. **4**:543–552.
23. **Tatti, K. M., C. H. Jones, and C. P. Moran.** 1991. Genetic evidence for interaction of σ^E with the *spoIIID* promoter in *Bacillus subtilis*. J. Bacteriol. **173**:7828–7833.
24. **Zheng, L., and R. Losick.** 1990. Cascade regulation of spore coat gene expression in *Bacillus subtilis*. J. Mol. Biol. **212**:645–660.

BIBLIOGRAPHY

Bibliography

1. **Aizawa, S.** 1996. Flagellar assembly in *Salmonella typhimurium.* Mol. Microbiol. **19**:1-5.

2. **Alex, L. A. and M. I. Simon.** 1994. Protein histidine kinases and signal transduction in prokaryotes and eukaryotes. Trends. Genet. **10**:133-138.

3. **Alper, S., L. Duncan, and R. Losick.** 1994. An adenosine nucleotide switch controlling the activity of a cell type-specific transcription factor in *B. subtilis.* Cell **77**:195-205.

4. **Anderson, M., Y. T. Yu, and L. Kroos.** Unpublished results.

5. **Arigoni, F., K. Pogliano, C. D. Webb, P. Stragier, and R. Losick.** 1995. Localization of protein implicated in establishment of cell type to sites of asymmetric division. Science **270**:637-640.

6. **Aza-Blanc, P., F. A. Ramirez-Weber, M. P. Laget, C. Schwartz, and T. B. Kornberg.** 1997. Proteolysis that is inhibited by Hedgehog targets Cubitus interruptus protein to the nucleus and convert it to a repressor. Cell **89**:1043-1053.

7. **Bagyan, I., J. Hobot, and S. Cutting.** 1996. A compartmentalized regulator of developmental gene expression in *Bacillus subtilis.* J. Bacteriol. **178**:4500-4507.

8. **Bai, U., I. Mandic-Mulec, and I. Smith.** 1993. SinI modulates the activity of SinR, a developmental switch protein of *Bacillus subtilis.* Genes Dev. **7**:139-148.

9. **Baldus, J. M., C. M. Buckner, and C. P. Moran.** 1995. Evidence that the transcriptional activator Spo0A interacts with two sigma factors in *Bacillus subtilis.* Mol. Microbiol. **17**:281-290.

10. **Baldus, J. M., B. D. Green, P. Youngman, and C. P. J. Moran.** 1994. Phosphorylation of *Bacillus subtilis* transcription factor Spo0A stimulates transcription from the *spoIIG* promoter by enhancing binding to weak 0A boxes. J. Bacteriol. **176**:296-306.

11. **Barak, I., J. Behari, G. Olmedo, P. Guzman, D. P. Brown, and P. Youngman.** 1996. Structure and function of the *Bacillus subtilis* SpoIIE protein and its localization at the sites of septum assembly. Mol. Microbiol. **19**:1047-1060.

12. **Bird, T. H., J. K. Grimsley, J. A. Hoch, and G. B. Spiegelman.** 1996. The *Bacillus subtilis* response regulator *spo0A* stimulates transcription of the *spoIIG* operon through modification of RNA polymerase promoter complex. J. Mol. Biol.

256:436-448.

13. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.

14. **Bramucci, M. G., B. D. Green, N. Ambulos, and P. Youngman.** 1995. Identification of a *Bacillus subtilis spo0H* allele that is necessary for suppression of the sporulation-defective phenotype of a *spo0A* mutation. J. Bacteriol. **177**:1630-1633.

15. **Bramucci, M. G., B. D. Green, N. Ambulos, and P. Youngman.** 1995. Identification of a *Bacillus subtilis* allele that is necessary for suppression of the sporulation phenotype of a *spo0A* mutation. J. Bacteriol. **177**:1630-1633.

16. **Burbulys, D., K. A. Trach, and J. A. Hoch.** 1991. Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. Cell **64**:545-552.

17. **Cutting, S., A. Driks, R. Schmidt, B. Kunkel, and R. Losick.** 1991. Forespore-specific transcription of a gene in the signal transduction pathway that governs pro-σ^K processing in *Bacillus subtilis*. Genes Dev. **5**:456-466.

18. **Cutting, S., V. Oke, A. Driks, R. Losick, S. Lu, and L. Kroos.** 1990. A forespore checkpoint for mother-cell gene expression during development in *Bacillus subtilis*. Cell **62**:239-250.

19. **Cutting, S., S. Roels, and R. Losick.** 1991. Sporulation operon *spoIVF* and the characterization of mutations that uncouple mother-cell from forespore gene expression in *Bacillus subtilis*. J. Mol. Biol. **221**:1237-1256.

20. **Diederich, B., J. F. Wilkinson, T. Magnin , S. M. A. Najafi, J. Errington, and M. D. Yudkin.** 1994. Role of interactions between SpoIIAA and SpoIIAB in regulating cell-specific transcription factor σ^F of *Bacillus subtilis.* Genes Dev. **8**:2653-2663.

21. **Dombroski, A. J., W. A. Walter, and C. A. Gross.** 1993. Amino-terminal amino acids modulate σ-factor DNA-binding activity. Genes & Dev. **7**:2446-2455.

22. **Dombroski, A. J., W. A. Walter, M. T. Record, D. A. Siegele, and C. A. Gross.** 1992. Polypeptides containing highly conserved regions of transcription initiation factor σ^{70} exhibit specificity of binding to promoter DNA. Cell **70**:501-512.

23. **Driks, A. and R. Losick.** 1991. Compartmentalized expression of a gene under the control of sporulation transcription factor σ^E in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **88**:9934-9938.

24. **Driks, A., S. Roels, B. Beall, C. P. Moran, and R. Losick.** 1994. Subcellular localization of proteins involved in the assembly of the spore coat of *Bacillus subtilis*. Genes & Dev. **8**:234-244.

25. **Duncan, L., S. Alper, F. Arigoni, R. Losick, and P. Stragier.** 1995.

Activation of cell-specific transcription by a serine phosphatase at the site of asymmetric division. Science **270**:641-644.

26. **Duncan, L. and R. Losick**. 1993. SpoIIAB is an anti-σ factor that binds to and inhibits transcription by regulatory protein σ^F from *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **90**:2325-29.

27. **Errington, J.** 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. **57**:1-33.

28. **Freese, E., J. E. Heinze, and E. M. Gelliers**. 1979. Partial purine deprivation causes sporulation of *Bacillus subtilis* in the presence of excess ammonia, glucose, and phosphate. J. Gen. Microbiol. **115**:193-205.

29. **Fuqua, C., S. C. Winan, and E. P. Greenberg**. 1996. Census and consensus in bacterial ecosystem: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Ann. Rev. Microbiol. **50**:527-551.

30. **Gaur, N. K., J. Oppenheim, and I. Smith**. 1991. The *Bacillus subtilis sin* gene, a regulator of alternate developmental processes, codes for a DNA-binding protein. J. Bacteriol. **173**:678-686.

31. **Gholamhoseinian, A. and P. J. Piggot**. 1989. Timing of *spoII* gene expression relative to septum formation during sporulation of *Bacillus subtilis*. J. Bacteriol. **171**:5747-5749.

32. **Gomez, M., S. Cutting, and P. Stragier**. 1995. Transcription of *spoIVB* is the only role of σ^G that is essential for pro-σ^K processing during spore formation in *Bacillus subtilis*. J. Bacteriol. **177**:4825-4827.

33. **Gorham, H. C., S. J. McGowan, P. R. Robson, and D. A. Hodgson**. 1996. Light-induced carotenogenesis in *Myxococcus xanthus*: light-dependent membrane sequestration of ECF sigma factor CarQ by anti-sigma factor CarR. Mol. Microbiol. **19**:171-186.

34. **Grossman, A.** 1995. Genetic networks controlling the initiation of sporulation and the development of genetic competence in *Bacillus subtilis*. Ann. Rev. Genetics **29**:477-508.

35. **Grossman, A. D., T. Lewis, N. Levin, and R. DeVivo**. 1992. Suppressors of a *spo0A* missense mutation and their effects on sporulation in *Bacillus subtilis*. Biochimie **74**:679-688.

36. **Guzman, P., J. Westpheling, and P. Youngman**. 1988. Characterization of the promoter region of the *Bacillus subtilis spoIIE* operon. J. Bacteriol. **170**:1598-1609.

37. **Halberg, R.** 1994. Ph.D. Thesis. Michigan State University, East Lansing.

38. **Halberg, R. and L. Kroos**. 1992. Fate of the SpoIIID switch protein during *Bacillus subtilis* sporulation depends on the mother-cell sigma factor, σ^K. J. Mol. Biol. **228**:840-849.

39. **Halberg, R. and L. Kroos**. 1994. Sporulation regulatory protein SpoIIID from *Bacillus subtilis* activates and represses transcription by both mother-cell-specific forms of RNA polymerase. J. Mol. Biol. **243**:425-436.

40. **Halberg, R., V. Oke, and L. Kroos**. 1995. Effects of *Bacillus subtilis* sporulation regulatory protein SpoIIID on transcription by σ^K RNA polymerase *in vivo* and *in vitro*. J. Bacteriol. **177**:1888-1891.

41. **Haldenwang, W. G.** 1995. The sigma factors of *Bacillus subtilis*. Microbiol. Rev. **59**:1-30.

42. **Harry, E. J., K. Pogliano, and R. Losick**. 1995. Use of immunofluorescence to visualize cell-specific gene expression during sporulation in *Bacillus subtilis*. J. Bacteriol. **177**:3386-3393.

43. **Harwood, C. R. and S. M. Cutting**. 1990. Molecular biological methods for *Bacillus*. John Wiley & Sons, Chichester, England.

44. **Healy, J., J. Weir, I. Smith, and R. Losick**. 1991. Post-transcriptional control of a sporulation regulatory gene encoding transcription factor σ^H in *Bacillus subtilis*. Mol. Microbiol. **5**:477-487.

45. **Hicks, K. A. and A. D. Grossman**. 1996. Altering the level and regulation of the major sigma subunit of RNA polymerase affects gene expression and development in *Bacillus subtilis*. Mol. Miocrobiol. **20**:201-212.

46. **Hoch, J.** 1994. The Phosphorelay Signal Transduction Pathway in the Initiation of Sporulation, p. 41-60. *In* Piggot, P.J., C.P. Moran and P. Youngman (ed.), Regulation of Bacterial Differentiation. American Society for Microbiology, Washington, D.C.

47. **Hofmeister, A.** 1997. Activation of the proprotein transcription factor pro-σ^E is associated with three changes in its subcellular localization during sporulation in *Bacillus subtilis*. J. Bacteriol. **submitted**:

48. **Hofmeister, A., A. Londono-Vallejo, E. Harry, P. Stragier, and R. Losick**. 1995. Extracellular signal protein triggering the proteolytic activation of a developmental transcription factor in *B. subtilis*. Cell **83**:219-226.

49. **Hughes, K. T., K. L. Gillen, M. J. Semon, and J. E. Karlinsey**. 1993. Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator. Science **262**:1277-1280.

50. **Ichikawa, H. and L. Kroos**. Unpublished data.

51. **Ireton, K. and A. Grossman**. 1992. Interaction among mutations that cause altered timing of gene expression during sporulation in *Bacillus subtilis*. J. Bacteriol. **174**:3185-3195.

52. **Ireton, K. and A. D. Grossman**. 1992. Coupling between gene expression and DNA synthesis early during development in Bacillus subtilis. Proc. Natl. Acad. Sci. USA **89**:8808-8812.

53. **Ireton, K. and A. D. Grossman**. 1994. A developmental checkpoint couples the initiation of sporulation to DNA replication in *Bacillus subtilis*. EMBO J. **13**:1566-1573.

54. **Ireton, K., N. W. Gunther, and A. D. Grossman**. 1994. *spo0J* is required for normal chromosome segregation as well as the initiation of sporulation in *Bacillus subtilis*. J. Bacteriol. **176**:5320-5329.

55. **Ireton, K., D. Z. Rudner, K. J. Siranosian, and A. D. Grossman**. 1993. Integration of multiple developmental signals in *Bacillus subtilis* through the *spo0A* transcription factor. Genes Dev. **7**:283-294.

56. **Ireton, K., D. Z. Rudner, K. J. Siranosian, and A. D. Grossman**. 1995. Krebs cycle function is required for activation of the Spo0A transcription factor. Proc. Natl. Acad. Sci. USA **92**:2845-2849.

57. **Jin, S. and A. L. Sonenshein**. 1994. Transcritional regulation of *Bacillus subtilis* citrate synthase genes. J. Bacteriol. **176**:4680-4690.

58. **Johnson, B. and A. Dombroski**. 1997. The role of the pro-sequence of *Bacillus subtilis* σ^K in controlling activity in transcription initiation. J. Biol. Chem. **in press**:

59. **Jonas, R. M., E. A. Weaver, T. J. Kenney, C. P. Moran, and W. G. Haldenwang**. 1988. The *Bacillus subtilis spoIIG* operon encodes both σ^E and a gene necessary for σ^E activation. J. Bacteriol. **170**:507-511.

60. **Ju, J., T. Luo, and W. Haldenwang**. 1997. *Bacillus subtilis* pro-σ^E fusion protein localizes to the forespore septum and fails to be processed when synthesized in the forespore. J. Bacteriol. **179**:4888-4893.

61. **Karow, M. L., P. Glaser, and P. Piggot**. 1995. Identification of a gene, *spoIIR*, that links the activation of σ^E to the transcriptional activity of σ^F during sporulation in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **92**:2012-2016.

62. **Katsukake**. 1994. Excretion of the anti-sigma factor through a flagella structure couples flagella gene expression with flagella assembly in *Salmonella typhymurium*. Mol. Gen. Genet. **243**:605-612.

63. **Kellner, E. M., A. Decatur, and C. P. Moran.** 1996. Two-stage regulation of an anti-sigma factor determines developmental fate during bacterial endospore formation. Mol. Microbiol. **21**:913-924.

64. **Kenney, T. J. and C. P. Moran**. 1987. Organization and regulation of an operon that encodes a sporulation-essential sigma factor in *Bacillus subtilis*. J. Bacteriol. **169**:3329-3339.

65. **Kenney, T. J., K. York, P. Youngman, and C. P. Moran**. 1989. Genetic evidence that RNA polymerase associated with σ^A uses a sporulation-specific promoter in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **86**:9109-9113.

66. **Kirchman, P. A., H. DeGrazia, E. M. Kellner, and J. C.P. Moran.** 1993. Forespore-specific disappearance of the sigma-factor antagonist SpoIIAB: implications for its role in determination of cell fate in *Bacillus subtilis*. Mol. Microbiol. **8**:663-671.

67. **Kobayashi, K., K. Shoji, T. Shimizu, K. Nakano, T. Sato, and Y. Kobayashi.** 1995. Analysis of a suppressor mutation *ssb* (*kinC*) of *sur0B20* (*spo0A*) mutation in *Bacillus subtilis* reveals that *kinC* encodes a histidine protein kinase. J. Bacteriol. **177**:176-182.

68. **Kroos, L. and S. Cutting.** 1994. Intercellular and intercompartmental communication during *Bacillus subtilis* sporulation, p. 155-180. *In* Piggot, P.J., C.P. Moran and P. Youngman (ed.), Regulation of Bacterial Differentiation. Amer. Soc. for Microbiol., Washington, DC.

69. **Kroos, L., B. Kunkel, and R. Losick.** 1989. Switch protein alters specificity of RNA polymerase containing a compartment-specific sigma factor. Science **243**:526-529.

70. **Kunkel, B., L. Kroos, H. Poth, P. Youngman, and R. Losick.** 1989. Temporal and spatial control of the mother-cell regulatory gene *spoIIID* of *Bacillus subtilis*. Genes Dev. **3**:1735-1744.

71. **Kunkel, B., R. Losick, and P. Stragier.** 1990. The *Bacillus subtilis* gene for the developmental transcription factor σ^K is generated by excision of a dispensable DNA element containing a sporulation recombinase gene. Genes Dev. **4**:525-535.

72. **Kunkel, B., K. Sandman, S. Panzer, P. Youngman, and R. Losick.** 1988. The promoter for a sporulation gene in the *spoIVC* locus of *Bacillus subtilis* and its use in studies of temporal and spatial control of gene expression. J. Bacteriol. **170**:3513-3522.

73. **Kutsukake, K., Y. Ohya, and T. Iino.** 1990. Transcriptional analysis of the flagellar regulon of *Salmonella typhimurium*. J. Bacteriol. **172**:741-747.

74. **LaBell, T. L., J. E. Trempy, and W. G. Haldenwang.** 1987. Sporulation-specific sigma factor, σ^{29}, of *Bacillus subtilis* is synthesized from a precursor protein, P^{31}. Proc. Natl. Acad. Sci. USA **84**:1784-1788.

75. **Lazazzera, B. A., J. M. Solomon, and A. D. Grossman.** 1997. An Exported peptide functions intracellularly to contribute to cell density signalling in *B. subtilis*. Cell **89**:917-925.

76. **LeDeaux, J. R. and A. D. Grossman.** 1995. Isolation and Characterization of kinC, a gene that encodes a sensor kinase homologous to the sporulation sensor kinases KinA and KinB in *Bacillus subtilis*. J. Bacteriol. **177**:166-175.

77. **LeDeaux, J. R., N. Yu, and A. D. Grossman.** 1995. Different roles for KinA, KinB, and KinC in the initiation of sporulation. J. Bacteriol. **177**:861-863.

78. **Levin, P. A. and R. Losick.** 1996. Transcription factor Spo0A switches the localization of the cell division protein FtsZ from a medial to a bipolar pattern in *Bacillus*

subtilis. Genes Dev. **10**:478-488.

79. **Lewis, P. J., S. R. Partridge, and J. Errington**. 1994. σ factors, asymmetry, and the determination of cell fate in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **91**:3849-3853.

80. **Lin, D. C. H., P. A. Levin, and A. D. Grossman**. 1997. Bipolar localization of a chromosome partitioning protein in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **94**:4721-4726.

81. **Linn, T. G., A. L. Greenleaf, R. G. Shorenstein, and R. Losick**. 1973. Loss of the sigma activity of RNA polymerase of *Bacillus subtilis* during sporulation. Proc. Natl. Acad. Sci. USA **70**:1865-1869.

82. **Londono-Vallejo, J. A.** 1997. Mutational analysis of the early forespore/mother-cell signalling pathway in *Bacillus subtilis*. Microbiology **143**:2753-2761.

83. **Londono-Vallejo, J. A. and P. Stragier**. 1995. Cell-cell signaling pathway activating a developmental transcription factor in *Bacillus subtilis*. Genes Dev. **9**:503-508.

84. **Lopez, J. M., A. Dromerick, and E. Freese**. 1981. Response of guanosine 5'-triphosphate concentration to nutritional changes and its significance for *Bacillus subtilis* sporulation. J. Bacteriol. **146**:605-613.

85. **Lopez, J. M., C. L. marks, and E. Freese**. 1979. The decrease of guanine nucleotides initiates sporulation of *Bacillus subtilis*. Biochem. Biophys. Acta. **587**:238-252.

86. **Losick, R. and P. Stragier**. 1992. Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*. Nature **355**:601-604.

87. **Losick, R., P. Youngman, and P. J. Piggot**. 1986. Genetics of endospore formation in *Bacillus subtilis*. Ann. Rev. Genet. **20**:625-669.

88. **Lu, S., S. Cutting, and L. Kroos**. 1995. Sporulation protein SpoIVFB from *Bacillus subtilis* enhances processing of the sigma factor precursor pro-σ^K in the absence of other sporulation gene products. J. Bacteriol. **177**:1082-1085.

89. **Lu, S., R. Halberg, and L. Kroos**. 1990. Processing of the mother-cell σ factor, σ^K, may depend on events occurring in the forespore during *Bacillus subtilis* development. Proc. Natl. Acad. Sci. USA **87**:9722-9726.

90. **Lu, S. and L. Kroos**. 1994. Overproducing the *Bacillus subtilis* mother-cell sigma factor precursor, pro-σ^K, uncouples σ^K-dependent gene expression from dependence on intercompartmental communication. J. Bacteriol. **176**:3936-3943.

91. **Mandic-Mulec, I., L. Doukhan, and I. Smith**. 1995. The *Bacillus subtilis* SinR protein is a repressor of the key sporulation gene *spo0A*. J. Bacteriol. **177**:4619-4627.

92. **Mandic-Mulec, I., N. Gaur, U. Bai, and I. Smith**. 1992. Sin, a stage-

specific repressor of cellular differentiation. J. Bacteriol. **174**:3561-3569.

93. **Margolis, P., A. Driks, and R. Losick**. 1991. Establishment of cell type by compartmentalized activation of a transcription factor. Science **254**:562-565.

94. **Mathee, K., C. J. Mcpherson, and D. E. Ohman**. 1997. Posttranslational control of the *algT* (*algU*)-encoded σ^{22} for expression of the alginate regulon in *Pseudomonas aeruginosa* and localization of its antagonist proteins MucA and MucB. J. Bacteriol. **179**:3711-3720.

95. **Min, K., C. M. Hilditch, B. Diederich, J. Errington, and M. D. Yudkin**. 1993. σ^F, the first compartment-specific transcription factor of *Bacillus subtilis*, is regulated by an anti-σ factor that is also a protein kinase. Cell **74**:735-742.

96. **Mitani, T., J. F. Heinze, and E. Freese**. 1977. Induction of sporulation in *Bacillus subtilis* by decoynine or hadacidin. Biochem. Biophys. Res. Commun. **77**:1118-1125.

97. **Miyao, A., G. Theeragool, M. Takeuchi, and Y. Kobayashi**. 1993. *Bacillus subtilis spoVE* gene is transcribed by -associated RNA polymerase. J. Bacteriol. **175**:4081-4086.

98. **Ohlsen, K. L., J. K. Grimsley, and J. A. Hoch**. 1994. Deactivation of the sporulation transcription factor Spo0A by the Spo0E protein phosphatase. Pro. Natl. Acad. Sci. USA **91**:1756-1760.

99. **Oke, V. and R. Losick**. 1993. Multilevel regulation of the sporulation transcription factor σ^K in *Bacillus subtilis*. J. Bacteriol. **175**:7341-7347.

100. **Palombella, V. J., O. J. Rando, A. L. Goldberg, and T. Maniatis**. 1994. The ubiquitin-proteasome pathway is required for processing the NF-κB precursor and the activation of NF-κB. Cell **78**:773-785.

101. **Parkinson, J. S.** 1993. Signal transduction schemes of bacteria. Cell **73**:857-871.

102. **Partridge, S. R. and J. Errington**. 1993. Importance of morphological events and intercellular interactions in the regulation of prespore-specific gene expression during sporulation in *Bacillus subtilis*. Mol. Microbiol. **8**:945-955.

103. **Partridge, S. R., D. Foulger, and J. Errington**. 1991. The role of σ^F in prespore-specific transcription in *Bacillus subtilis*. Mol. Microbiol. **5**:757-767.

104. **Perego, M., P. Glaser, and J. A. Hoch**. 1996. Aspartyl-phosphate phosphatases deactivate the response regulator components of the sporulation signal transduction system in *Bacillus subtilis*. Mol. Microbiol. **19**:1151-1157.

105. **Perego, M., C. Hanstein, K. M. Welsh, T. Djavakhishvili, P. Glaser, and J. A. Hoch**. 1994. Multiple protein-aspartate phosphatases provide a mechanism for the integration of diverse signals in the control of development in *B. subtilis*. Cell **79**:1047-1055.

106. **Perego, M. and J. A. Hoch.** 1991. Negative regulation of *Bacillus subtilis* sporulation by the *spo0E* gene product. J. Bacteriol. **173**:2514-2520.

107. **Perego, M. and J. A. Hoch.** 1996. Cell-cell communication regulates the effects of protein aspartate phosphatases on phosphorelay controlling development in *Bacillus subtilis.* Proc. Natl. Acad. Sci. USA **93**:1549-1553.

108. **Perego, M., J.-J. Wu, G. B. Spiegelman, and J. A. Hoch.** 1991. Mutational dissociation of the positive and negative regulatory properties of the Spo0A sporulation transcription factor of *Bacillus subtilis*. Gene **100**:207-212.

109. **Peters, H. K., H. C. Carlson, and W. G. Haldenwang.** 1992. Mutational analysis of the precursor-specific region of *Bacillus subtilis* σ^E. J. Bacteriol. **174**:4629-4637.

110. **Peters, H. K. and W. G. Haldenwang.** 1994. Isolation of a *Bacillus subtilis spoIIGA* allele that suppresses processing-negative mutations in the pro-σ^E gene (*sigE*). J. Bacteriol. **176**:7763-7766.

111. **Pogliano, K., E. Harry, and R. Losick.** 1995. Visualization of the subcellular location of sporulation proteins in *Bacillus subtilis* using immunofluorescence microscopy. Mol. Microbiol. **18**:459-470.

112. **Pogliano, K., A. Hofmeister, and R. Losick.** 1997. Disappearance of the σ^E transcription factor from the forespore and the SpoIIE phosphatase from the mother cell contributes to establishment of cell-specific gene expression during sporulation in *Bacillus subtilis*. J. Bacteriol. **179**:3331-3341.

113. **Predich, M., G. Nair, and I. Smith.** 1992. *Bacillus subtilis* early sporulation genes *kinA, spo0F,* and *spo0A* are transcribed by RNA polymerase containing σ^H. J. Bacteriol. **174**:2771-2778.

114. **Rather, P. N., R. Coppolecchia, H. DeGrazia, and J. C.P. Moran .** 1990. Negative regulator of σ^G-controlled gene expression in stationary-phase *Bacillus subtilis*. J. Bacteriol. **172**:709-715.

115. **Resnekov, O., S. Alper, and R. Losick.** 1996. Subcellular localization of proteins governing the proteolytic activation of a developmental transcription factor in *Bacillus subtilis*. Genes to Cells **1**:529-542.

116. **Ricca, E., S. Cutting, and R. Losick.** 1992. Characterization of *bofA*, a gene involved in intercompartmental regulation of pro-σ^K processing during sporulation in *Bacillus subtilis*. J. Bacteriol. **174**:3177-3184.

117. **Sakai, J., E. A. Duncan, R. B. Rawson, X. Hua, M. S. Brown, and J. L. Goldstein.** 1996. Sterol-regulated release of SREBP-2 from cell membrane requires two sequential cleavages, one within a transmembrane domain. Cell **85**:1037-1048.

118. **Sandman, K., L. Kroos, S. Cutting, P. Youngman, and R. Losick.** 1988. Identification of the promoter for a spore coat protein gene in *Bacillus subtilis* and

studies on the regulation of its induction at a late stage of sporulation. J. Mol. Biol. **200**:461-473.

119. **Sato, T., K. Harada, Y. Ohta, and Y. Kobayashi**. 1994. Expression of the *Bacillus subtilis spoIVCA* gene, which encodes a site-specific recombinase, depends on the *spoIIGB* product. J. Bacteriol. **176**:935-937.

120. **Satola, S. W., J. M. Baldus, and C. P. Moran**. 1992. Binding of Spo0A stimulates *spoIIG* promoter activity in *Bacillus subtilis*. J. Bacteriol. **174**:1448-1453.

121. **Schmidt, R., A. Decatur, P. Rather, C. Moran, and R. Losick**. 1994. *Bacillus subtilis* Lon protease prevents inappropriate transcription of genes under the control of the sporulation transcription factor σ^G. J. Bacteriol. **176**:6528-6537.

122. **Setlow, P.** 1989. Forespore-specific genes of *Bacillus subtilis*: function and regulation of expression. 211-221.

123. **Sharpe, M. E. and J. Errington**. 1996. The *Bacillus subtilis soj-spo0J* locus is required fora centromere-like function involved in prespore chromosome partitioning. Mol. Microbiol. **21**:501-509.

124. **Sharrock, R. A., S. Rubenstein, M. Chan, and T. Leighton**. 1984. Intergenic suppression of *spo0* phenotypes by the *Bacillus subtilis* mutation *rvtA*. Mol. Gen. Genet. **194**:260-264.

125. **Shazand, K., N. Frandsen, and P. Stragier**. 1995. Cell-type specificity during development in *Bacillus subtilis*: the molecular and morphological requirements for σ^E activation. EMBO J. **14**:1439-1445.

126. **Shcheptov, M., G. Chyu, I. Bagyan, and S. Cutting**. 1997. Characterization of *csgA*, a new member of the forespore-expressed regulon from *Bacillus subtilis*. Gene **184**:133-140.

127. **Shurr, M. J., H. Yu, J. M. Martinez-Salazar, J. C. Boucher, and V. Deretic**. 1996. Control of AlgU, a member of the σ^E-like family of stress sigma factor, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. J. Bacteriol. **178**:4997-5004.

128. **Siranosian, K. J., K. Ireton, and A. D. Grossman**. 1993. Alanine dehydrogenase (*ald*) is required for normal sporulation in *Bacillus subtilis*. J. Bacteriol. **175**:6789-6796.

129. **Smith, I., I. Mandic-Mulec, and N. Gaur**. 1991. The role of negative control in sporulation. Res. Microbiol. **142**:831-839.

130. **Solomon, J. M., B. A. Lazazzera, and A. D. Grossman**. 1996. Purification and characterization of an extracellular peptide factor that affects two different developmental pathways in *Bacillus subtilis*. Genes Dev. **10**:2014-2024.

131. **Solomon, J. M., R. Magnuson, A. Srivastava, and A. d. Grossman**. 1995. Convergent sensing pathways mediate response to two extracellular competence factors in *Bacillus subtilis*. Genes Dev. **9**:547-558.

132. **Stevens, C. M. and J. Errington**. 1990. Differential gene expression during sporulation in *Bacillus subtilis*: structure and regulation of the *spoIIID* gene. Molec. Microbiol. **4**:543-552.

133. **Stock, J. B., A. J. Ninfa, and A. M. Stock**. 1989. Protein phosphorylation and regulation of adaptive response in bacteria. Microbiol. Rev. **53**:450-490.

134. **Stragier, P.** 1992. Establishment of forespore-specific gene expression during sporulation of *Bacillus subtilis*. **47**:297-310.

135. **Stragier, P., C. Bonamy, and C. Karmazyn-Campelli**. 1988. Processing of a sporulation sigma factor in *Bacillus subtilis*: How morphological structure could control gene expression. Cell **52**:697-704.

136. **Stragier, P., B. Kunkel, L. Kroos, and R. Losick**. 1989. Chromosomal rearrangement generating a composite gene for a developmental transcription factor. Science **243**:507-512.

137. **Stragier, P. and R. Losick**. 1996. Molecular genetics of sporulation in *Bacillus subtilis*. Ann. Rev. Genetics **30**:297-341.

138. **Strauch, M., V. Webb, G. Spiegelman, and J. A. Hoch**. 1990. The Spo0A protein of *Bacillus subtilis* is a repressor of the *abrB* gene. Proc. Natl. Acad. Sci. USA **87**:1801-1805.

139. **Strauch, M. A. and J. A. Hoch**. 1993. Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. Mol. Microbiol. **7**:337-342.

140. **Strauch, M. A., J. J. Wu, R. H. Jonas, and J. A. Hoch**. 1993. A positive feedbackloop controls transcription of the *spo0F* gene, a component of the sporulation phosphorelay in *Bacillus subtilis*. Mol. Microbiol. **7**:967-974.

141. **Sun, D., R. M. Cabrera-Martinez, and P. Setlow**. 1991. Control of transcription of the *Bacillus subtilis spoIIIG* gene, which codes for the forespore specific transcription factor σ^G. J. Bacteriol. **173**:2977-2984.

142. **Sun, D., P. Stragier, and P. Setlow**. 1989. Identification of a new σ-factor involved in compartmentalized gene expression during sporulation of *Bacillus subtilis*. Genes & Dev. **3**:141-149.

143. **Tjian, R. and R. Losick**. 1974. An immunological assay for the sigma subunit of RNA polymerase in extracts of vegetative and sporulation *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **71**:2872-2876.

144. **Trach, K. A. and J. Hoch**. 1993. Multisensory activation of the phosphorelay initiating sporulation in *Bacillus subtilis*: identification and sequence of the protein kinase of the alternate pathway. Mol. Microbiol. **8**:69-79.

145. **Van Hoy, B. E. and J. A. Hoch**. 1990. Characterization of the *spoIVB* and *recN* loci of *Bacillus subtilis*. J. Bacteriol. **172**:1306-1311.

146. **Wang, H., R. Sato, M. S. Brown, X. Jia, and J. L. Goldstein**. 1994. SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. Cell **77**:53-62.

147. **Webb, C. D., A. Telema, S. Gordon, A. Straught, A. Belmont, D. C. H. Lin, A. D. Grossman, A. Wright, and R. Losick**. 1997. Bipolar localization of the replication origin regions of chromosomes in vegetative and sporulating cells of *B. subtilis*. Cell **88**:667-674.

148. **Wu, J., M. G. Howard, and P. J. Piggot**. 1989. Regulation of transcription of the *Bacillus subtilis spoIIA* locus. J. Bacteriol. **171**:692-698.

149. **Wu, J. and A. Newton**. 1997. Regulation of the *Caulobacter* flagellar gene hierarchy; not just for motility. Mol. Microbiol. **24**:233-239.

150. **Wu, J. J., P. J. Piggot, K. M. Tatti, and C. P. Moran**. 1991. Transcription of the *Bacillus subtilis spoIIA* operon. Gene **101**:113-116.

151. **Wu, L. J. and J. Errington**. 1994. *Bacillus subtilis* SpoIIIE protein required for DNA segregation during assymetric cell division. Science **264**:572-575.

152. **Xie, Z. D., C. D. Hershberger, S. Shankar, R. W. Ye, and A. M. Chakrabarty**. 1996. Sigma factor-anti-sigma factor interaction in alginate synthesis: inhibition of AlgT by MucA. J. Bacteriol. **178**:4990-4996.

153. **York, K., T. J. Kenny, S. Satola, and C. P. Moran**. 1992. Spo0A controls the σ^A-dependent activation of *Bacillus subtilis* sporulation-specific transcription unit *spoIIE*. J. Bacteriol. **174**:2648-2658.

154. **Youngman, P., J. B. Perkins, and R. Losick**. 1984. Construction of a cloning site near one end of Tn*917* into which foreign DNA may be inserted without affecting transposition in *Bacillus subtilis* or expression of the transposon-borne *erm* gene. Plasmid **12**:1-9.

155. **Yu, Y. T. and L. Kroos**. Unpublished results.

156. **Zhang, B., R. Daniel, J. Errington, and L. Kroos**. 1997. *Bacillus subtilis* SpoIIID protein binds to two sites in the *spoVD* promoter and represses transcription by σ^E RNA polymerase. J. Bacteriol. **179**:972-975.

157. **Zhang, B. and L. Kroos**. 1997. A feedback loop regulates the switch from one sigma factor to the next in the cascade controlling *Bacillus subtilis* mother cell gene expression. J. Bacteriol. **179**:6138-6144.

158. **Zhang, L., M. L. Higgins, P. J. Piggot, and M. L. Karow**. 1996. Analysis of the role of prespore gene expression in the compartmentalization of mother-cell gene expression during sporulation of *Bacillus subtilis*. J. Bacteriol. **178**:2813-2817.

159. **Zheng, L., R. Halberg, S. Roels, H. Ichikawa, L. Kroos, and R. Losick**. 1992. Sporulation regulatory protein GerE from *Bacillus subtilis* binds to and can activate or repress transcription from promoters for mother-cell-specific genes. J. Mol.

Biol. **226**:1037-1050.

160. **Zuber, P. and R. Losick**. 1987. Role of AbrB in Spo0A- and Spo0B-dependent utilization of a sporulation promoter in *Bacillus subtilis*. J. Bacteriol. **169**:2223-2230.