Isolation and Characterization of Multicopy Suppressors of a *spo0K* Mutant in *Bacillus subtilis*

by

John Robert LeDeaux

B.S., University of Virginia

(1987)

Submitted to the Department of Biology in Partial Fulfillment

of the Requirements for the Degree of

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at the

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November, 1994

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Abstract

Upon entering stationary phase, cells of *Bacillus subtilis* are capable of entering one of two different developmental pathways, depending on the growth conditions. These developmental pathways include sporulation, which is the ability to form an environmentally-resistant spore, and the development of competence, which is the ability to internalize exogenous DNA. The Spo0K oligopeptide permease is necessary for both the initiation of sporulation and the development of competence and appears to be acting early in both of these processes. To better understand how both of these developmental processes are regulated, I conducted a screen for genes which when present on a multicopy plasmid could bypass the need for *spo0K* in either of these developmental pathways. I characterized three such multicopy suppressors of *spo0K*. *kinC* (*mskA*) encodes a sensor kinase homologous to the sporulation sensor kinases KinA and KinB. These sensor kinases autophosphorylate and serve as phosphate donors (indirectly) for the Spo0A sporulation transcription factor. The immediate recipient of phosphate from the sensor kinases is the response regulator Spo0F; Spo0B transfers phosphate from Spo0F to Spo0A. Spo0A~P represses transcription of *abrB* and activates transcription of several sporulation genes. While KinC is not needed for sporulation under normal circumstances, it appears to be the first of the three sensor kinases to serve as a phosphate donor for Spo0A as judged by derepression of *abrB*. Also, in the absence of *spo0F* and *spo0B*, KinC appears to be the kinase responsible for phosphorylation of the *rvtA11* and *sof-1* forms of Spo0A. *mskB*, a multicopy suppressor of the *spo0K* sporulation defect, encodes a protein similar to SinI, which inhibits the SinR repressor of *spo0A* transcription. *mskD*, a multicopy suppressor of the *spo0K* competence defect, encodes a homolog of the ClpX ATPase, and a model for how overexpressed MskD may be affecting competence is presented.

Thesis Supervisor: Dr. Alan D. Grossman

Title: Associate Professor of Biology

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I am extremely grateful to my advisor Alan Grossman, without whose conscientious guidance and generous support I could not have done this work.

I also want to acknowledge the many interesting and insightful discussions I've had with my labmates, without which my understanding of development in *B. subtilis* would be much less than it is, and also for putting up with the music I listen to (most of the time), and for introducing me to the music they listen to.

I would also like to thank Yasuo Kobayashi and Ruth Schmidt for communicating DNA sequence data prior to publication.

Last but most, I want to thank my long-suffering helpmate Amy and my not-as-long-suffering daughter Shira, to both of whom this thesis is dedicated.

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Preface

Some of the material presented in Chapter 2 has been published by Rudner et al. (Rudner et al., 1991). Most of the material in Chapter 3 and Chapter 4 is in press (LeDeaux and Grossman, 1994; LeDeaux et al., 1994).

Chapter 1:

Introduction

Fundamental questions concerning signal transduction in cellular developmental processes include: What are the signals that induce developmental changes within the cell? How are these signals sensed? Once the signals are sensed, how do they lead to the changes in gene expression that cause the developmental changes to occur? The gram-positive soil bacterium *Bacillus subtilis* provides an excellent system for studying these questions (Harwood and Cutting, 1990; Sonenshein et al., 1993; Piggot et al., 1994).

When cells of *B. subtilis* enter stationary phase and receive and integrate certain extra- and intracellular signals, they can embark on a number of different developmental pathways, including pathways that lead to sporulation and competence. This introduction will provide an overview of these two processes and how they relate to the work presented in this thesis.

Sporulation is the process by which vegetatively growing cells produce heat-resistant, metabolically-dormant spores. Whereas vegetatively growing cells divide symmetrically, cells that have initiated sporulation divide asymmetrically. The smaller compartment is called the forespore and will eventually become the spore proper; the larger cell is called the mother cell. The mother cell eventually engulfs the forespore, builds a protective protein coat around the spore, and then lyses, releasing the mature spore into the environment.

Each stage of sporulation is assigned a number: Stage I is condensation of the DNA, Stage II is placement of the asymmetric septum, Stage III is engulfment of the forespore by the mother cell, Stages IV through VI are the construction of

protective layers around the engulfed forespore, and Stage VII is lysis of the mother cell and release of the mature spore into the environment. A mutant not able to sporulate is called *spo*, assigned a stage number indicating at which stage it is blocked, and then is assigned a letter particular for the mutated gene (e.g., *spoIIA*). Sporulation mutants which do not get as far as Stage II are *spo0* mutants. (There are no known mutations defining stage I.)

A major focus of the field is how cells change from growing vegetatively to initiating sporulation. The *spo0* genes studied so far are all involved in initiating that change. The sporulation transcription factor Spo0A is absolutely required for the cells to initiate sporulation (Piggot and Coote, 1976; Hoch, 1993). Spo0A needs to be phosphorylated to become active (Hoch, 1993), and Spo0A~P activates transcription of several *spo* genes (*spoIIA* (Trach et al., 1991), *spoIIE* (York et al., 1992), *spoIIG* (Satola et al., 1991; Satola et al., 1992; Bird et al., 1993; Baldus et al., 1994) and represses transcription of *abrB* (Perego et al., 1988; Strauch et al., 1990), which itself represses other sporulation genes (Zuber and Losick, 1987; Robertson et al., 1989; Strauch et al., 1990; Weir et al., 1991).

Before cells initiate sporulation, they receive and integrate many extracellular and intracellular signals. Many signals appear to affect the accumulation of Spo0A~P, as judged by the ability of gain-of-function mutations in *spo0A* to bypass the need for these signals in activating transcription of *spoII* genes. These signals include starvation for nutrients (Ireton et al., 1993), high cell density (Grossman and Losick, 1988; Ireton et al., 1993), lack of DNA damage (Ireton and Grossman, 1992a), replication of DNA (Ireton and Grossman, 1994), and a functioning Krebs cycle (Ireton et al., 1994).

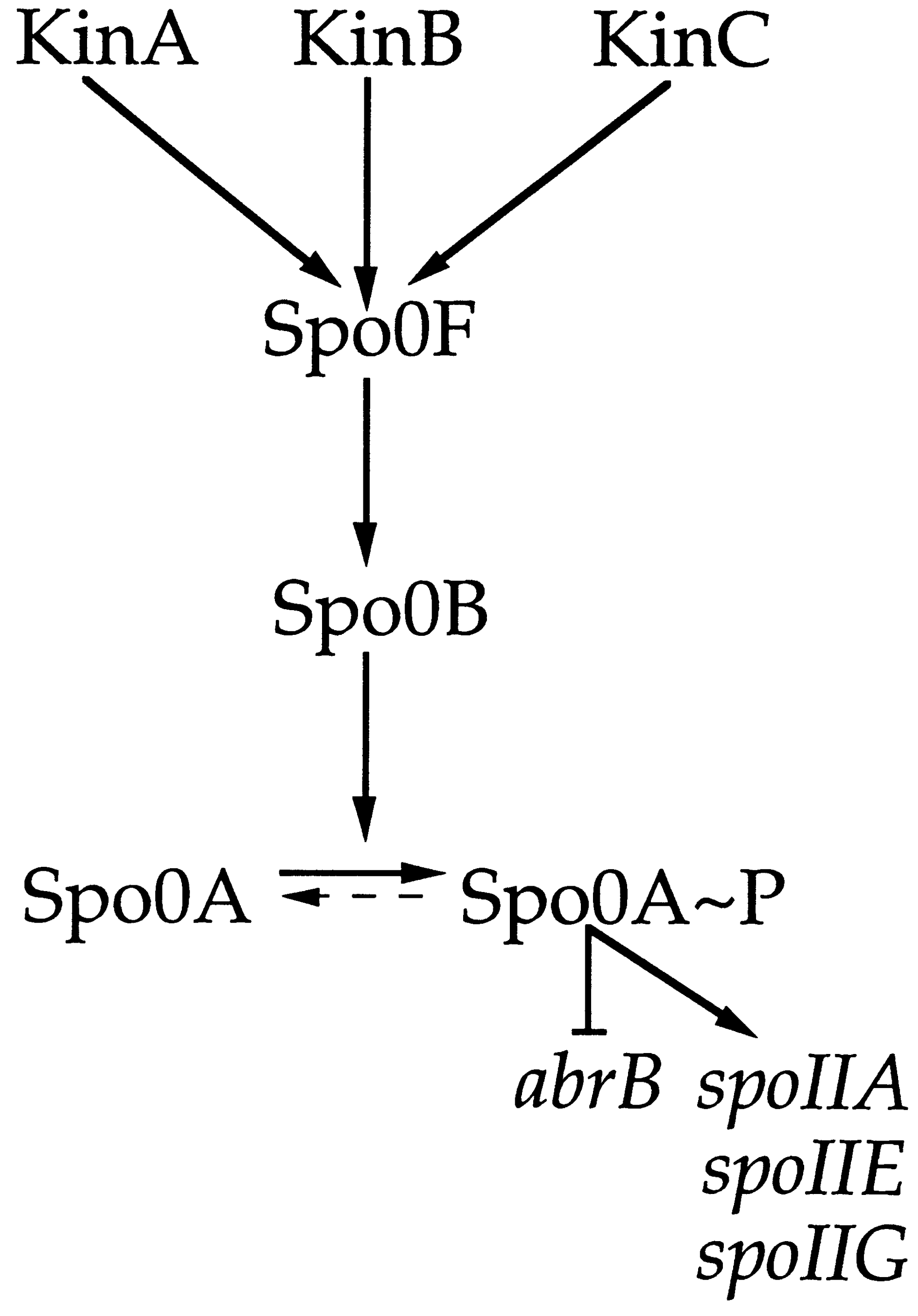
Spo0A belongs to a family of transcriptional regulators known generally as response regulators. Usually, response regulators receive phosphate from cognate autophosphorylated histidine protein kinases known as sensor kinases.

A sensor kinase and its cognate response regulator are known together as a two-component system (reviewed in Albright et al., 1989; Parkinson and Kofoid, 1992; Parkinson, 1993). There are estimated to be ~50 two-component systems in *E. coli* (Stock et al., 1989b), and while two-component systems have been studied extensively in bacteria, they have also been recently identified in plants (Chang et al., 1993) and yeast (Ota and Varshavsky, 1993). Sensor kinases are histidine protein kinases that autophosphorylate under certain conditions. The phosphorylated kinase serves as a phosphate-donor for its partner response regulator. In bacteria, response regulators are generally involved in regulation of gene expression.

The conserved response regulator domain consists of a hydrophobic core comprised of a five-stranded parallel beta sheet surrounded by five alpha-helices, based on the three-dimensional X-ray crystallographic structure of the response regulator CheY (Stock et al., 1989a). The three most highly conserved acidic residues are found at the C-terminal edge of the beta sheet in close proximity to each other (Stock et al., 1989a) and include the single aspartate that is phosphorylated (Sanders et al., 1989).

Unlike canonical response regulators, Spo0A does not usually receive phosphate directly from its cognate sensor kinases, KinA (Perego et al., 1989; Antoniewski et al., 1990), KinB (Trach and Hoch, 1993) and KinC (Kobayashi et al., 1994; LeDeaux and Grossman, 1994; and Chapter 4). Rather, the sporulation sensor kinases first serve as a phosphate donor for the response regulator Spo0F. This has been shown biochemically for KinA (Perego et al., 1989; Burbulys et al., 1991), and there is genetic evidence that indicates this is also true for KinB (Trach and Hoch, 1993) and KinC (see Chapter 4). Spo0F~P then donates phosphate to the phosphotransferase Spo0B, which then serves as a phosphate donor for Spo0A (Burbulys et al., 1991) (Figure 1.1). Together the sporulation sensor

Figure 1.1. The phosphorelay controls the accumulation of Spo0A~P. Under certain conditions, the sensor kinases KinA, KinB, and KinC autophosphorylate and serve as phosphate-donors for the response regulator Spo0F. The phosphotransferase Spo0B transfers phosphate from Spo0F to the response regulator and sporulation transcription factor Spo0A (Burbulys et al., 1991). Spo0A~P represses transcription of *abrB* (Perego et al., 1988; Strauch et al., 1990) and activates transcription of several *spoII* genes (Satola et al., 1991; Trach et al., 1991; Satola et al., 1992; York et al., 1992; Bird et al., 1993; Baldus et al., 1994).


KinA
KinB
KinC
Spo0F
Spo0B
Spo0A
Spo0A~P
abrB
spoIIA
spoIIE
spoIIG

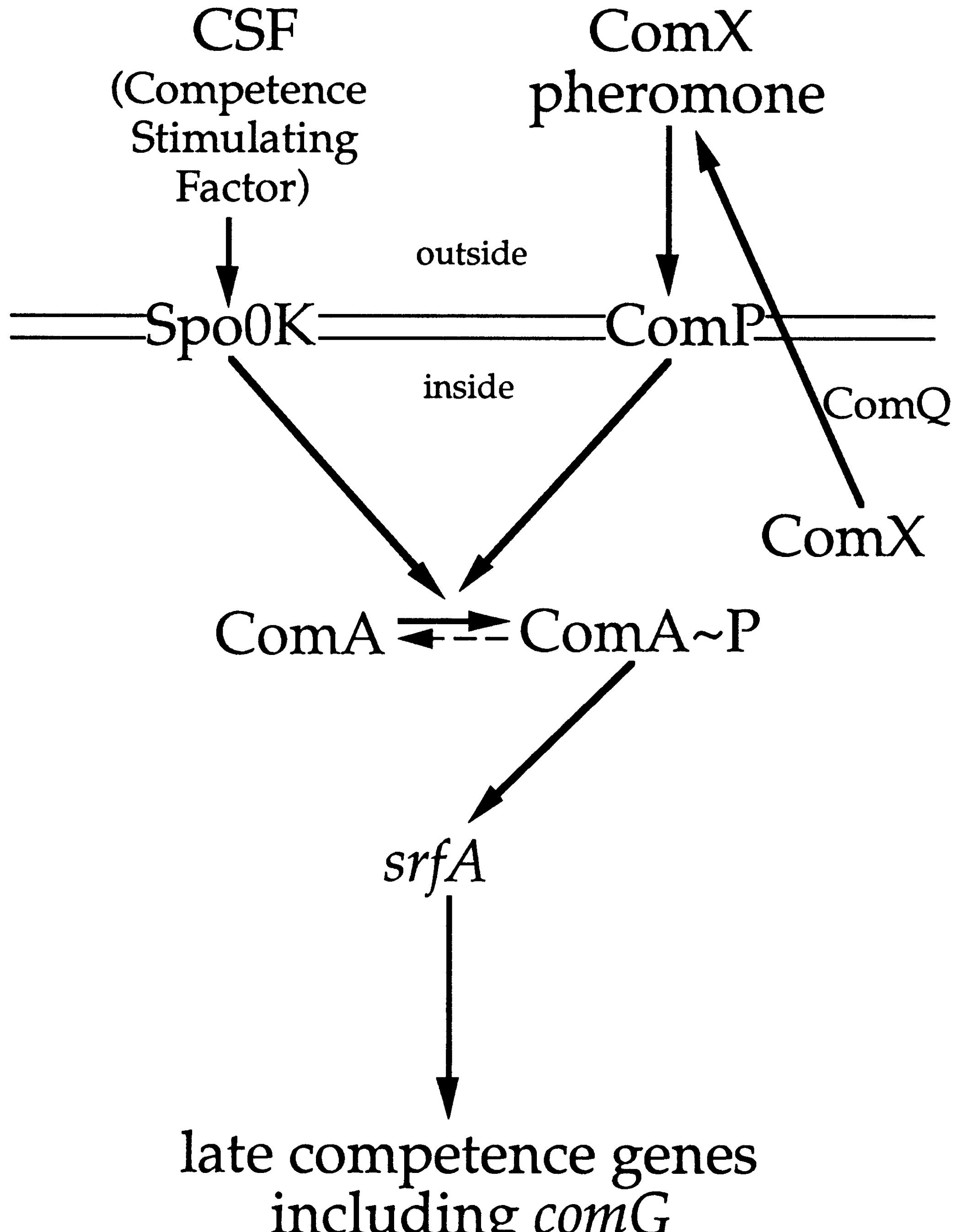
kinases, Spo0F, and Spo0B constitute a "phosphorelay" (Burbulys et al., 1991). This phosphorelay provides several possible checkpoints for regulating the accumulation of Spo0A~P (Burbulys et al., 1991; Grossman, 1991).

Another key player involved in the accumulation of Spo0A~P is Spo0K, which is necessary for efficient sporulation (Perego et al., 1991; Rudner et al., 1991). The *spo0K* operon consists of five open reading frames, and the product of each of these reading frames is very similar to the corresponding protein of oligopeptide permease from *Salmonella typhimurium* and *Escherichia coli* (Perego et al., 1991; Rudner et al., 1991; and Chapter 2). Spo0K is necessary for transport of oligopeptides into the cell (Perego et al., 1991), and belongs to a large family of conserved proteins known as ABC transporters, where "ABC" stands for "ATP-binding cassette", a motif common to all these transporters (reviewed in Ames, 1986; Higgins, 1992).

Spo0K is also necessary for the development of genetic competence. Competence is the ability to internalize exogenous DNA (reviewed in Dubnau, 1989; Dubnau, 1993). Only a fraction (~10%) of cells within the population becomes competent, and this fraction has a different buoyant density than the noncompetent fraction of cells. Competent cells have membrane-bound proteins that bind and nick exogenous DNA. Single-stranded fragments of ~ 10 kb are brought into the cell. These membrane-bound proteins are products of the "late" competence genes, and are only expressed in the competent fraction of the population. However, before the late competence genes are expressed, "early" competence genes must become active.

The early competence genes include *spo0K*, *comQ*, *comX*, *comP*, *comA*, and *srfA* (Figure 1.2). *comX* encodes a precursor for the ComX extracellular pheromone that is required for competence (Magnuson et al., 1994a), and *comQ* is necessary for production of the pheromone (Magnuson et al., 1994a).

Figure 1.2. The early competence pathway acts to regulate expression of *srfA*. Modified from Solomon et al., 1994.


CSF
(Competence
Stimulating
Factor)
ComX
pheromone
outside
Spo0K
ComP
inside
ComQ
ComX
ComA
ComA~P
srfA
late competence genes
including comG

comP encodes a membrane bound sensor kinase (Weinrauch et al., 1990) that is involved in sensing the ComX pheromone (Solomon et al., 1994). The cognate response regulator for ComP is ComA. ComA~P is necessary for expression of *srfA*, which is required for the development of competence. The role of Spo0K in initiating competence development is associated with Competence Stimulating Factor (CSF) (Solomon et al., 1994). CSF is at least in part an oligopeptide and is necessary for competence and full expression of *srfA* (Magnuson et al., 1994b). Spo0K, ComP, and ComA seem to be required in the development of competence only in their positive roles as direct or indirect activators of *srfA* expression, since expression of *srfA* from a heterologous promoter causes constitutive competence and bypasses the need for *comQ*, *spo0K*, *comP* and *comA* (Hahn and Dubnau, 1991; Nakano and Zuber, 1991).

To determine which of the five genes of the *spo0K* operon were required for sporulation, competence, or peptide transport, nonpolar mutations were made in each of the first four *spo0K* open reading frames (Chapter 2). Under some conditions, each of the first four genes are needed to a similar extent in sporulation, competence and oligopeptide transport as the entire operon. The last open reading frame is needed to a lesser extent for all three functions.

To identify other genes which are involved in the initiation of sporulation and the development of competence, I isolated genes which when overexpressed could bypass the need for *spo0K* for either of these processes. This screen is described in detail in Chapter 3. *kinC*, which when present on a multicopy plasmid bypasses the need for *spo0K* for the initiation of sporulation (Chapter 4), was originally isolated in this screen.

One of the main results presented in this thesis is that while KinA and KinB are responsible for the accumulation of Spo0A~P necessary to activate transcription of *spoII* genes (Trach and Hoch, 1993), KinC is responsible for the

early accumulation of Spo0A~P necessary for normal regulation (repression) of *abrB* (Chapter 4). Another important result is that the three sporulation kinases, KinA, KinB and KinC, have different roles under different growth conditions (Chapter 4).

Several point mutations in *spo0A* lead to suppression or partial suppression of the sporulation defect of *spo0K*, *kinA kinB* double, *spo0F*, or *spo0B* mutants. An important result of this work is that the sensor kinase KinC is necessary for this suppression, presumably because these mutant forms of Spo0A are capable of taking phosphate directly from KinC~P (Chapter 4). After I had finished these experiments, we learned that Y. Kobayashi had independently isolated *kinC* in a screen for genes responsible for the suppression activity of one of these point mutations in spo0A (Kobayashi et al., 1994).

Two other genes isolated as multicopy suppressors of *spo0K* are *mskB* and *mskD*. *mskB* partially suppresses the sporulation defect of a *spo0K* mutant, and encodes a protein similar to SinI (Bai et al., 1993). SinI inhibits SinR (Bai et al., 1993); SinR itself represses *spo0A* transcription (I. Smith, personal communication, see Chapter 5).

mskD partially suppresses the competence defect of a *spo0K* mutant (Chapter 5) and encodes a homolog of ClpX (Gottesman et al., 1993). ClpX belongs to a large family of ATPases that direct the activity of the ClpP protease (Gottesman and Maurizi, 1992; Squires and Squires, 1992). MecB, which is a homolog of the ClpC ATPase (Krüger et al., 1994; Msadek et al., 1994), and MecA, which has some similarity to ClpP (Kong and Dubnau, 1994), appear to act together to negatively regulate the activity of the late competence transcription factor ComK (Hahn et al., 1994; Kong and Dubnau, 1994; Msadek et al., 1994; van Sinderen et al., 1994; van Sinderen and Venema, 1994). An intriguing model for how MskD may be acting to increase expression of late competence genes is that

MskD when overexpressed interferes with MecA and/or MecB so that neither can regulate the activity of ComK (Chapter 5).

Chapter 2:

Analysis of nonpolar single gene mutations within the *spo0K* operon

spo0K is necessary for the efficient initiation of sporulation, the development of competence, and oligopeptide permease activity (Perego et al., 1991; Rudner et al., 1991). This chapter describes work characterizing the extent to which each of the *spo0K* open reading frames is required for each of these activities.

Cloning and sequencing *spo0K*. As part of our work on signal transduction and the initiation of sporulation, we set out to isolate insertion mutations that would cause *kinA* mutants to have a more severe defect in sporulation (Ireton and Grossman, 1992b). In the same screen, we were also able to isolate sporulation mutations that could be suppressed by the overexpression of *kinA* from a heterologous promoter. During the course of this work, we isolated a Tn*917lac* insertion mutation that was in *spo0K*, *spo0K*::Tn*917lac*. The transposon has a promoterless *lacZ* gene, and the orientation of the insertion is such that *lacZ* is not expressed.

spo0K was originally defined by the mutant allele *spo0K141* (Z31 of Coote (Coote, 1972) and Piggot and Coote (Piggot and Coote, 1976)). Definitive evidence that the transposon was in the *spo0K* locus came from cloning and characterizing DNA surrounding the transposon. We demonstrated that clones of DNA adjacent to the transposon could rescue the *spo0K141* mutation by recombination and that other clones could disrupt the *spo0K* locus when integrated into the chromosome. Clones were used to sequence the locus and to further characterize the function of *spo0K*. This work is described in detail elsewhere (Rudner et al., 1991).

Homology with Opp. The *spo0K* operon contains five open reading frames. These open reading frames are very similar to those of the oligopeptide permease (*opp*) operon of *S. typhimurium*, and both operons have the same organization (Figure 2.1). The percentage identity between individual gene products ranges from 34% to 53% (Figure 2.1). Both *spo0K* and *opp* belong to a large conserved family of genes/operons of ATP-dependent transport systems (Higgins, 1992). This family includes *his*, *mal*, and *pst* transport systems in bacteria, that import histidine, maltose, and phosphate, respectively; STE6, which exports a-factor pheromone in yeast; and MDR, which exports drugs, and CFTR, which channels chloride ions in mammals. All of these systems share a region of homology called an ATP-binding cassette, so they are known as ABC transporters. The assignment of *spo0K* to this family is based on its sequence homology and its function as an oligopeptide permease.

Based on its homology to the gene products of *opp*, the following roles have been assigned to each of the gene products in the *spo0K* operon (Perego et al., 1991; Rudner et al., 1991): Spo0KA is the ligand binding protein, Spo0KB and Spo0KC form a complex in the membrane through which the ligand is transported, and Spo0KD and Spo0KE are the ATP-binding proteins that are thought to provide energy for transport. However, there are some interesting differences between components of Spo0K and Opp; for example, while the ligand binding protein OppA is a periplasmic protein (Hiles et al., 1987), the ligand binding protein Spo0KA is probably tethered to the membrane by a lipid anchor (Perego et al., 1991).

Figure 2.1. Homology between the *spo0K* operon of *B. subtilis* and the *opp* operon of *S. typhimurium*. Percent identity and similarity are as determined by the BestFit program (Devereux et al., 1984).

	Spo0KA	Spo0KB	Spo0KC	Spo0KD	Spo0KE
% Identity ; Similarity	34 ; 54	48 ; 73	43 ; 67	51 ; 69	53 ; 69
	OppA	OppB	OppC	OppD	OppF

Phenotypes associated with *spo0K* mutations. *spo0K* null mutants are deficient in the ability to initiate sporulation, initiate competence development and transport oligopeptides.

Sporulation. *spo0K* null mutants do not sporulate efficiently, sporulating between ~1% to ~10% as efficiently as wild type.

Competence. *spo0K* null mutants take up exogenous DNA 2-3 orders of magnitude less efficiently than wild type. Consistent with this, *srfA-lacZ* and *comG-lacZ* fusions show decreased expression in a *spo0K* null mutant as compared to wild type (Figures 2.4 and 2.5). (*srfA* encodes an early competence gene. *comG* is a late competence gene that is thought to encode part of the DNA-uptake complex (Dubnau, 1993).)

Oligopeptide transport. There are several results that indicate that Spo0K can act as an oligopeptide permease, as its homology to Opp indicates. *spo0K* null mutants are resistant to the toxic oligopeptide phosphinothricyl-alanyl-alanine, also known as PTT or bialaphos (Perego et al., 1991), and is resistant to bialaphos at concentrations up to and including 100 μg/ml. Also, Spo0K is required for utilization of peptides as a source of required amino acids. For example, Spo0K$^+$ cells that are auxotrophic for Met and Phe will grow on minimal medium in the presence of the tripeptide Met-Leu-Phe; *spo0K* mutants with the same auxotrophies will not (Solomon, 1994). Also, proline auxotrophs will grow in the presence of Pro-Gly-Gly but *pro spo0K* double mutants will not (Mathiopoulos et al., 1991). Interestingly, Spo0K will not transport the dipeptide Pro-Gly (at least not to the extent to rescue a Pro auxotroph) (Mathiopoulos et al., 1991), whereas Opp from Salmonella will (Abouhamad et al., 1991).

***spo0K* nonpolar deletions.** To determine the relative contribution of each *spo0K* cistron to the phenotypes associated with null alleles of the entire operon, I made what I presume are in-frame deletions in each of the first four open reading

frames, a single deletion encompassing the last two open reading frames, and two null mutations in *spo0KE*. Table 2.1 lists the plasmids I used to make these deletions, and the sizes of the deletions. These deletions are diagrammed in Figure 2.2.

To make each deletion, I first made a plasmid that contained the deletion in the gene of interest within sequence that overlapped either the 5' or 3' end of the *spo0K* operon (see Figure 2.2 and Table 2.1). I transformed each of these integrative plasmids into a wild type strain (JH642). If the deletion actually gives rise to a Spo$^-$ phenotype, in the cases where the plasmid extends past the 3' end of the operon (pJL34 for *ΔspoOKC*, pJL9 for *ΔspoOKD*, and pJL19 for *ΔspoOKDE*, see Figure 2.2), then integration of the plasmid could give rise to two different classes of recombinants depending on the crossover point (see Figure 2.3, which uses pJL34 as an example). If the plasmid crossed over upstream of the deletion, the cells should be Spo$^-$ (case A in Figure 2.3); if the plasmid crossed over downstream of the deletion, the cells should be Spo$^+$ (case B in Figure 2.3). In all three cases, transforming the plasmid in question gave rise to Spo$^-$ and Spo$^+$ transformants, indicating that these three deletions did indeed lead to a Spo$^-$ phenotype.

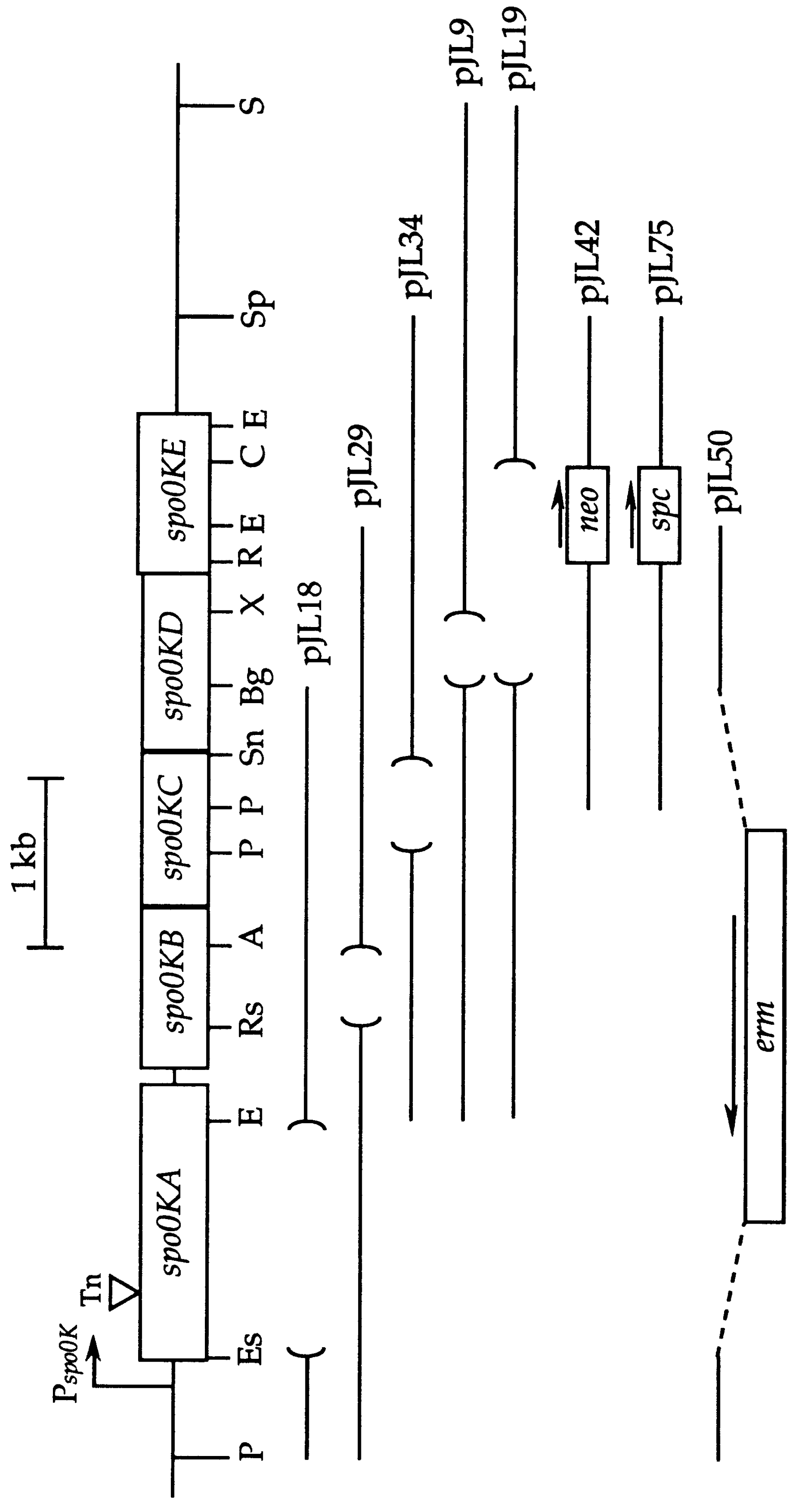
All single crossover events of plasmids that have inserts that extend past the 5' end of the operon (pJL18 for *ΔspoOKA* and pJL29 for *ΔspoOKB*) should have resulted in cells that were phenotypically Spo$^+$, whether those crossovers occurred upstream or downstream of the deletion in the plasmid. However, Spo$^-$ transformants could result from gene conversion events that replaced the wild type copy of the gene with the deletion version on the plasmid. There were Spo$^-$ transformants in both cases, indicating that these deletions also resulted in a Spo$^-$ phenotype.

TABLE 2.1 *spo0K* deletion mutations.

Gene	Deletion[a]	Fraction of ORF deleted	Plasmid used
spo0KA	1371 bp	457aa/545aa	pJL18
spo0KB	447 bp	139aa/311aa	pJL29
spo0KC	573 bp	191aa/305aa	pJL34
spo0KD	408 bp	136aa/358aa	pJL9
spo0KDE	1341 bp		pJL19
spo0KE	541 bp	*neo* insertion	pJL42
spo0KE	"	*spc* insertion	pJL75
spo0KABCD	3184 bp	*erm* insertion	pJL50

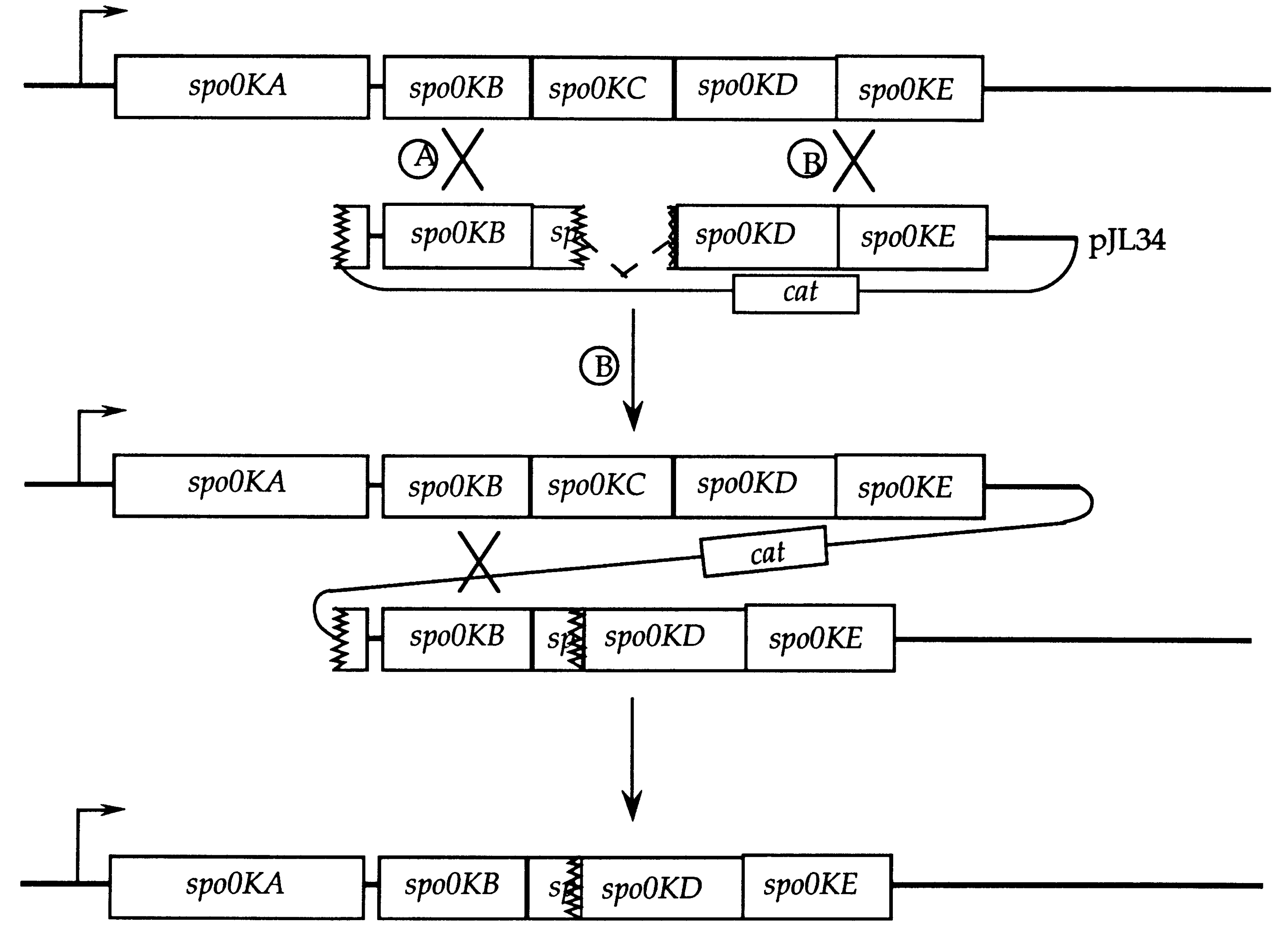
[a]Deletions in each of the first four open reading frames should be in-frame.

Figure 2.2. Map of *spo0K* region and plasmids used to make *spo0K* mutations. Restriction site abbreviations: P, PvuII; Es, EspI; E, EcoRI; Rs, RsrII; A, ApaI; Sn, SnaBI; Bg, BglII; X, XcmI; R, EcoRV; C, ClaI; Sp, SplI; S, SphI. The location of the likely promoter site of *spo0K* is indicated by an arrow and P*spo0K* (Rudner et al., 1991). The location of the Tn*917lac* insertion is indicated by a triangle and Tn (Rudner et al., 1991).



1 kb
P_{spo0K}
Tn
spo0KA
spo0KB
spo0KC
spo0KD
spo0KE
P
Es
E
Rs
A
P
P
Sn
Bg
X
R
E
C
E
Sp
S
pJL18
pJL29
pJL34
pJL9
pJL19
pJL42
pJL75
pJL50
neo
spc
erm

Figure 2.3. Making *ΔspoOKC*, as an example of how each of the *spo0K* deletion mutations was constructed. pJL34 contains sequence overlapping the 3' terminus of the *spo0K* operon, and a deletion in the *spo0KC* gene (see Table 2.1 and Figure 2.2). Integrating the plasmid into the chromosome of a wild type strain could give rise to two classes of Cm^R recombinants: If a single crossover event occurs upstream of the deletion on the plasmid (A), the recombinant would have *Δspo0KC* under control of the *spo0K* promoter (result not shown), and therefore, it would be Spo^-. However, if a single crossover event occurs downstream of the deletion on the plasmid (B), the recombinant would have *spo0K*$^+$ under control of the *spo0K* promoter (shown) and would be Spo^+. To make a strain that contains only *Δspo0KC* and no wild type copy of *spo0KC*, a Spo^+ recombinant is grown under conditions where the presence of *cat* was not required (in the absence of chloramphenicol) and plated on sporulation plates. A second recombination event that excises the plasmid and leaves behind *Δspo0KC* under control of the *spo0K* promoter would give rise to a Spo^- Cm^S recombinant.



spo0KA
spo0KB
spo0KC
spo0KD
spo0KE
A
B
spo0KB
spo0KD
spo0KE
pJL34
cat
B
spo0KA
spo0KB
spo0KC
spo0KD
spo0KE
cat
spo0KB
spo0KD
spo0KE
spo0KA
spo0KB
spo0KD
spo0KE

(I assume that gene conversion events also occurred in the transformations of the plasmids to delete the downstream genes, but they were phenotypically indistinguishable from recombinants that were the result of a single crossover event.)

To make strains that had only the deletion of the gene of interest and no wild type copy of that gene present, I screened for a second recombination event that excised the plasmid and left behind the deletion (Step 2 in Figure 2.3). To do that, one or more Spo^+ recombinants from the transformation of each plasmid was isolated and grown under conditions where the presence of the plasmid was not required (i.e., in the absence of chloramphenicol), and plated on non-selective sporulation plates. These plates were screened for the presence of Spo^- colonies which were then further screened for Cm^S, indicating the loss of the plasmid.

The deletions in *spo0KA*, *spo0KB*, *spo0KD*, and *spo0KDE* have been shown to be present in the chromosome by PCR analysis, but PCR analysis of the *spo0KC* mutant has so far been unsuccessful (Solomon, 1994).

Complementation assays. None of the deletions made in the first four genes of the *spo0K* operon are polar on downstream genes. A complementation test was performed on the *Δspo0KA* mutation by putting *spo0KA* under control of the *spo0K* promoter at the *amyE* locus using pJL28 (see Chapter 7) and scoring for the ability to sporulate on sporulation plates. (This same construct was used to show that the *spo0KA*::Tn*917lac* insertion is polar on downstream genes in the *spo0K* operon.)

A complementation test was also performed on the *Δspo0KB* and *Δspo0KC* mutations to determine if they were polar on downstream genes. Two plasmids were made, one that had only *spo0KB* under control of the IPTG-inducible promoter Pspac (pJL32) and one that had both *spo0KB* and *spo0KC* under the control of Pspac (pJL79). Both of the constructs were put at the nonessential

amyE locus in both the *ΔspoOKB* and *ΔspoOKC* strains. Both constructs rescued the *ΔspoOKB* strain in an IPTG-dependent manner, indicating that the *ΔspoOKB* deletion is not polar on *spoOKC* or *spoOKD* and *spoOKE*. In the presence of IPTG, the Pspac-*spoOKB* construct did not rescue the *ΔspoOKC*, but the Pspac-*spoOKBC* construct did, indicating that the *ΔspoOKC* deletion was not polar on *spoOKD* and *spoOKE*, and that two copies of *spoOKB* could not substitute for a loss of *spoOKC*.

The *ΔspoOKD* deletion is not completely polar on *spoOKE* because the *ΔspoOKD* allele causes a less severe phenotype than the *ΔspoOKDE* allele (see below).

Phenotypes associated with deletions of particular *spoOK* genes. The *ΔspoOKA*, *ΔspoOKB*, and *ΔspoOKC* nonpolar deletions and the *ΔspoOKDE* deletion cause phenotypes that are indistinguishable from a *spoOK* null mutant in terms of sporulation (Table 2.2), *srfA-lacZ* (Figures 2.4) and *comG-lacZ* (Figures 2.5) expression, and resistance to bialaphos, where bialaphos was used at 50 μg/ml. The *spoOK* null mutation is one where the first four open reading frames of *spoOK* have been replaced with the selectable *erm* gene, see pJL50 of Figure 2.2. *ΔspoOKD* is virtually indistinguishable from *spoOK* null mutants in terms of sporulation or bialaphos resistance consistent with results previously reported (Perego et al., 1991), but a *ΔspoOKD* mutation had a less severe effect on *srfA-lacZ* (Figure 2.4) and *comG-lacZ* (Figures 2.5) expression as compared to a *spoOK* null mutant.

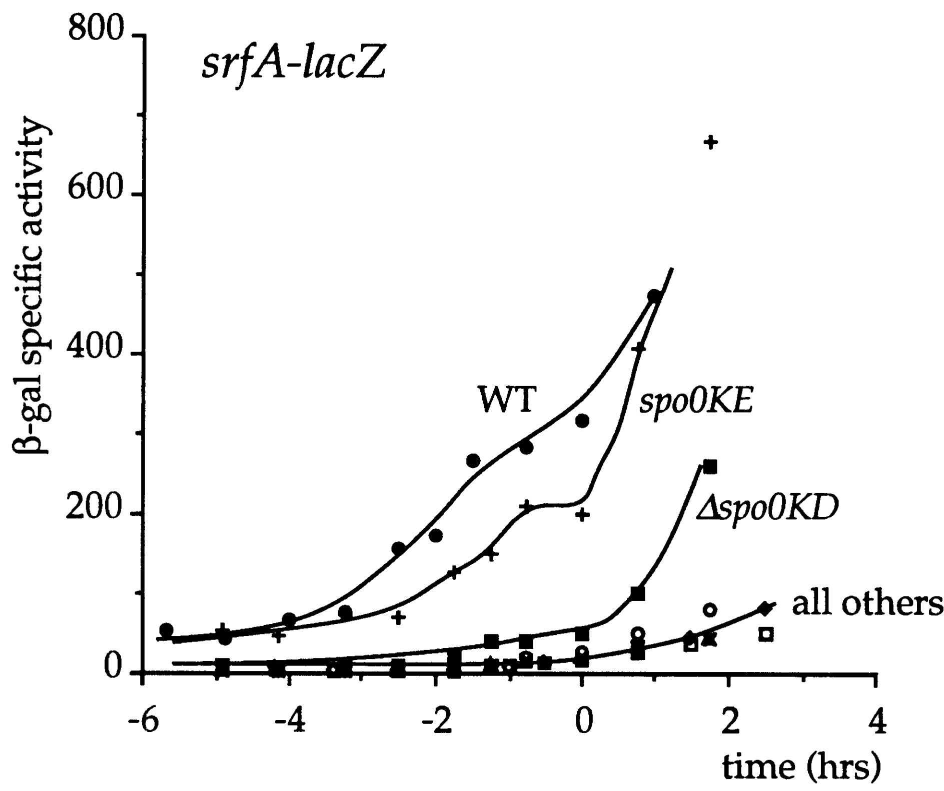
spoOKE null mutants sporulate 5 to 20 times better than *spoOK* null mutants. A *spoOKE* insertion was previously reported to give a Com$^-$ defect, where the competence of the cells was measured at a single point during growth ~2 hours prior to entry into stationary phase (Rudner et al., 1991), but a multiple point transformation assay with a *spoOKE* deletion-insertion mutant indicates competence levels more in line with those expected from the expression of

TABLE 2.2 Sporulation frequency of different *spo0K* mutants in DS medium.

strain	genotype	spores/ml[a]	spores/cell	relative to wild type
JH642	WT	2.4×10^8	5.1×10^{-1}	1
JRL131	*ΔspoOKA*	2.5×10^6	1.0×10^{-2}	2.0×10^{-2}
JRL189	*ΔspoOKB*	2.3×10^6	9.1×10^{-3}	1.8×10^{-2}
JRL322	*ΔspoOKC*	2.5×10^6	8.1×10^{-3}	1.6×10^{-2}
JRL43	*ΔspoOKD*	1.5×10^6	5.7×10^{-3}	1.1×10^{-2}
JRL221	*ΔspoOKE::neo*	2.9×10^7	1.1×10^{-1}	2.1×10^{-1}
JRL135	*ΔspoOKDE*	7.3×10^5	2.5×10^{-3}	5.0×10^{-3}
KI418	*spoOKA*::Tn*917*	5.9×10^5	2.4×10^{-3}	4.8×10^{-3}
JRL358	*ΔspoOK::erm*	3.7×10^6	1.3×10^{-2}	2.5×10^{-2}

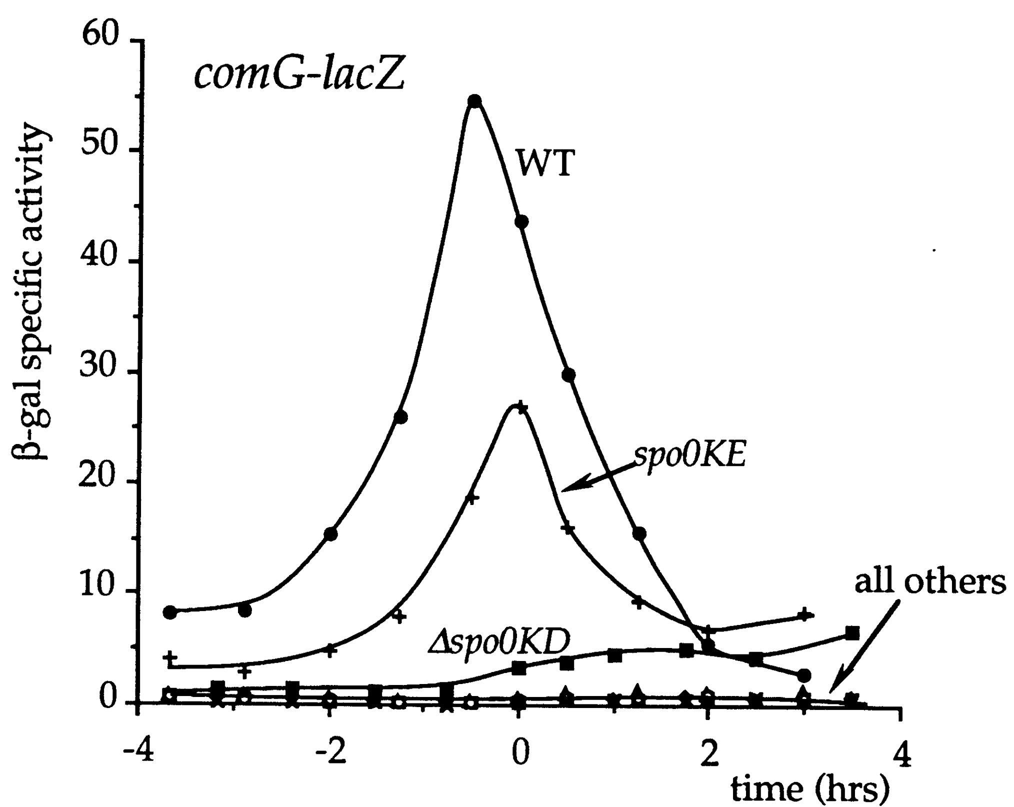
[a]Data shown are from a single representative experiment, and similar results were obtained in at least three independent experiments. The number of viable cells/ml ranged from 2.1×10^8 to 4.7×10^8.

Figure 2.4. *srfA-lacZ* expression in different *spo0K* mutants. Strains were grown in S7 minimal medium with 1% glucose and 0.1% glutamate and amino acids for auxotrophic requirements as described in Chapter 7. The time of exit from exponential growth is defined as time 0 (T_0). The *srfA-lacZ* fusion is located in single copy at the *amyE* locus and is associated with either the *cat* marker or with the *cat* marker that had been converted to *cat::spc* (Cm^S Spc^R) using pJL62. filled circles, wild type (JRL293); +'s, *Δspo0KE::spc*, (JRL685); filled squares, *Δspo0KD* (JRL489); triangles, *Δspo0KA* (JRL486); open squares, *Δspo0KB* (JRL1105); diamonds, *Δspo0KC* (JRL1082); open circles, *Δspo0KDE* (JRL492); and x's, *Δspo0KABCD::erm* (JRL494).



srfA-lacZ
β-gal specific activity
800
600
400
200
0
-6
-4
-2
0
2
4
time (hrs)
WT
spo0KE
Δspo0KD
all others

Figure 2.5. *comG-lacZ* expression in different *spo0K* mutants. Strains were grown in S7 minimal medium with 1% glucose and 0.1% glutamate and amino acids for auxotrophic requirements as described in Chapter 7. The *comG-lacZ* fusion is located in single copy at the *amyE* locus and is associated with the *neo* marker. filled circles, wild type (JRL275); +'s, *Δspo0KE::spc*, (JRL682); filled squares, *Δspo0KD* (JRL488); triangles, *Δspo0KA* (JRL485); open squares, *Δspo0KB* (JRL1102); diamonds, *Δspo0KC* (JRL1103); open circles, *Δspo0KDE* (JRL1104); and x's, *Δspo0KABCD::erm* (JRL493).



comG-lacZ
β-gal specific activity
60
50
40
30
20
10
0
-4
-2
0
2
4
time (hrs)
WT
spo0KE
Δspo0KD
all others

comG-lacZ seen in Figure 2.5 (data not shown). These same insertions were sensitive to all but the lowest levels of bialaphos (0.02 - 0.1 µg/ml) tested, but at these same levels wild type cells were still sensitive. This indicates that *spo0KE* mutants transport bialaphos but not quite as well as wild type cells.

There may exist conditions under which the phenotypes associated with *spo0KE* mutants are more severe than reported above (unpublished results), but in every case looked at, phenotypes associated with a *spo0KE* mutation were less severe than those associated with a complete *spo0K* null mutation.

Why is Spo0KE not needed to the same extent as the other Spo0K proteins for the initiation of sporulation, the development of competence and oligopeptide transport? It is clear that OppF, the Spo0KE homolog in *S. typhimurium*, is necessary for oligopeptide transport. Also, it has been shown for the maltose and histidine ABC transporters that the stoichiometry of ATP-binding proteins is such that there are two for every membrane pore (Davidson and Nikaido, 1991; Kerppola et al., 1991). It may be possible that Spo0KD can substitute for Spo0KE, that is, a Spo0KD homodimer may substitute for the proposed Spo0KD-Spo0KE heterodimer. However, since a *spo0KD* mutant has a more severe phenotype than a *spo0KE* mutant, a putative Spo0KE homodimer can not substitute for the proposed Spo0KD-Spo0KE heterodimer. Spo0KD and Spo0KE are 42% identical.

It may be the case that an ATP-binding protein from some other ABC transporter is substituting for Spo0KE. What makes this idea particularly intriguing is the recent characterization of *app*, a cryptic oligopeptide permease of *B. subtilis*, which is ~5 kb upstream of *spo0K* (Koide and Hoch, 1994; Solomon, 1994). Like *spo0K*, the gene products of *app* are homologous to those of *opp* of *S. typhimurium*. Each gene of *app* is named according to which gene of *opp* it is most similar. Unlike *spo0K*, the order of the genes within the operon is different: The

genes encoding the two ATP-binding proteins are first, followed by the gene encoding the ligand binding protein, and ending with the genes encoding the two membrane pore proteins, so the gene order is *appD, appF, appA, appB* and *appC*. The mutation which causes App to be inactive in wild type cells is a frameshift mutation in *appA*, which encodes the ligand binding protein. A revertant that restores the open reading frame suppresses the sporulation, competence and some of the transport phenotypes of a *spo0K* mutant (the *app* revertant will transport a tetrapeptide and a pentapeptide as well as the wild type strain, but not a tripeptide) (Koide and Hoch, 1994). It is not known if the frame-shift mutation in *appA* in wild type cells is polar on *appB* and *appC*, but it almost certainly is not affecting expression of the upstream genes encoding the two ATP-binding proteins, AppD and AppF. Since AppF is 54% identical to Spo0KE (Koide and Hoch, 1994), it is possible that AppF could substitute for Spo0KE. This idea is readily testable.

There is precedent for one part of an ABC transporter to work with another ABC transporter. The ligand-binding protein of the dipeptide permease Dpp can work with the membrane proteins of Spo0K to transport dipeptides: a mutation in *dpp* which is not polar on the gene encoding the ligand-binding protein of Dpp will still allow transport of dipeptides into the cell, but this transport is dependent on *spo0K* (F. Slack and A. L. Sonenshein, personal communication). Another example is the ability of UgpC, the single ATP-binding protein of the *sn*-glycerol-3-phosphate (G3P) ABC transporter Ugp, to substitute for MalK, the single ATP-binding protein of the maltose ABC transporter. This is measured by the growth of *malK* mutants on plates where maltose is the sole carbon source under conditions where UgpC is expressed (Hekstra and Tommassen, 1993). Conversely, MalK was able to substitute for UgpC as measured by growth of

ugpC mutants on plates where G3P was the sole phosphate source (Hekstra and Tommassen, 1993).

Chapter 3:

Isolation of multicopy suppressors of *spo0K*

I was interested in finding genes which when overexpressed could bypass either the sporulation defect or the competence defect (or both) caused by *spo0K* mutations. Spo0K appears to act early in both of these processes. So, for example, overexpression of the sporulation sensor kinase KinA suppresses the sporulation defect, but not the competence defect of a *spo0K* mutant (Rudner et al., 1991). This indicates that Spo0K may act upstream of KinA, and hence upstream of Spo0A, in the initiation of sporulation. Identifying genes which when overexpressed bypass the need for *spo0K* in the initiation of sporulation might also identify genes that regulate the phosphorelay. Similarly, expression of the early-acting competence gene *srfA* from a heterologous promoter suppresses the competence defect of a *spo0K* mutant (Hahn and Dubnau, 1991; Nakano and Zuber, 1991).

Rationale. Identification of genes based on phenotypes caused by increased expression is a relatively general approach that is easier and can sometimes be used in place of isolating gain-of-function mutations. In addition, genes that might be difficult to identify based on phenotypes caused by loss-of-function mutations are sometimes easier to identify based on phenotypes caused by overexpression.

To isolate genes which when present on a multicopy plasmid, and presumably overexpressed, bypass the need for *spo0K* in either the initiation of sporulation or the development of competence, I transformed a *spo0K* mutant with two size-selected multicopy plasmid libraries (see below). Because *spo0K* mutants have a decreased frequency of transformation (Roggiani et al., 1990;

Rudner et al., 1991; and Chapter 2), I constructed a conditional *spo0K* mutation in which to transform the multicopy plasmid libraries. The multicopy plasmid library could be transformed into the mutant under permissive conditions, when the cells are Spo0K$^+$, and screened under nonpermissive conditions, when the cells are Spo0K$^-$. The conditional allele replaced the normal *spo0K* promoter with the LacI-repressible/IPTG-inducible promoter Pspac such that the Pspac-*spo0K* fusion was in single copy in the chromosome and was the only copy of *spo0K* (for details of the construction of the Pspac-*spo0K* allele, see Figure 7.1 and Chapter 7). Strains containing the Pspac-*spo0K* fusion as the only copy of *spo0K* could be made competent under permissive conditions (in the presence of IPTG) and transformants screened under nonpermissive conditions (in the absence of IPTG).

Making the multicopy plasmid libraries. Two different multicopy plasmid libraries were made in the *B. subtilis*/*E. coli* shuttle vector pHP13 (Haima et al., 1987; Bron, 1990). pHP13 has two drug markers suitable for selection in *B. subtilis*: *erm* which confers MLS^R and *cat* which confers Cm^R. In *B. subtilis,* pHP13 has a copy number of ~5-6 per cell (Haima et al., 1987; Bron, 1990). Chromosomal DNA from JH642 was partially digested with Sau3A and electrophoresed on an agarose gel. DNA was isolated from the size range of approximately 2 to 4.5 kb (library A) and approximately 4.5 to 9 kb (library B), based on size markers run in parallel. Size fractionated DNA was ligated into pHP13 that had been linearized with BamHI and treated with phosphatase, and transformed into *E. coli* selecting for resistance to chloramphenicol. Transformants were pooled and plasmid DNA was prepared for transformation into *B. subtilis*.

Approximately 80 to 90% of the plasmids in library A had inserts and the chance that any particular fragment of the average size of 3.2 kb is present in the

library is ~87%, assuming a random distribution of Sau3A sites, no bias in selection in *E. coli*, and an average insert size of ~3.2 kb (probably an overestimate). For library B, ~60 to 70% of the plasmids had inserts, and assuming an average size of 5 kb, the chances that a particular fragment of average size is represented is ~50%.

Plasmid marker rescue transformation. *B. subtilis* takes up transforming DNA as a linear single strand, making establishment of a multicopy circular plasmid rather inefficient. However, incoming DNA is able to recombine with homologous sequences very efficiently. To facilitate introduction and establishment of a multicopy plasmid library in *B. subtilis*, I constructed a helper plasmid, pJL52, that could be used in combination with the cloning vector pHP13. The helper plasmid, pJL52, was made by deleting from BamHI to NcoI in pHP13. This deletes part of *cat*, and pJL52 does not confer Cm^R but still confers MLS^R. When a competent cell containing pJL52 takes up a plasmid monomer from a pHP13-based library and linearizes it, pJL52 provides the homology necessary for the plasmid to recombine and form a closed circular plasmid. One can select for these recombination events using the Cm^R marker present on pHP13 that is not present on pJL52. This general process is known as plasmid marker rescue transformation or homology assistance (Gryczan et al., 1980; Bron, 1990).

Conditions for the sporulation suppressor screen. Since *spo0K* mutations cause a more severe sporulation defect in the PB2 strain background than in the JH642 background (Rudner et al., 1991), it seemed that a screen for multicopy suppressors of the *spo0K* sporulation defect might be more sensitive in the PB2 background. Accordingly, I constructed a PB2 derivative that contained the Pspac-*spo0K* fusion, the helper plasmid pJL52, and a *spoIIA-lacZ* transcriptional fusion (Ireton and Grossman, 1994). This strain, JRL459, was grown in the

presence of MLS to maintain pJL52. JRL459 was made competent in S7 minimal medium (+ tryptophan at 40 μg/ml) in 1 mM IPTG to allow expression of *spo0K*. Competent cells were transformed with the pHP13-based multicopy plasmid libraries and Cm^R transformants were selected on 2xSG plates containing chloramphenicol or containing chloramphenicol and Xgal (120 μg/ml). Transformants that had a more pronounced Spo^+ morphology, or that were darker blue on Xgal (indicating increased expression of *spoIIA-lacZ*) compared to the parent strain were chosen for further analysis.

Conditions for the competence suppressor screen. The conditions used to find suppressors of the competence defect of a *spo0K* mutant were similar to those used to find suppressors of the sporulation defect. First, I constructed a PB2 derivative that contained the Pspac-*spo0K* fusion, the helper plasmid pJL52, and a *comG-lacZ* transcriptional fusion; *comG* is a late competence gene the expression of which coincides with competence (Dubnau, 1993). This strain, JRL454, was made competent in the presence of MLS, to maintain pJL52, and in 1 mM IPTG, to allow expression of *spo0K*. Competent cells were transformed with the pHP13-based libraries. Because transformants did not grow well when plated directly on minimal medium plates containing chloramphenicol and Xgal (120 μg/ml), they were first selected on 2xSG plates containing chloramphenicol and then replica plated onto minimal medium plates containing chloramphenicol and Xgal. Transformants that appeared to have increased expression of *comG-lacZ*, as judged by a darker blue color on Xgal indicator plates, were chosen for further analysis. (The initial plating onto 2xSG plates containing chloramphenicol also allowed screening of more transformants for the sporulation suppressor screen.)

Isolation of multicopy suppressors of the *spo0K* sporulation defect. I screened approximately 11,000 transformants (~4,300 from library A and ~6,700

from library B) for suppression of the *spo0K* sporulation defect, and found 9 that contained plasmids that reproducibly suppressed the sporulation defect caused by the conditional *spo0K* mutation (Table 3.1). To test if these plasmids suppressed the sporulation defect caused by a true *spo0K* null mutation (*Δspo0KABCD*::*erm*), I isolated plasmid DNA from the candidates and introduced it into wild type (PB2), a Pspac-*spo0K* mutant (JRL408), and a *Δspo0K*::*erm* mutant (JRL417). All 9 plasmids suppressed the sporulation defect caused by the *Δspo0K*::*erm* mutation as well as that caused by the Pspac-*spo0K* mutation, as judged by colony morphology on sporulation plates.

Restriction mapping of the 9 plasmids showed that there were just two classes of plasmids that contained different inserts, exemplified by the plasmids pLK2 and pLK11 (Table 3.1). The genes contained on these plasmids were called *mskA* and *mskB*, respectively, where *msk* stands for multicopy suppressor of *spo0K*. These two plasmids suppressed or partially suppressed, the sporulation defect, but not the competence defect, caused by null mutations in *spo0K*. As described in the next chapter, *mskA* encodes a sensor kinase most similar to the sporulation sensor kinases *kinA* and *kinB*, and has since been renamed *kinC*. *mskB* is described in Chapter 5, and appears to encode an inhibitor of SinR, a repressor of *spo0A* transcription (I. Smith, personal communication).

Isolation of multicopy suppressors of the *spo0K* competence defect. I screened approximately 3,700 transformants (~3,400 from library A and 272 from library B) for suppression of the *spo0K* competence defect, and found 3 that contained plasmids that reproducibly suppressed the competence defect caused by the conditional *spo0K* mutation (Table 3.1). Restriction mapping of the 3 plasmids showed that there were again two classes of inserts in the plasmids, exemplified by plasmids pLK4 and pLK6. pLK4 seemed to be unstable in *E. coli*

and consequently was not studied further. Analysis of pLK6 is described in Chapter 5.

TABLE 3.1. Summary of screen for multicopy suppressors of the sporulation (Spo) or competence (Com) defects of a *spo0K* mutant.

Class	*spo0K* defect suppressed	Library	Original[a] plasmid	Strain in which isolated	Screen used to isolate plasmid
pLK2	Spo	B	pLK2	JRL459	*spoIIA-lacZ*
"			pLK3	JRL459	*spoIIA-lacZ*
"			pLK7	JRL459	Spo^+
"			pLK8	JRL454	Spo^+
"			pLK9	JRL459	Spo^+
"			pLK12	JRL454	Spo^+
pLK11	Spo	A	pLK10[b]	JRL454	Spo^+
"			pLK11	JRL454	Spo^+
"			pLK15	JRL454	Spo^+
pLK6	Com	A	pLK6	JRL454	*comG-lacZ*
pLK4	Com	B	pLK4	JRL454	*comG-lacZ*
"			pLK5	JRL454	*comG-lacZ*

[a] Five transformants originally isolated did not show suppression upon retesting, and one other isolate contained a plasmid that did not confer suppressing activity upon reintroduction into the parent strain.

[b] The insert in pLK10 contains a 135 bp deletion relative to pLK11 (see Chapter 5).

Chapter 4:

Characterization of *kinC*, and the different roles of the sensor kinases KinA, KinB, and KinC in sporulation

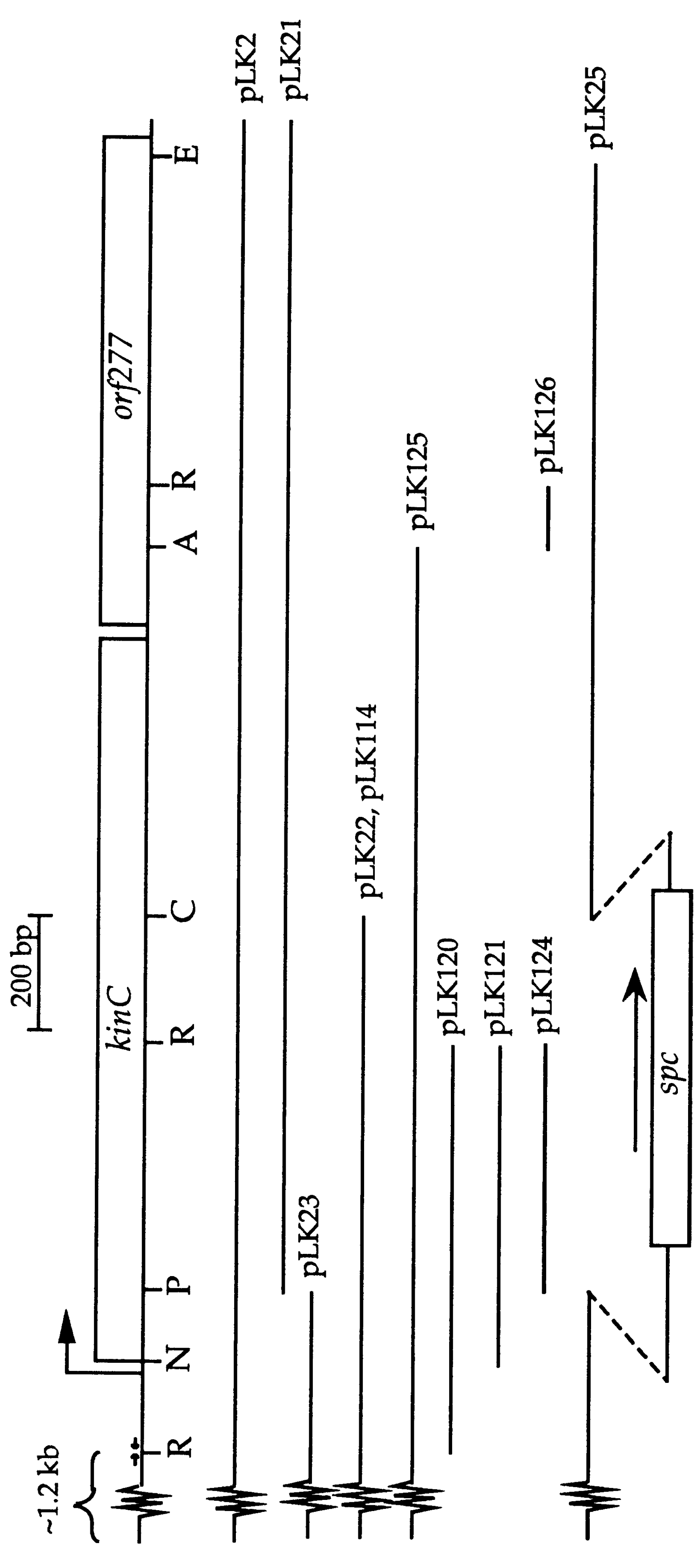
As described in the previous chapter, I isolated two multicopy plasmids that contain genes that suppress (pLK2) or partially suppress (pLK11) the sporulation defect caused by a *spo0K* mutation. pLK2 had the more dramatic effect and the gene responsible for this effect encodes a previously unknown histidine protein kinase, *kinC*. In this chapter, I describe the isolation and characterization of *kinC*, as well as the different roles of the three sensor kinases involved in sporulation of *Bacillus subtilis*, KinA, KinB, and KinC. These sensor kinases autophosphorylate and serve as phosphate-donors to the response regulator Spo0F; the phosphotransferase Spo0B then transfers phosphate from Spo0F to the response regulator and sporulation transcription factor Spo0A (see Figure 1.1). This system is known as the phosphorelay (Burbulys et al., 1991).

Although KinC is not necessary for the initiation of sporulation, it is the first of the three kinases to have an effect on the accumulation of Spo0A~P, as judged by derepression of *abrB* transcription; *abrB* is repressed by Spo0A~P (Perego et al., 1988; Strauch et al., 1990). There exist mutant forms of Spo0A encoded by the *spo0A* alleles *rvtA11* (Sharrock et al., 1984) and *sof-1* (Kawamura and Saito, 1983; Hoch et al., 1985; Spiegelman et al., 1990) that suppress *spo0F* and *spo0B* mutations; in the absence of Spo0F or Spo0B, KinC is the major kinase that donates phosphate to the *sof-1* and *rvtA11* forms of Spo0A.

pLK2 contains a gene (*kinC*) encoding a histidine protein kinase homologous to KinA and KinB. Various fragments from the insert in pLK2 were subcloned into the multicopy vector (pHP13) to give pLK21, pLK22, pLK23, and pLK125 (Figure 4.1, Table 7.2). These subclones were used to identify the region responsible for suppression of the *spo0K* sporulation phenotype. pLK22 and pLK23 did not suppress the *spo0K* mutant. The insert in pLK125 extends from the left end of pLK2 (as drawn in Figure 4.1) to the ApaI site and completely suppressed the *spo0K* sporulation phenotype. pLK21 contains the right end of the insert in pLK2 and ends at the PstI site and partially suppressed the sporulation phenotype. These results indicate that the suppressing activity is located upstream of the ApaI site (Figure 4.1).

I determined the DNA sequence from the EcoRV site upstream of *kinC* (previously *mskA*) to the right end of the pLK2 insert (Figure 4.2). Analysis of the sequence revealed two open reading frames. The predicted protein product of the first open reading frame is 428 amino acids and is homologous to histidine protein kinases. Of all the proteins in the combined databases, KinC is most similar to the two histidine protein kinases involved in sporulation, KinA (Perego et al., 1989; Antoniewski et al., 1990) and KinB (Trach and Hoch, 1993). In the carboxy-terminal 213 amino acids of the proteins, KinC has 111 residues (51%) that are identical to KinA, and 81 (36%) that are identical to KinB (Figure 4.3). The ability to suppress the *spo0K* sporulation defect was localized to the *kinC* open reading frame (see above). pLK21, which partially suppresses the sporulation defect, is truncated in the 5' end of the open reading frame and probably produces a protein fragment that is likely expressed from a plasmid promoter.

Figure 4.1. Map of the *kinC* region and plasmids. Restriction site abbreviations: R, EcoRV; N, NdeI; P, PstI; C, ClaI; A, ApaI; E, EcoRI. pLK2, pLK21, pLK22, pLK23 and pLK125 are present in multicopy in *Bacillus subtilis*; pLK25, pLK114, pLK120, pLK121, pLK124, and pLK126 are integrative plasmids.



~1.2 kb
200 bp
kinC
orf277
R
N
P
R
C
A
R
E
pLK2
pLK21
pLK23
pLK22, pLK114
pLK125
pLK120
pLK121
pLK124
pLK126
pLK25
spc

Figure 4.2. Sequence of *kinC*. DNA sequence was determined from both strands as described in Chapter 7. An inverted repeat surrounding the EcoRV site upstream of *kinC* is indicated by a pair of arrows. A putative -10 region for a sigma-A promoter is shown with a thick underline. The transcriptional start site is indicated with an arrow at nucleotide 158. Putative ribosome binding sites for KinC and Orf277 are underlined, and putative translational start sites for KinC and Orf277 are boldfaced. Sequence complementary to primer LKP16, which was used for primer extension analysis, is shown with a single arrow. This sequence has been assigned the databank accession number L34803.

```
                          EcoRV
   1  CTAAAAAAGGCGGAGTGATATCATCTCCGCCTTTTTTGCGTGCCAATTTTTGATTACCGCCGCCTAAGATAAGACATCAAGATATTTGGT

  91  AATGAATGATTTGGGATACTTTACATATTTTACTCAATTATTTGTCGAAGAATGGTACAATAAGTAGAGAAACACAAGCGGCAGGTGGTC
 181  ATATGAGAAAATATCAAGCTCGTATCATTTCCATCATTTTGGCAATGATTTTTATAATGTTTTGGGATTATTTATTTTATTTTATAGGCA
KinC  M  R  K  Y  Q  A  R  I  I  S  I  I  L  A  M  I  F  I  M  F  W  D  Y  L  F  Y  F  I  G  K
 271  AAAACCCGATTAATTGGCCTGTTGATATTGTGTATACTGCAGTCACGCTGGTAAGTGTCTGGATGTTGGCTTATTATATTGATGAGAAAC
        N  P  I  N  W  P  V  D  I  V  Y  T  A  V  T  L  V  S  V  W  M  L  A  Y  Y  I  D  E  K  Q
 361  AGCAGCTGGTTAAGAAAATGAAGGATAACGAATGGAAGTATAAACAGCTTTCTGAAGAAAAAAACCGCATCATGGATAATTTGCAGGAAA
        Q  L  V  K  K  M  K  D  N  E  W  K  Y  K  Q  L  S  E  E  K  N  R  I  M  D  N  L  Q  E  I
 451  TCGTATTTCAAACGAATGCAAAAGGTGAAATTACATATTTAAACCAAGCGTGGGCATCTATAACCGGGTTTTCAATCAGTGAATGTATGG
        V  F  Q  T  N  A  K  G  E  I  T  Y  L  N  Q  A  W  A  S  I  T  G  F  S  I  S  E  C  M  G
 541  GAACAATGTATAACGATTACTTCATAAAAGAAAAGCACGTAGCCGACCACATTAACACCCAAATCCAAAACAAAGCGTCTTCTGGCATGT
        T  M  Y  N  D  Y  F  I  K  E  K  H  V  A  D  H  I  N  T  Q  I  Q  N  K  A  S  S  G  M  F
 631  TTACGGCAAAATACGTGACAAAAAACGGCACGATTTTTTGGGGAGAAGTTCATTATAAACTTTACTATGACCGGGATGACCAATTTACAG
        T  A  K  Y  V  T  K  N  G  T  I  F  W  G  E  V  H  Y  K  L  Y  Y  D  R  D  D  Q  F  T  G
 721  GCAGCCTGGGTACAATGTCAGATATCACTGAGCGGAAAGAGGCTGAAGATGAGCTCATTGAGATTAATGAACGGCTGGCGAGGGAATCCC
        S  L  G  T  M  S  D  I  T  E  R  K  E  A  E  D  E  L  I  E  I  N  E  R  L  A  R  E  S  Q
 811  AGAAACTATCAATCACAAGTGAACTTGCCGCAGGTATTGCTCATGAGGTCAGAAACCCTTTAACATCTGTCAGCGGTTTCCTCCAGATTA
        K  L  S  I  T  S  E  L  A  A  G  I  A  H  E  V  R  N  P  L  T  S  V  S  G  F  L  Q  I  M
 901  TGAAAACACAATATCCGGACAGAAAAGACTATTTTGACATCATCTTTTCAGAGATTAAAAGAATCGATTTAGTGCTCAGCGAGCTGCTGC
        K  T  Q  Y  P  D  R  K  D  Y  F  D  I  I  F  S  E  I  K  R  I  D  L  V  L  S  E  L  L  L
 991  TGCTTGCAAAACCGCAGGCAATCACATTTAAAACACACCAGCTTAATGAGATCTTGAAACAAGTCACGACATTGCTTGATACCAATGCAA
        L  A  K  P  Q  A  I  T  F  K  T  H  Q  L  N  E  I  L  K  Q  V  T  T  L  L  D  T  N  A  I
1081  TTCTGTCCAATATCGTCATAGAGAAAAATTTCAAAGAGACAGATGGCTGTATGATTAATGGAGACGAAAATCAGCTGAAGCAGGTCTTTA
        L  S  N  I  V  I  E  K  N  F  K  E  T  D  G  C  M  I  N  G  D  E  N  Q  L  K  Q  V  F  I
1171  TCAACATCATTAAAAACGGAATTGAGGCAATGCCAAAGGGCGGTGTCGTAACCATTTCAACTGCTAAAACCGCCTCTCATGCAGTGATAA
        N  I  I  K  N  G  I  E  A  M  P  K  G  G  V  V  T  I  S  T  A  K  T  A  S  H  A  V  I  S
1261  GCGTAAAGGATGAAGGAAACGGCATGCCGCAGGAAAAGCTGAAGCAGATTGGCAAACCTTTTTATTCAACAAAAGAAAAGGGCACTGGAC
        V  K  D  E  G  N  G  M  P  Q  E  K  L  K  Q  I  G  K  P  F  Y  S  T  K  E  K  G  T  G  L
1351  TGGGACTTCCCATTTGTTTGAGAATCCTGAAGGAACATGACGGGGAATTGAAAATCGAAAGTGAAGCTGGAAAAGGCAGCGTCTTTCAAG
        G  L  P  I  C  L  R  I  L  K  E  H  D  G  E  L  K  I  E  S  E  A  G  K  G  S  V  F  Q  V
1441  TGGTTTTGCCTTTAAAATCAGACAGCTGAGAGGAGAAAAATAAAGTGAACTCGCTTCTGTTTGTATACGGGACATTAAGAAAGCATGAAA
        V  L  P  L  K  S  D  S  *        Orf277  M  N  S  L  L  F  V  Y  G  T  L  R  K  H  E  K
1531  AAAACCATCATTTGCTGGCACAATCGGCATGTATCAATGAGCAGGCGAGAACAAAGGGAAGTTTGTTTGCTGCAAAAGAGGGGCCCACAG
        N  H  H  L  L  A  Q  S  A  C  I  N  E  Q  A  R  T  K  G  S  L  F  A  A  K  E  G  P  T  V
1621  TTGTTTTCAATGATGAAGATGAAGGCTATATATATGGCGAAGTATATGAAGCAGATGAATTGTGTATACATAAGCTCGATCAATTTTTTC
        V  F  N  D  E  D  E  G  Y  I  Y  G  E  V  Y  E  A  D  E  L  C  I  H  K  L  D  Q  F  F  Q
1711  AAGGATATCATAAACAAACGGTGTTTGTAGAAACGGATGTCGGGATTAAAATTGCGCTTATTTATTTTATGAACAAAGACGGGTGTGCCG
        G  Y  H  K  Q  T  V  F  V  E  T  D  V  G  I  K  I  A  L  I  Y  F  M  N  K  D  G  C  A  G
1801  GTTTTACGAAAATAAGCAGCGGCGACTGGAAAGAACATCAGATGATCAGCAAATCGAAAAATCCCATTTATTATTTTGCCTATGGATCAT
        F  T  K  I  S  S  G  D  W  K  E  H  Q  M  I  S  K  S  K  N  P  I  Y  Y  F  A  Y  G  S  C
1891  GCATGGATAATGCCCGCTTTCAAAAAGCGGGAGTCGATCACTATTTTCAAGATCCAGTAGGAAGAGCTGTTTTAAAAGGATACACAACCC
        M  D  N  A  R  F  Q  K  A  G  V  D  H  Y  F  Q  D  P  V  G  R  A  V  L  K  G  Y  T  T  R
1981  GCTTCACGCTAAAAAGGGAAGACGGTTCAAGAGCGGACATGTTGGAAGACGGAGGAACAACAGAAGGCGTTTTATACCGTATCCCTTATT
        F  T  L  K  R  E  D  G  S  R  A  D  M  L  E  D  G  G  T  T  E  G  V  L  Y  R  I  P  Y  S
2071  CTGCTCTCTCCTATCTATATAAAAGGGAGGGCGTCGAATCTCTTACGTATCGGCCGGCATTTGTAGACGTTGAAGCTGGCGGAAGGCACT
        A  L  S  Y  L  Y  K  R  E  G  V  E  S  L  T  Y  R  P  A  F  V  D  V  E  A  G  G  R  H  Y
2161  ACAAAGACTGTTTAACCTTTCTCGTCCTCCAAAAAGAAGCGGAAATTGCCCCGCCTCAGCACTATCAGATTGAAATCGAACGCGGAGCGG
        K  D  C  L  T  F  L  V  L  Q  K  E  A  E  I  A  P  P  Q  H  Y  Q  I  E  I  E  R  G  A  E
2251  AGCTGTATTTGTCGCCTGAGTTTACTGAAAAGCTCAAGCGGCATATGAATTCGCTGCCAAAAGGATAACACTGTAACAAAAGAATATGAT
        L  Y  L  S  P  E  F  T  E  K  L  K  R  H  M  N  S  L  P  K  G  *
2341  AAAATTTGATC  2351
```

Figure 4.3. Comparison of the C-terminal region of KinC to the C-terminal regions of KinA and KinB. The sequences are aligned according to the PILEUP program of the UWGCG software package (Devereux et al., 1984). The first residue shown is amino acid 389, 204, and 205 for KinA, KinC, and KinB respectively. In the regions shown, KinC is 51% identical to KinA and 36% identical to KinB. The residues in bold are conserved in almost every sensor kinase known (Stock et al., 1989b; Parkinson and Kofoid, 1992), and in KinC, include the histidine at position 223, which is the histidine thought to become phosphorylated, the asparagine at position 331, and the two glycine rich regions at positions 363-367 and 387-391.

```
KinA  ...KSEKLSI AGQLAAGIAH EIRNPLTAIK GFLQLM.... KPTMEGNEHY  431
         |:||||  .::||||||| |:|||||.:. ||||:|      |.  .:...|
KinC  LARESQKLSI TSELAAGIAH EVRNPLTSVS GFLQIM.... KTQYPDRKDY  250
        .|:|:.|  .||||::|| ||||||| |. ||:|::      . | ... ||
KinB  .LIHSEKMTI VSELAASVAH EVRNPLTVVR GFVQLLFNDE TLQNKSSADY  252

KinA  FDIVFSELSR IELILSELLM LAKPQQNAVK EYLNLKKLIG EVSALLETQA  481
      |||:|||:.| |:|:||||||: |||||. ..| .. .|..:: :|..||:|.|
KinC  FDIIFSEIKR IDLVLSELLL LAKPQAITFK TH.QLNEILK QVTTLLDTNA  299
       .:::||:.|  : ::.:.|  :||.| .. |     |..::| :...|: . |
KinB  KKLVLSELDR AQGIITNYLD MAKQQLYE.K EVFDLSALIK ETSSLMVSYA  301

KinA  NLNGIFIRTS Y.EKDSIYIN GDQNQLKQVF INLIKNAVES MPDG.GTVDI  529
       |..|.| .. : |.|:. || ||:||||||| ||:|||::|. ||.| |.|.|
KinC  ILSNIVIEKN FKETDGCMIN GDENQLKQVF INIIKNGIEA MPKG.GVVTI  348
       ...:.:| :     ...: :|  ||...|||.  ||::||:||| :|.| |:: |
KinB  NYKSVTVEAE ..TEPDLLIY GDATKLKQA. INLMKNSIEA VPHGKGMIHI  348

KinA  IITEDEHSVH VTVKDEGEGI PEKVLNRIGE PFLTTKEKGT GLGLMVTFNI  579
       .....  .  :.||||:|: |:. |..||. ||..|||||| |||| :.:.|
KinC  STAKTASHAV ISVKDEGNGM PQEKLKQIGK PFYSTKEKGT GLGLPICLRI  398
      |. :.:    : |.:.|:| || .:..:..:|. |:|| |..|| ||||.:.:.|
KinB  SAKRNGHTIM INITDNGVGM TDHQMQKLGE PYYSLKTNGT GLGLTVTFSI  398

KinA  IENHQGVIHV DSHPEKGTAF KISFPKK*    606
      :.:|:| :.: :|.::||..| .: :| |
KinC  LKEHDGELKI ESEAGKGSVF QVVLPLKSDS* 428
      :..|.|.:.: :|. .||...  : || .
KinB  IEHHHGTISF NSSFQKGTTV TIKLPADLPH* 428
```

Just downstream of *kinC* there is an open reading frame predicted to encode a protein of 277 amino acids, Orf277 (Figure 4.2). The *orf277* gene product does not appear to be similar to any other protein in the database. In particular it does not seem to be a member of the response regulator family of proteins (sensor proteins and their cognate response regulators often occur in the same operon (Albright et al., 1989)), nor is it similar to KapB, the product of the gene downstream of *kinB* (Trach and Hoch, 1993).

This same locus was also sequenced independently by Kobayashi et al. (1994), and they named it *kinC* also. Trach and Hoch (1993) briefly mention an open reading frame they call *kinC* that is homologous to *kinA* and *kinB*, but it is not clear whether this is the same as the locus I describe in this report.

Genetic mapping of *kinC*. I used generalized transduction with PBS1 to determine the chromosomal map location of *kinC*. A PBS1 lysate was made from JRL660 (*ΔkinC::spc*) and used to transduce the mapping kit strains (Dedonder et al., 1977) to $Spec^R$. Initial results indicated that *kinC* was ~15 to ~20% cotransduced with $pyrD^+$. A series of two factor crosses indicated that *kinC* was ~50% linked to *spo0E* and ~50% linked to *kinA* by transduction. To map *kinC* with greater resolution, I did a three factor cross with *ΔkinC::spc*, *spo0E11*, and *kinA*::Tn*917*. Strain KI644 ($kinC^+$, *spo0E11*, *kinA*::Tn*917*) was transduced to $Spec^R$ with a PBS1 lysate grown on JRL660 (*ΔkinC::spc*, $spo0E^+$, $kinA^+$). $spo0E^+$ was cotransduced with *ΔkinC::spc* in 107 out of 199 transductants and $kinA^+$ was cotransduced with *ΔkinC::spc* in 147 out of 199 transductants. Every $spo0E^+$ transductant was also $kinA^+$ (MLS^S), indicating that the gene order is *spo0E - kinA - kinC*.

Effects of overexpression of *kinC* in different *spo0* mutants. I tested the ability of multicopy *kinC* (pLK2) to suppress the sporulation defect caused by *spo0* mutations. In all cases, the sporulation frequency was measured in

otherwise isogenic strains containing the specific sporulation mutation and either pLK2 or the cloning vector, pHP13. pLK2 significantly suppressed the sporulation defect caused by *spo0K, spo0J, spo0F,* and *spo0B* mutations (Table 4.1). In addition, it also suppressed the sporulation defect of a *kinA* mutant (Table 4.1). Multicopy *kinC* did not suppress the sporulation defect of the *spo0A9V* mutant nor that of the *spo0E11* mutant. Because multicopy *kinC* is able to significantly bypass the need for *spo0F* and *spo0B* in sporulation, it appears that KinC, at least when overexpressed, is able to act directly on Spo0A.

The effects of pLK2 on the *spo0B* mutant makes it possible to determine the likely target of Spo0E *in vivo. In vitro* experiments have shown that Spo0E is a phosphatase that removes phosphate from Spo0A~P (Ohlsen et al., 1994). *spo0E11* is a nonsense mutation that produces an N-terminal fragment of Spo0E and inhibits sporulation (Perego and Hoch, 1991), most likely due to increased phosphatase activity (Ohlsen et al., 1994).

If the *in vivo* target of Spo0E is one of the components of the phosphorelay (Spo0F or Spo0B), then *spo0E* mutations should have little or no effect in the absence of a functioning phosphorelay. On the other hand, if the *in vivo* target of Spo0E is Spo0A, as suggested by the *in vitro* results (Ohlsen et al., 1994), then *spo0E* mutations are likely to have effects in the absence of the phosphorelay, if they can be measured. pLK2 partially suppressed the sporulation defect of a *spo0F spo0B* double mutant (Table 4.1) and this suppression was enhanced by a null mutation in *spo0E* (Table 4.1; *spo0F spo0B Δspo0E::spc*). Since the *spo0E* mutation causes a phenotype in the absence of Spo0F and Spo0B, Spo0E is probably inhibiting Spo0A directly, consistent with the *in vitro* results.

TABLE 4.1 Suppression of *spo0* mutants by multicopy *kinC*.

relevant genotype of strain harboring plasmid	sporulation frequency[a]	
	pHP13 (vector)	pLK2 (multicopy *kinC*)
WT (JH642)	0.41	0.81
Δspo0K358::erm	6.0×10^{-3}	0.63
kinA::Tn*917*	9.8×10^{-2}	0.28
spo0A9V	$<9.0 \times 10^{-8}$	$<8.2 \times 10^{-8}$
spo0J93	2.1×10^{-4}	0.21
spo0E11	2.5×10^{-3}	6.9×10^{-3}
spo0FΔS	$<2.4 \times 10^{-7}$	9.8×10^{-3}
spo0BΔPst	$<4.1 \times 10^{-7}$	1.1×10^{-2}
spo0FΔS spo0BΔPst	$<1.8 \times 10^{-7}$	2.5×10^{-2}
spo0FΔS spo0BΔPst Δspo0E::spc	8.5×10^{-7}	0.22

[a]Cells were grown in DS medium with chloramphenicol and the sporulation frequency was determined as described in Chapter 7: Materials and Methods.

***kinC* null mutants have no significant effect on sporulation.** To characterize the phenotypes caused by loss of *kinC*, I constructed a strain (JRL660) containing a deletion-insertion mutation in *kinC*, *ΔkinC::spc* (Figure 4.1; Chapter 7). The *ΔkinC::spc* mutation had little or no effect on the sporulation frequency compared to wild type (Table 4.2). Sporulation conditions tested included 2xSG medium, DS medium, minimal exhaustion medium (Table 4.2), and minimal medium with decoyinine (data not shown). In addition, the *ΔkinC::spc* mutation did not significantly affect the sporulation defect caused by mutations in *kinA*, *kinB* (Table 4.2), *spo0K* or *spo0J* (data not shown). That is, double mutants with *ΔkinC::spc* had sporulation phenotypes similar to the single mutants. Also, the *ΔkinC::spc* mutation had little or no effect on competence development or expression of *comG-lacZ* compared to otherwise isogenic *kinC*$^+$ cells (Srivastava, 1994).

KinC is required for the residual sporulation seen in a *kinA kinB* double mutant in the rich sporulation medium, 2xSG. *kinA kinB* double mutants had a much more severe sporulation defect than either single mutant under all conditions tested (Table 4.2) consistent with previous findings (Trach and Hoch, 1993). In 2xSG medium the *kinA kinB* double mutant consistently produced approximately 10^3 spores/ml, at least 100- to 1000-fold more than in DS medium or minimal medium (Table 4.2). This small but reproducible level of sporulation in 2xSG medium was entirely dependent on *kinC* as the *kinA kinB kinC* triple mutant produced <10 spores/ml in 2xSG medium (Table 4.2). *kinC*, and not any other gene downstream of *kinC*, was responsible for this phenotype, since a disruption of *orf277* had little or no effect on the sporulation of the *kinA kinB* double mutant (Table 4.2).

TABLE 4.2 Relative sporulation frequency of different *kin* mutants in different media.

strain	genotype[b]	relative sporulation frequency[a] 2xSG[c]	DSM[c]	minimal[c]
JH642	WT	1	1	1
AG522	*kinA*	0.10	8.0×10^{-2}	1.2
NY120	*kinB*	0.67	0.13	7.1×10^{-2}
JRL920	*kinC*	0.77	0.45	0.91
NY121	*kinA kinB*	1.9×10^{-6}	$<5 \times 10^{-8}$	$<3 \times 10^{-7}$
JRL1046	*kinA kinC*	3.3×10^{-2}	6.8×10^{-2}	0.41
JRL1004	*kinB kinC*	0.43	0.15	1.0×10^{-2}
JRL1007	*kinA kinB kinC*	$<3 \times 10^{-8}$	$<2 \times 10^{-7}$	$<3 \times 10^{-7}$
JRL1081	*kinA kinB orf277*	3.2×10^{-6}	-	-

[a] Relative sporulation frequency is the number of spores/ml as a fraction of the number of cells/ml, normalized to the control (JH642) in a given experiment.

[b] The JH642 strain is *trpC2 pheA1* (Perego et al., 1988), and all the other strains shown are isogenic with JH642. The *kinA* allele is *kinA*::Tn*917* (Sandman et al., 1987; Antoniewski et al., 1990), the *kinB* allele is *ΔkinBkapB*::*spc* (Materials and Methods), the *kinC* allele is *kinC*::pLK124 (Figure 4.1), and the *orf277* allele is *orf277*::pLK126 (Figure 4.1).

[c] 2xSG is a rich sporulation medium and contains nutrient broth and 0.1% glucose (Leighton and Doi, 1971). DSM is the nutrient sporulation medium of Schaeffer (Schaeffer et al., 1965). The minimal medium was S7 medium (Vasantha and Freese, 1980) as used previously (Jaacks et al., 1989), except that glucose was used at 0.1%.

Phenotypes caused by *kinA* or *kinB* null mutations depend on the sporulation medium. The ability of KinC to affect sporulation in a *kinA kinB* double mutant is dependent on the sporulation medium. How does the sporulation medium affect the roles of KinA and KinB in the initiation of sporulation? *kinA* null mutants sporulated at a frequency of approximately 4 - 10% of wild type when grown in DS (nutrient broth) medium or the richer 2xSG (twice the nutrient broth as DS medium plus 0.1% glucose) medium (Table 4.2), consistent with published data (Sandman et al., 1987; Perego et al., 1989; Antoniewski et al., 1990; Rudner et al., 1991; Lee et al., 1992). However, when sporulation was induced by exhaustion of glucose from cultures grown in defined minimal medium with glucose (0.1%) as the carbon source, the *kinA* mutant was able to sporulate at or near wild type frequencies (Table 4.2). In contrast, the sporulation frequency of the *kinB* mutant was approximately normal in 2xSG medium and approximately 5 - 10% of wild type in DS medium or minimal medium (Table 4.2). The phenotype of *kinB* in DS medium was somewhat variable and seemed to depend on the specific preparation of DS medium, and is consistent with effects reported by others (Trach and Hoch, 1993). On the other hand, the sporulation defect of the *kinB* mutant in minimal glucose medium was highly reproducible, but was observed only if the culture had undergone at least four or five doublings after inoculation and before entry into stationary phase.

The requirement for a certain number of cell divisions before observing the Spo^- phenotype of the *kinB* mutant indicates that an activator of sporulation had accumulated in the preculture that was used as the inoculum, and this factor must be diluted below a certain level to see the *kinB* phenotype. This factor could be a metabolite or a gene product that could substitute for KinB, perhaps another kinase.

Taken together, these results indicate that under certain conditions either KinA or KinB is the major sporulation kinase, and that the different sporulation sensor kinases can respond to different nutritional conditions. These effects could reflect differences in expression of *kinA* and *kinB*, or differences in regulation of kinase activity, or both.

KinC is required for *sof-1* and *rvtA11* to suppress the sporulation defect of *spo0B* mutants. The *sof-1* and *rvtA11* alleles of *spo0A* bypass or partially bypass the sporulation defect caused by mutations in *spo0F* and *spo0B*, presumably because these altered forms of Spo0A will accept phosphate from kinases independently of Spo0F and Spo0B. To determine whether KinC is one such kinase, I transformed the *ΔkinC::spc* mutation into a *spo0B* mutant that had either the *sof-1* or *rvtA11* alleles of Spo0A. The *kinC* mutation completely abolished the suppressing effect of either allele (Table 4.3). In addition, *kinC* was required for *sof-1* and *rvtA11* to suppress *spo0F* mutants (data not shown). *kinC*, and not *orf277* or any other gene downstream of *kinC*, was responsible for this phenotype. Disruption of *orf277* by integrating pLK126 (Figure 4.1) by single crossover had no effect on the ability of *rvtA11* to suppress *spo0F221* (data not shown). These results indicate that in the absence of Spo0F or Spo0B, KinC is the major kinase that donates phosphate to the *sof-1* and *rvtA11* forms of Spo0A.

Additional results suggest that KinC can also act on Spo0F, and in some conditions, might prefer Spo0F over Spo0A. The incomplete suppression of the *spo0B* mutant by *sof-1* or *rvtA11* was due to the presence of *spo0F*$^+$. *rvtA11* allowed *spo0F spo0B* double mutants to sporulate much better than the otherwise isogenic *spo0B* single mutant and this suppression was

TABLE 4.3 Suppression of *spo0BΔPst* by *sof-1* or *rvtA11* requires *kinC*.

strain	relevant genotype	sporulation frequency[a]
JH642	WT	0.59
JRL791	*ΔkinC::spc rvtA11*	0.51
JRL790	*ΔkinC::spc sof-1*	0.70
JRL766	*spo0BΔPst*	$<4.1 \times 10^{-8}$
JRL763	*spo0BΔPst sof-1*	3.6×10^{-4}
JRL767	*spo0BΔPst sof-1 ΔkinC*	1.4×10^{-7}
JRL764	*spo0BΔPst rvtA11*	2.1×10^{-3}
JRL768	*spo0BΔPst rvtA11 ΔkinC*	$<2 \times 10^{-8}$
JRL783	*spo0FΔS spo0BΔPst rvtA11*	0.73
JRL792	*spo0FΔS spo0BΔPst rvtA11 ΔkinC*	$<1 \times 10^{-7}$
JRL770	*spo0BΔPst rvtA11 kinA*	1.2×10^{-5}
JRL794	*spo0FΔS spo0BΔPst rvtA11 kinA*	0.34

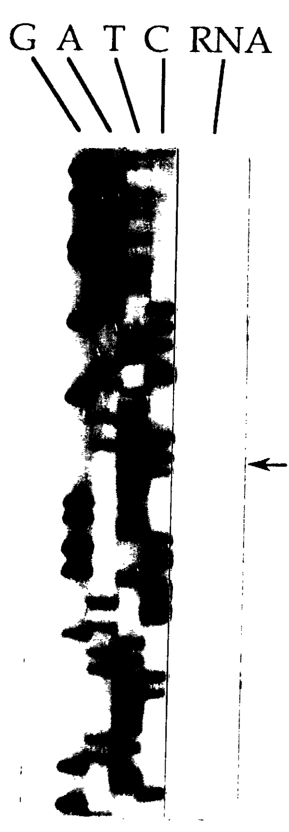
[a]Cells were grown in 2xSG and sporulation frequency was determined as described in Materials and Methods.

completely dependent on *kinC* (Table 4.3). Null mutations in *kinA* significantly reduced the sporulation frequency of the *spo0B rvtA11* strain, but had no significant effect on sporulation of the otherwise isogenic *spo0F spo0B rvtA11* strain (Table 4.3). Together, these results indicate that KinC probably interacts with Spo0F and that the presence of Spo0F inhibits the ability of KinC to donate phosphate to $Spo0A^{rvtA11}$. Furthermore, the absence of KinA probably leaves more Spo0F unphosphorylated, diverting more KinC from $Spo0A^{rvtA11}$ and decreasing the ability of the cells to sporulate. These results suggest that KinC normally can interact with Spo0F and probably contributes to the initiation of sporulation.

Localization of the promoter and primer extension analysis. Preliminary analysis indicated that the *kinC* promoter was between the EcoRV and NdeI sites upstream of the *kinC* open reading frame (Figure 4.1). This was determined by integrating various plasmids into a *spo0F rvtA11* strain (KI1521). If the insert in the plasmid is internal to the *kinC* transcription unit, then integrating the plasmid would disrupt *kinC* and the strain would become Spo^-. If the plasmid extends past the 5' end of the transcription unit (or past the 3' end of *kinC*), then integrating the plasmid would not disrupt *kinC* and the cells will remain Spo^+. Integration of pLK121 and pLK124 resulted in a Spo^- phenotype, while integration of pLK120 maintained the Spo^+ phenotype. These results indicated that the promoter region of *kinC* was probably between the upstream endpoint of pLK120 (EcoRV) and the upstream endpoint of pLK121 (NdeI).

I did primer extension analysis, using primer LKP16 (Figure 4.2; Chapter 7), to localize the 5' end of the *kinC* mRNA. RNA was prepared from strains grown in 2xSG medium. Despite repeated attempts, I could not detect *kinC*-specific RNA from wild type cells. However, a *kinC* transcript was easily

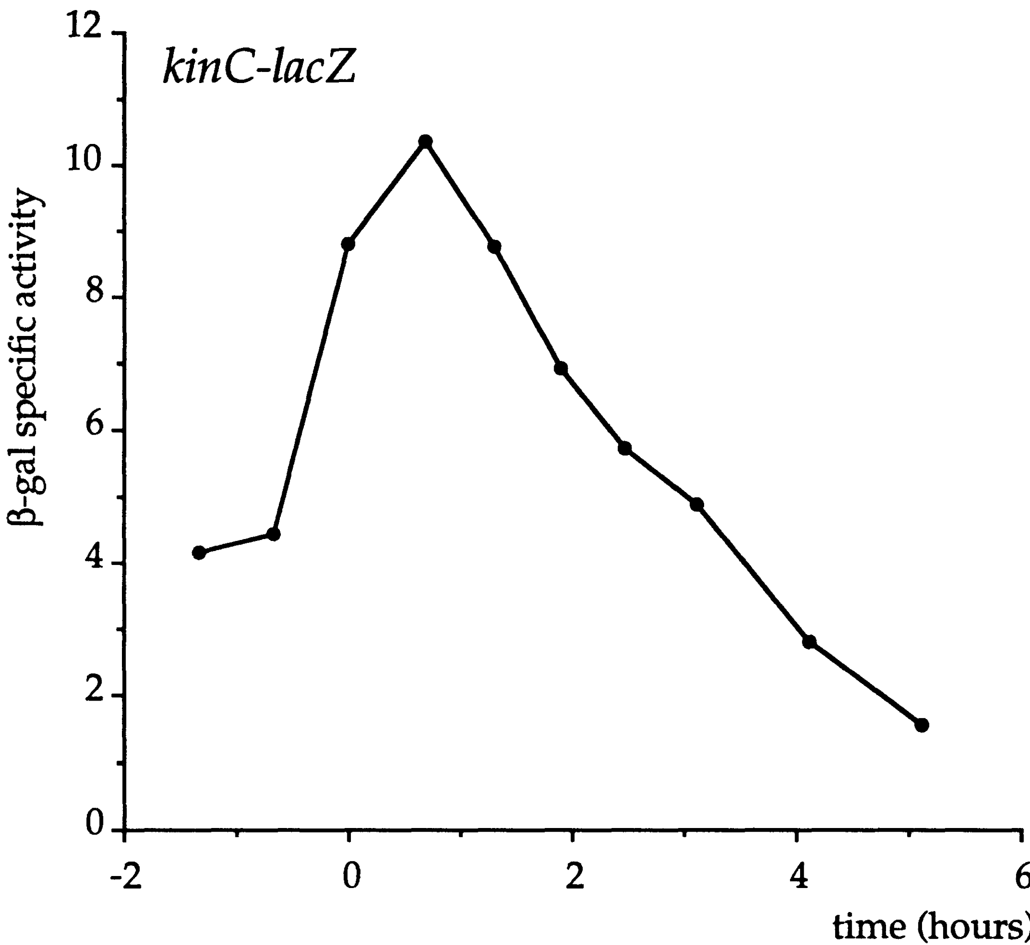
Figure 4.4. Primer extension analysis of the *kinC* mRNA. RNA was made as described in Chapter 7 and extensions done with primer LKP16 (see Fig. 4.2). The sequencing ladder was made from pLK2 using primer LKP16.


G A T C RNA

detected from a strain (JRL951) with *kinC* in multicopy (Figure 4.4). The 5' end of the mRNA is ~25 nucleotides upstream of the *kinC* start codon (Figures 4.2 and 4.4). Just upstream of the putative start site is a sequence that matches in 5 of 6 positions the consensus for the -10 region of sigma-A promoters (Figure 4.2). However, sequences located in the -35 region do not show striking resemblance to the -35 consensus for sigma-A promoters, perhaps explaining the evidently low level of expression of *kinC*. Just upstream of the putative -10 region is a perfect "0A box", TGNCGAA, the consensus binding site for Spo0A (Strauch et al., 1990). Based on this, one might expect Spo0A or Spo0A~P to act as a repressor of *kinC*. However, a *spo0A* null mutation caused at most a small but negative effect on *kinC-lacZ* expression (see below), indicating that Spo0A or Spo0A~P may act to positively regulate expression of *kinC*. Kobayashi et al. (1994) report that a *spo0A* mutation had a more noticeable negative effect on *kinC* expression as measured by primer extension analysis.

***kinC* is expressed as the cells enter stationary phase.** To determine how *kinC* is normally expressed, I made a *kinC-lacZ* fusion and recombined it into the chromosome at the *thrC* locus using pLK114 (Figure 4.1; Chapter 7). The *kinC-lacZ* fusion contains all of the sequences upstream of the *kinC* open reading frame that are present in pLK2. Expression of *kinC-lacZ* was low during growth in 2xSG medium and increased as cells approached stationary phase (Figure 4.5). The low level of β-galactosidase specific activity made it difficult to reliably determine quantitative effects of various regulatory mutations on *kinC-lacZ* expression. However, there seemed to be no significant effect of *spo0A*, *spo0B*, *spo0F*, *spo0H*, *spo0K*, *kinA*, *abrB*, *comP*, *comA*, or *sinR* mutations on the expression of the *kinC-lacZ* fusion (data not shown).

Figure 4.5. Expression of *kinC-lacZ*. Strain JRL812 (*thrC::kinC-lacZ*) was grown in 2xSG medium and samples were taken at the indicated times for determination of β-galactosidase specific activity. The time of exit from exponential growth is defined as time 0 (T_0). Similar results were obtained with cells grown in DS medium except that specific activities were lower (data not shown).


kinC-lacZ
β-gal specific activity
time (hours)
12
10
8
6
4
2
0
-2
0
2
4
6

KinC is required for normal regulation of *abrB* expression. Transcription of the stationary phase regulatory gene *abrB* is repressed by Spo0A~P (Perego et al., 1988; Strauch et al., 1990), but a *kinA kinB* double mutant has little or no effect on expression of an *abrB-lacZ* fusion (Trach and Hoch, 1993). To see if KinC is involved in expression of *abrB*, I measured expression of an *abrB-lacZ* fusion in strains containing different combinations of *kinA*, *kinB*, or *kinC* mutations.

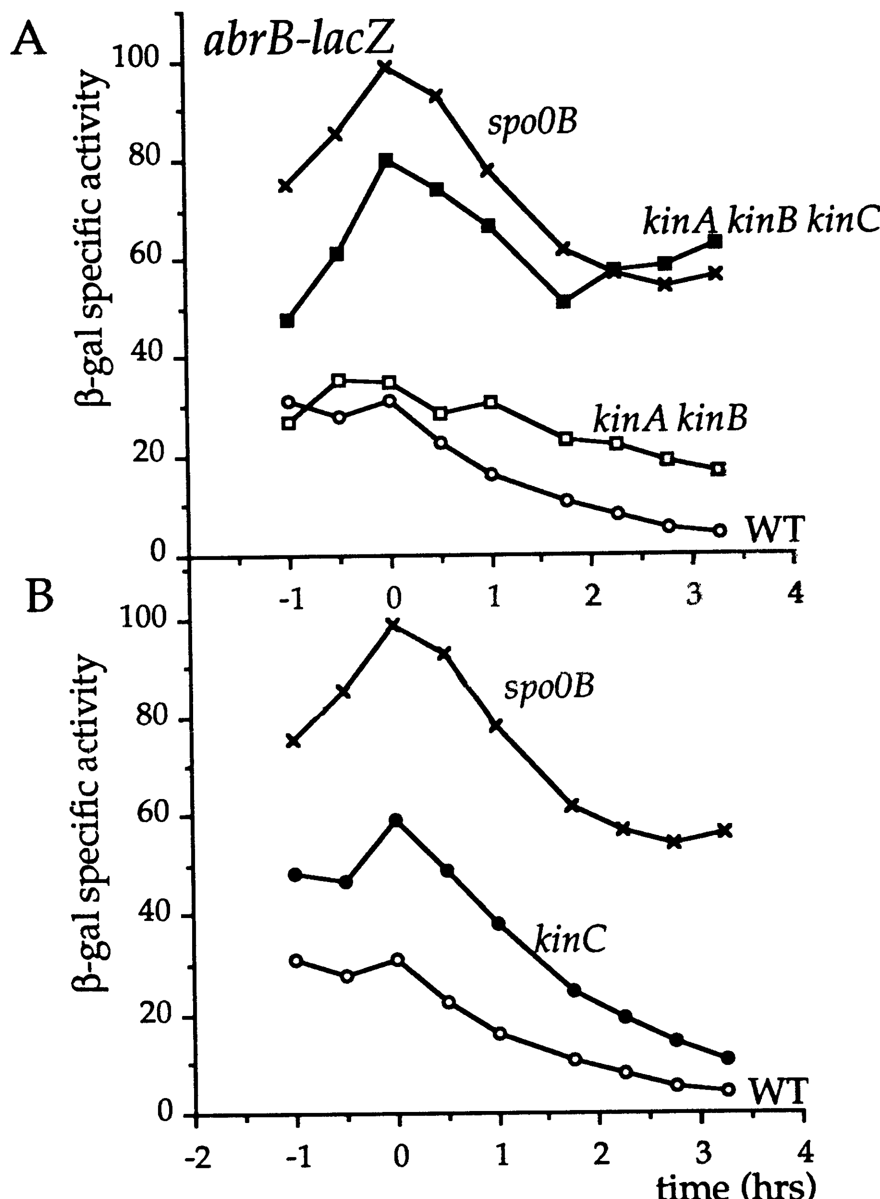
KinC was largely responsible for most of the regulation of *abrB-lacZ* via the phosphorelay in the *kinA kinB* double mutant. Expression of *abrB* in the *kinA kinB* double mutant in DS medium was similar to that in wild type, as previously reported (Trach and Hoch, 1993), although the amount of β-galactosidase at times after entry into stationary phase (T_0) was reproducibly ~2-fold higher in the double mutant (Figure 4.6A). The amount of β-galactosidase accumulated before entry into stationary phase in the double mutant was less than that in a *spo0B* mutant (Figure 4.6). This indicates that there must be at least one other source of phosphate, and that phosphate must be transferred to Spo0A by the Spo0B phosphotransferase (Trach and Hoch, 1993). KinC was a major source of that phosphate in the absence of KinA and KinB, as expression of *abrB* was higher in the *kinA kinB kinC* triple mutant than in the *kinA kinB* double mutant (Figure 4.6A). The initial accumulation of β-galactosidase in the triple mutant was somewhat lower than that in a *spo0B* mutant, suggesting that there might be yet another minor source of phosphate for Spo0A.

A *kinC* mutation also caused increased expression of *abrB* in otherwise wild type (*kinA*$^+$ *kinB*$^+$) cells. Expression of *abrB* was reproducibly higher in the *kinC* mutant before and at the time of entry into stationary phase (Figure 4.6B). Shortly after entry into stationary phase, β-galactosidase specific activity

Figure 4.6. *abrB-lacZ* expression of different kinase mutants in DS medium. The time of exit from exponential growth is defined as time 0 (T_0). The *abrB-lacZ* fusion used is a transcriptional fusion located in single copy at the *amyE* locus and was a gift from Mark Strauch (Perego et al., 1988; Strauch et al., 1990). The *cat* marker associated with the fusion was converted to *cat*::*neo* (Cm^S Neo^R) using pIK105 as described (Ireton and Grossman, 1992b).

A. open circles, wild type (JRL1018); open squares, *kinA kinB* (JRL1036); closed squares, *kinA kinB kinC* (JRL1039); x's, *spo0B* (JRL1058).

B. open circles, wild type (JRL1018); closed circles, *kinC* (JRL1035); x's, *spo0B* (JRL1058). The data shown are from the same experiment as shown in A, and the data points for wild type and *spo0B* are the same.



A
abrB-lacZ
β-gal specific activity
100
80
60
40
20
0
spo0B
kinA kinB kinC
kinA kinB
WT
-1
0
1
2
3
4
B
β-gal specific activity
100
80
60
40
20
0
spo0B
kinC
WT
-2
-1
0
1
2
3
4
time (hrs)

began to decrease, similar to the decrease seen in wild type cells, This substantive decrease was due to KinA and KinB, as there was much less of a decrease in the triple kinase mutant (Figure 4.6A).

These results on the effects of kinase mutations on *abrB* expression indicate that different kinases that contribute to the phosphorelay and production of Spo0A~P may be active at different times during growth and stationary phases. The contribution of KinC seems to be greater during entry into stationary phase, while KinA and KinB appear to contribute more after entry into stationary phase. In addition, media conditions may also affect which kinase is more important for the developmental process. Both the growth stage and media effects could be at the level of expression or activity of the kinases, or both, but there is not yet enough information available concerning regulation of expression or activity of the kinases to distinguish these possibilities.

Chapter 5:

Additional multicopy suppressors of *spo0K*

In Chapter 3, I described the isolation of two multicopy suppressors of the sporulation defect associated with *spo0K* mutants, and two multicopy suppressors of the competence defect associated with *spo0K* mutants. The multicopy suppressor of the *spo0K* sporulation defect, *kinC*, on which I have focused most of my attention, was described in Chapter 4.

In this chapter, I describe the other two multicopy suppressors of *spo0K* that I have isolated and characterized. When expressed on a multicopy plasmid, *mskB* partially suppresses the sporulation defect of a *spo0K* mutant. *mskB* encodes a protein similar to SinI. SinI inactivates SinR (Bai et al., 1993); SinR is a repressor of *spo0A* transcription (I. Smith, personal communication) and of some genes regulated by Spo0A (Mandic-Mulec et al., 1992). Based on these findings, multicopy MskB may be activating sporulation by inhibiting SinR.

When expressed on a multicopy plasmid, *mskD* causes a two- to threefold increase in *comG-lacZ* expression whether in a wild type or *spo0K* background. *mskD* encodes the *B. subtilis* homolog of *E. coli* ClpX. In *E. coli*, ClpX is an ATPase that directs the ATP-dependent protease ClpP to specific substrates (Gottesman et al., 1993). It has recently been shown that the *B. subtilis* proteins MecA and MecB are similar to ClpP (Kong and Dubnau, 1994) and the ClpC ATPase (Krüger et al., 1994; Msadek et al., 1994), respectively. MecA and MecB act negatively in the development of competence by inhibiting ComK (Hahn et al., 1994; van Sinderen et al., 1994; van Sinderen and Venema, 1994). ComK is a transcription factor needed for expression of late competence genes that include *comG*. A simple model for the ability of overexpressed MskD (ClpX) to increase

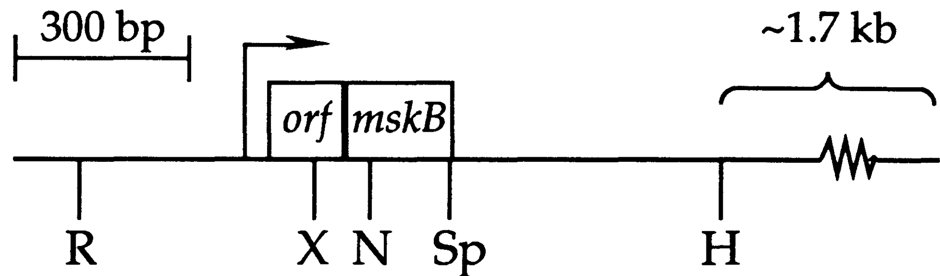
comG expression is that MskD interferes with MecA or MecB such that they can no longer regulate ComK.

mskB, a multicopy suppressor of the sporulation defect of *spo0K*

Isolation of *mskB* on a multicopy plasmid. *mskB* was isolated in multicopy plasmids that partially suppressed the sporulation defect of a conditional *spo0K* mutant (see Chapter 3). There were three plasmids that were similar in their restriction maps and multicopy suppressing ability: pLK10, pLK11, and pLK15. They were similar except that where pLK10 had a ~1.1 kb HindIII fragment that contained the suppressing activity, pLK11 and pLK15 had a ~1.3 kb HindIII fragment. (Because pLK11 and pLK15 were identical by restriction mapping, of the two, I focused on pLK11.) Both pLK10 and pLK11 caused markedly filamentous growth in both wild type and *spo0K* cells as judged under a light microscope. Various subclones of the two plasmids were made to localize the regions responsible for the multicopy suppression activity and the cause of filamentous growth (Figure 5.1). In every case, partial suppression of the *spo0K* Spo$^-$ defect correlated with filamentous growth (Figure 5.1)

Sequence and map location of pLK11 and pLK10 inserts. The sequence of the ~1.3 kb Sau3A-HindIII fragment from pLK11 responsible for the suppressing activity was determined. When I compared the MskB open reading frame (see below) to the non-redundant DNA database translated in all six frames, I found that this region had already been sequenced as part of the *B. subtilis* chromosome sequencing project (Glaser et al., 1993). Sequence data from that project is shown in Figure 5.2.

Figure 5.1. Map of the *mskB* region and plasmids. Restriction site abbreviations: R, EcoRV; X, XcmI; N, NdeI; Sp, SpeI; H, HindIII. Every plasmid insert shown is subcloned into the pHP13 *B. subtilis*/ *E. coli* shuttle vector (Haima et al., 1987; Bron, 1990), except for the insert of pLK115, which is in the integrative pJH101 vector (Ferrari et al., 1983). pLK63 is a subclone of pLK11, and pLK65 is a subclone of pLK10. "*orf*" refers to *orf45* (see text). The frame shift mutation in *orf45* in pLK122 is marked with "-1" and an X. The frame shift mutation in *mskB* in pLK123 is marked with "+2" and an X. "*spo0K* suppression and filamentous growth?" refers to the ability of the multicopy plasmid to suppress the *spo0K* sporulation defect as determined by colony morphology on sporulation plates and the ability of the plasmid to induce filamentous growth as determined by light microscopy.



spo0K suppression and filamentous growth?

yes pLK10
yes pLK11
no pLK63/pLK65
yes pLK66
yes pLK64
no pLK116
no pLK117
no pLK118
no pLK119
-1
yes pLK122
+2
no pLK123
pLK115
spc

Figure 5.2. Sequence of *mskB*. The sequence shown corresponds to the complementary strand of GenBank accession number X73124 (Glaser et al., 1993), bases 33592 to 34818. A direct repeat surrounding the sequence that was deleted in pLK10 is indicated by a pair of arrows. A putative -10 region for a sigma-A promoter is shown with a thick underline. The transcriptional start site is indicated with an arrow at nucleotide 398. Putative ribosome binding sites for Orf45 and MskB as well as Ipa-32r (Glaser et al., 1993) are underlined and putative translational start sites are boldfaced. Sequence complementary to primer LKP14, which was used for primer extension analysis, is shown with a single arrow. This sequence is essentially the same as sequence from only one or the other strand from the same region that I determined independently.

```
   1  GATCTTTTTTTTCATCTGACGTTTTTCGAGAAGGTACAGCAGAAAATCGTCCTTGCTGAC
  61  AAAATGAACATAAAATGCTCCTTTGCTGTACCCGGCGGCTCTGCTGATATCTTCTACAGA
 121  AACCGGATCATACGCTCGGTCAATCAACAAATTCACGCCAGCGTCTATAAAACTTTCAAA
 181  TGTTGCTTCTCTTTTAATTTGAAATTTGTTTTTTTTCATATTTTTCTCCTGGCGGTAATG
 241  ATTACCCTTATCTTACACCGGATCAATCTTTAGCGGTATTTATTTCTTTAAACGGTTATG
 301  CTATTCGGTTTCCCAGGGGTCATTTTTAATAGGCTAAATGATTTATTTTCATTTACATAC

 361  TATCCAGTCAGTATTTAATTGTTTATTATTATAAATAAGGTCCATTGGTTATATTAGTCA

 421  TTTTTTTAGGGAATAGGTAGGTGGTTTTTCCTTCAGTTTGTGAACACCGAGCAACTATGT
                          Orf45  M  V  F  P  S  V  C  E  H  R  A  T  M  C
                                                   XcmI
 481  GTGAAATTTGTTGTACTCGCCTCCATCATCGCGCTGGCTTGTCTTTTATTGCCCGCATGT
        E  I  C  C  T  R  L  H  H  R  A  G  L  S  F  I  A  R  M  L
 541  TGCAGGAAAACATTTTATACAATTGGAGGTTACTATGAAAACTCATGTTAAAAAAGATTT
        Q  E  N  I  L  Y  N  W  R  L  L  *
                                            MskB  M  K  T  H  V  K  K  D  L
                          NdeI
 601  GGACAAAGGTTGGCATATGTTAATTCAAGAAGCTAGATCCATTGGATTAGGCATTCATGA
       D  K  G  W  H  M  L  I  Q  E  A  R  S  I  G  L  G  I  H  D
 661  TGTGAGGCAGTTTTTAGAATCTGAGACAGCATCAAGAAAGAAAAACCACAAAAAAACCGT
       V  R  Q  F  L  E  S  E  T  A  S  R  K  K  N  H  K  K  T  V
 721  CCGGCAAGACTAGTCCGAACAGGCGGATCTATTTACCTCTGGCTTTTCTATCACCTGATC
       R  Q  D  *
 781  TGAGTTTTCACCATATCCTCTATTCAAAAGCAAATCGCTGCGCTGAGCTTGAGGACAAAC
 841  GGTGTGTCAGCTAACTACGACAGACAGCCGGTGATGCGATCACCTCTTGGCACATGCATT
 901  GTCATAAAGAGGTGATGCGAACATGATAACCGTGCTCTGGTAAACAGCAGCTTTGCTGAG
 961  AACAAGCTGCATTCGGTGAGGTTTTGAAAGCAAACTCGTTTATACACGGCCGAGCTTGAA
1021  AACCTTCGCCTGTCTTTAGACCACTGTGTTCACCTCTTCACGACTTCCTTCTTTTTTCAT
1081  CCTCCAGCTATTGTACAACGCATAAATCGGACTTTCAGTACATACCTATTGGTGTACCTT
1141  TTTGCTTACGTTTAGCGAATAAAAGGAGGAAAGCCATGTACAAAGCTGATTATAAACAGA
                                          Ipa-32r  M  Y  K  A  D  Y  K  Q  I
                                    HindIII
1201  TTGCTGCAACGCCGTCTTTTCAAGCTT  1227
        A  A  T  P  S  F  Q  A
```

mskB maps to the *B. subtilis* chromosome between *sacX* and *sacT*, at 331.5°. This is based on the data from the chromosome sequencing project (Glaser et al., 1993). (This is another instance of the relative ease with which one can map genes to the *B. subtilis* chromosome by using the Ireton and Grossman method © of mapping by sequencing (Ireton, 1993).)

Sequence from the pLK10 insert indicated that pLK10 has a 135 bp deletion relative to pLK11. The deletion is flanked by a 9 bp direct repeat (Figure 5.2), and a single homologous recombination event at this repeat would result in a loss of the intervening DNA.

Within the sequence determined from the pLK11 insert, two small open reading frames were found. The first one may encode a protein 45 residues long, and was imaginatively called *orf45*, and the second one, which encodes MskB (see below) is 52 residues long. These open reading frames are shown in Figures 5.1 and 5.2.

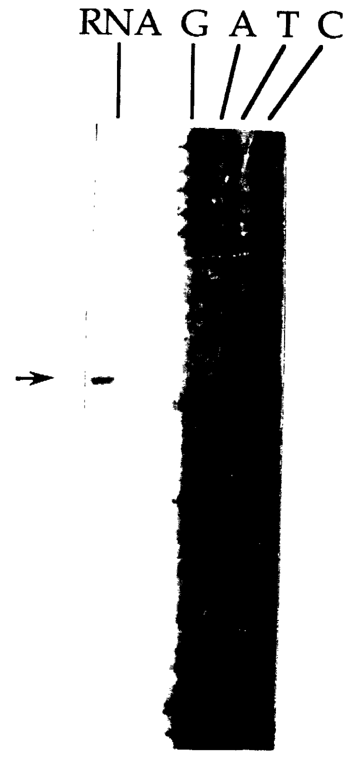
The second open reading frame ends 446 bp upstream of the putative coding sequence *ipa-32r* (Glaser et al., 1993) (Figure 5.2). There are no obvious transcriptional terminators between *mskB* and *ipa-32r*, so it is possible that they are in the same operon.

Localizing suppressing activity. The multicopy suppressing activity and the cause of filamentous growth was due to the more downstream of the two small open reading frames found in the pLK64 insert. To determine which of the two open reading frames was responsible for this activity, frameshift mutations were made in each of the two open reading frames. The frameshift mutation in the upstream open reading frame was made by digesting pLK64 with XcmI, excising the 1-base 3'-overhang with the Klenow fragment of DNA polymerase I and religating. The frameshift mutation in the downstream open reading frame was made by digesting pLK64 with NdeI, creating a 2-base 5'-overhang that was

filled in using the Klenow fragment of DNA polymerase I and religated (see Figures 5.1 and 5.2). The mutation in the upstream open reading frame did not affect the ability of the plasmid to suppress the *spo0K* defect or to cause filamentous growth, whereas the mutation in the downstream open reading frame did (Figure 5.1). Based on this, the downstream open reading frame was identified as the one responsible for the multicopy suppression of *spo0K* and filamentous growth and was called *mskB* (Figure 5.1). It is not clear if the upstream open reading frame is actually translated or not, but it is transcribed (see below).

***mskB* promoter localization.** To localize the 5' end of the *mskB* mRNA, primer extension analysis was performed using primer LKP14 (see Figure 5.2, Chapter 7) and RNA prepared from strains grown in 2xSG medium. I could not detect *mskB*-specific transcripts from wild type cells. However, I could easily detect an *mskB* transcript from a strain harboring pLK11 (Figure 5.3). The 5' end of the mRNA is ~43 nucleotides upstream of the start codon of the more upstream open reading frame (Figures 5.2 and 5.3). Just upstream of this start site is a sequence that matches in 5 of 6 positions the consensus sequence for the -10 region of sigma-A promoters (Figure 5.2).

Figure 5.3. Primer extension analysis of the *mskB* mRNA. RNA was made as described in Chapter 7 and extensions were done with primer LKP14 (see Fig. 5.2). The sequencing ladder was made from pLK11 using primer LKP14.


RNA G A T C

Multicopy *mskB* phenotypes. *mskB* when present on a multicopy plasmid (pLK10 or pLK11) partially suppressed the sporulation defect associated with *spo0K*, *spo0J93*, and *spo0E11*, and completely suppressed the sporulation defect associated with *kinA* (Table 5.1), but did not suppress the sporulation defect associated with *spo0F*, *spo0B* or *spo0A* (data not shown). Also, pLK11 in wild type cells caused decreased *comG-lacZ* expression (Solomon, 1994).

Homology with SinI. The putative product of *mskB* is a protein of 52 residues. This protein shows some similarity to repressor SinR (sin stands for sporulation inhibitor), and the SinR inhibitor, SinI (Figure 5.4). SinR represses transcription of *spo0A* (I. Smith, personal communication) and *spoIIA* (Mandic-Mulec et al., 1992). SinR exists as a tetramer in solution and consists of two domains: an N-terminal DNA-binding domain and a C-terminal multimerization domain (Gaur et al., 1991; Bai et al., 1993). SinI is similar to the multimerization domain of SinR (Bai et al., 1993), and MskB is similar to SinI and SinR in this domain (Figure 5.4). It has been shown that SinI binds to SinR and in so doing, antagonizes the ability of SinR to bind to its target DNA sequences (Bai et al., 1993). Mutations in *sinR* and overexpression of SinI both lead to filamentous growth and decreased expression of *comG* (Bai et al., 1993), and furthermore, a *sinR* mutation partially suppresses the sporulation defect of a *spo0K* mutant (T. Leighton, personal communication, and unpublished results) and also suppresses the sporulation defect of a *kinA* mutant (Mandic-Mulec et al., 1992).

TABLE 5.1 Suppression of *spo0* mutants by multicopy *mskB*.

relevant genotype of strain harboring plasmid	relative sporulation frequency[a]	
	vector (pHP13)	multicopy *mskB*[b]
WT (JH642)	1	ND
ΔspoOK::erm	2.6×10^{-2}	0.18
kinA::Tn*917*	0.15	0.81
spo0J93	3.6×10^{-5}	3.0×10^{-4}
spo0E11	2.5×10^{-3}	9.3×10^{-3}

[a]Cells were grown in DS medium with chloramphenicol. Relative sporulation frequency is the number of spores/ml as a fraction of the number of cells/ml, normalized to the control (JH642 pHP13) in a given experiment.

[b]The multicopy *mskB* plasmid used in the *ΔspoOK::erm*, *spo0J93* and *spo0E11* strains was pLK10. The multicopy *mskB* plasmid used in the *kinA* strain was pLK11.

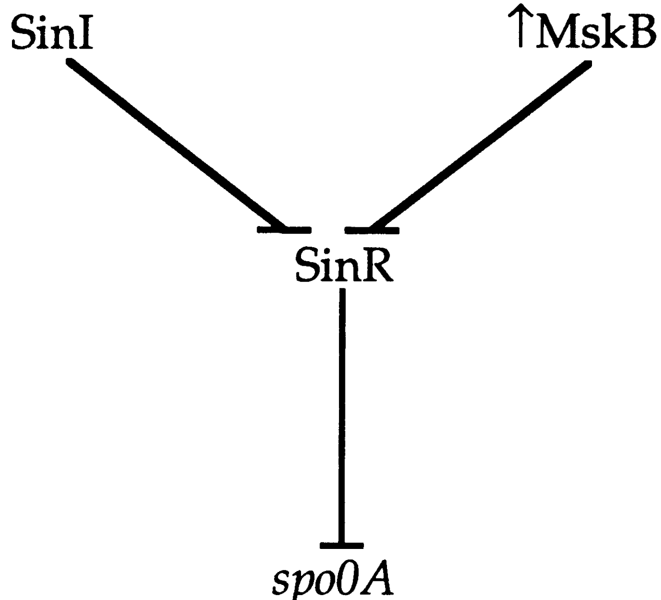
Figure 5.4. Comparison of MskB to SinI and SinR. The sequences are aligned according to the PILEUP program of the UWGCG (Devereux et al., 1984). The region of SinR shown is thought to be part of the region involved in the multimerization of SinR (Bai et al., 1993).

```
MskB    1 MKTHVKK ..DLDKGWHM LIQEARSIGL GIHDVRQFLE  35
          ||   ..    :||.:|   |: ||:. .: : .::|.:|
SinI    1 MKNAKQE HFELDQEWVE LMVEAKEANI SPEEIRKYLL  37
           |:..:  . :|| || . |: :|....: |..::|.:|
SinR   64 EKHETEY DGQLDSEWEK LVRDAMTSGV SKKQFREFLD 100
           | ...     :||.:|.. |:.:|.. |: : .:.|:||:
MskB    1 MKTHVKK ..DLDKGWHM LIQEARSIGL GIHDVRQFLE  35
```

Together, these results readily suggest a simple model for the action of multicopy *mskB*, both as a suppressor of the sporulation defect of *spo0K* and as a cause of filamentous growth. Like SinI, perhaps MskB binds to and inhibits the repression activity of SinR, which leads to increased expression of *spo0A* and *spoII* genes and filamentous growth (Figure 5.5). The increase in expression of Spo0A leads to increased amounts of Spo0A~P, bypassing or partially bypassing the need for some stage 0 sporulation genes.

ΔmskB. *sinI* mutants usually exhibit a sporulation defect resulting in sporulation frequencies of ~10% of wild type, and this defect is suppressed by mutations in *sinR* (Bai et al., 1993). To determine if loss of *mskB* causes a similar sporulation defect, I constructed a strain (JRL871) containing a deletion-insertion mutation in *mskB*, *ΔmskB::spc* (Figure 5.1; Chapter 7). The *ΔmskB::spc* mutation had little or no effect on the sporulation frequency compared to wild type (data not shown). This could be because *mskB* is not normally involved in sporulation. Alternatively, since *sinI* mutants spontaneously acquire mutations in *sinR* at a higher than normal frequency (I. Smith, personal communication), it is possible that the *ΔmskB::spc* strain I isolated is carrying a mutation in *sinR* which is suppressing an *mskB* sporulation defect. This latter possibility is easily testable.

Figure 5.5. MskB may interact with SinR. SinI (Bai et al., 1993) and perhaps overexpressed MskB binds to and inhibits SinR. SinR represses transcription of *spo0A*.


SinI
↑MskB
SinR
spo0A

mskD, a multicopy suppressor of the competence defect of *spo0K*

Multicopy *mskD* acts later than *srfA* in the development of competence. pLK6 caused a ~2-3-fold increase in expression of *comG-lacZ* in both the wild type background and in the Pspac-*spo0K* mutant as compared to the parent vector (Figure 5.6A), and a similar fold increase in a *spo0K* null mutant (Figure 5.6B). However, the activity of pLK6 that causes this increase in expression must occur later than that of *srfA* in the development of competence, as pLK6 had no effect on the *srfA-lacZ* expression in a wild type, Pspac-*spo0K*, or *spo0K* null background (Figure 5.7). The IPTG-inducible Pspac promoter must allow some transcription even in the absence of IPTG, but less than wild type levels, since both *comG-lacZ* and *srfA-lacZ* are expressed at levels between those observed in wild type and *Δspo0K::erm* strains (Figures 5.6 and 5.7).

Isolation and localization of *mskD* on a multicopy plasmid. pLK6 caused a marked increase in *comG-lacZ* expression in the Pspac-*spo0K* background as judged by the darker blue color of the colonies harboring pLK6 on Xgal indicator plates. To determine where in the 2.7 kb insert in pLK6 this activity was located, I made various subclones of the pLK6 insert into the multicopy vector pHP13 (Figure 5.8), and transformed them into the Pspac-*spo0K*, *comG-lacZ* strain, JRL454. Since pLK68 bypassed the *spo0K* mutation, but pLK67 and pLK69 did not (Figure 5.8), sequence containing the EcoRI site was necessary for the suppressing activity of pLK6.

MskD is the *B. subtilis* homolog of ClpX. Partial sequence around the EcoRI site revealed a single large open reading frame that I call MskD.

Figure 5.6. Overexpression of *mskD* increases expression of *comG-lacZ* in wild type and *spo0K* strains. Strains were grown in S7 minimal medium with 1% glucose and 0.1% glutamate and amino acids for auxotrophic requirements as described in Chapter 7. The *comG-lacZ* fusion is a transcriptional fusion located in single copy at the *amyE* locus (Magnuson et al., 1994a). pHP13 is the parent vector, pLK6 is the *mskD* multicopy plasmid.

A. open circles, wild type, pHP13 (JRL515); closed circles, wild type, pLK6 (JRL1131); open squares, Pspac-*spo0K*, pHP13 (JRL455); closed squares, Pspac-*spo0K*, pLK6 (JRL1132).

B. open triangles, *Δspo0K::erm*, pHP13 (JRL523); closed triangles, *Δspo0K::erm*, pLK6 (JRL1133).

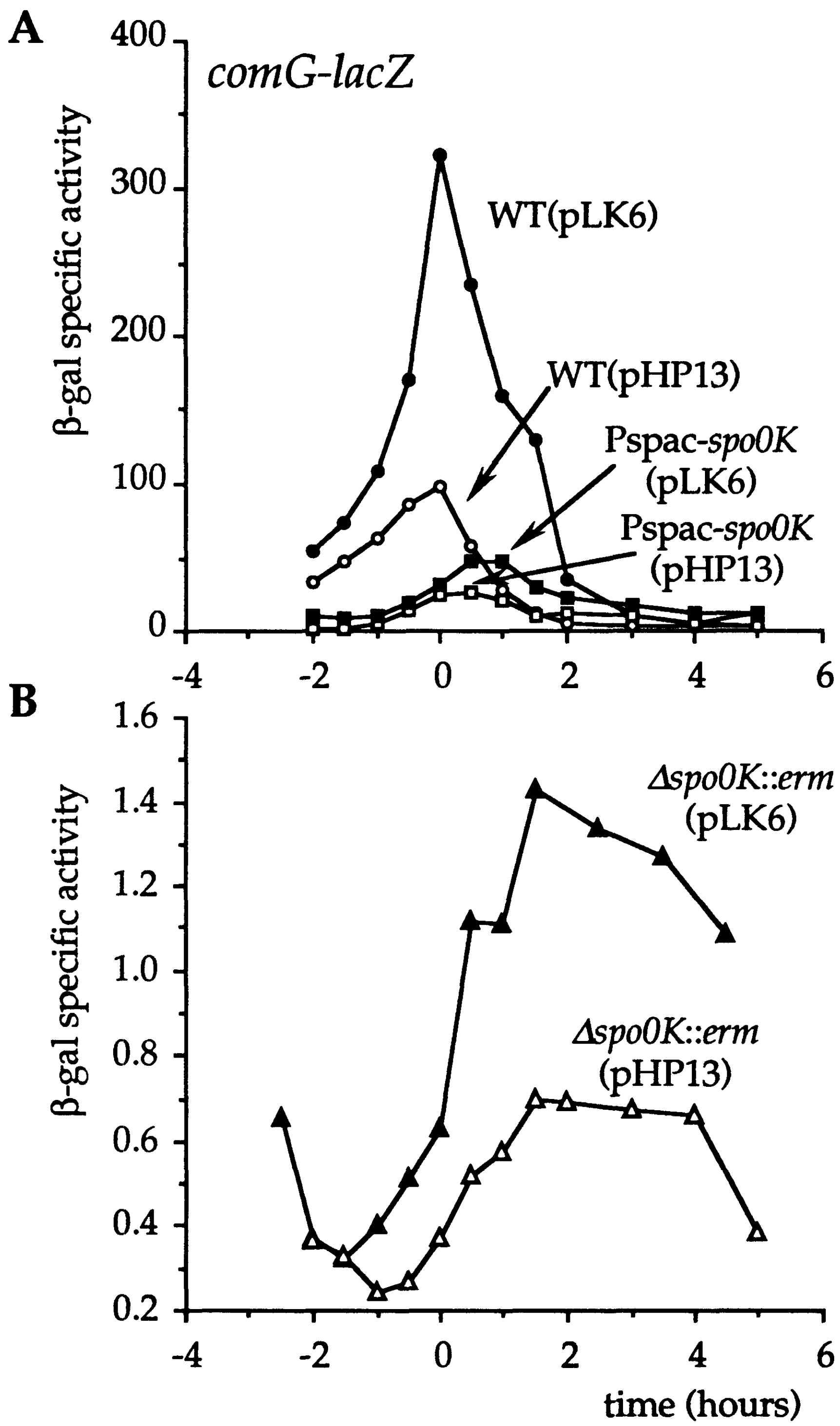
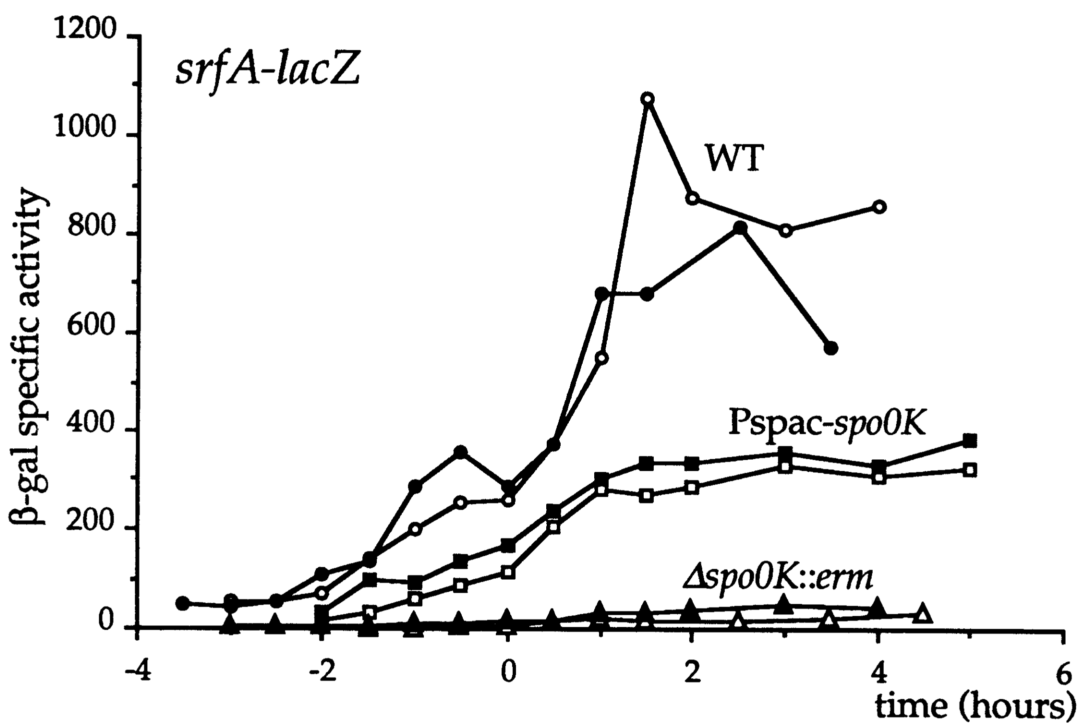
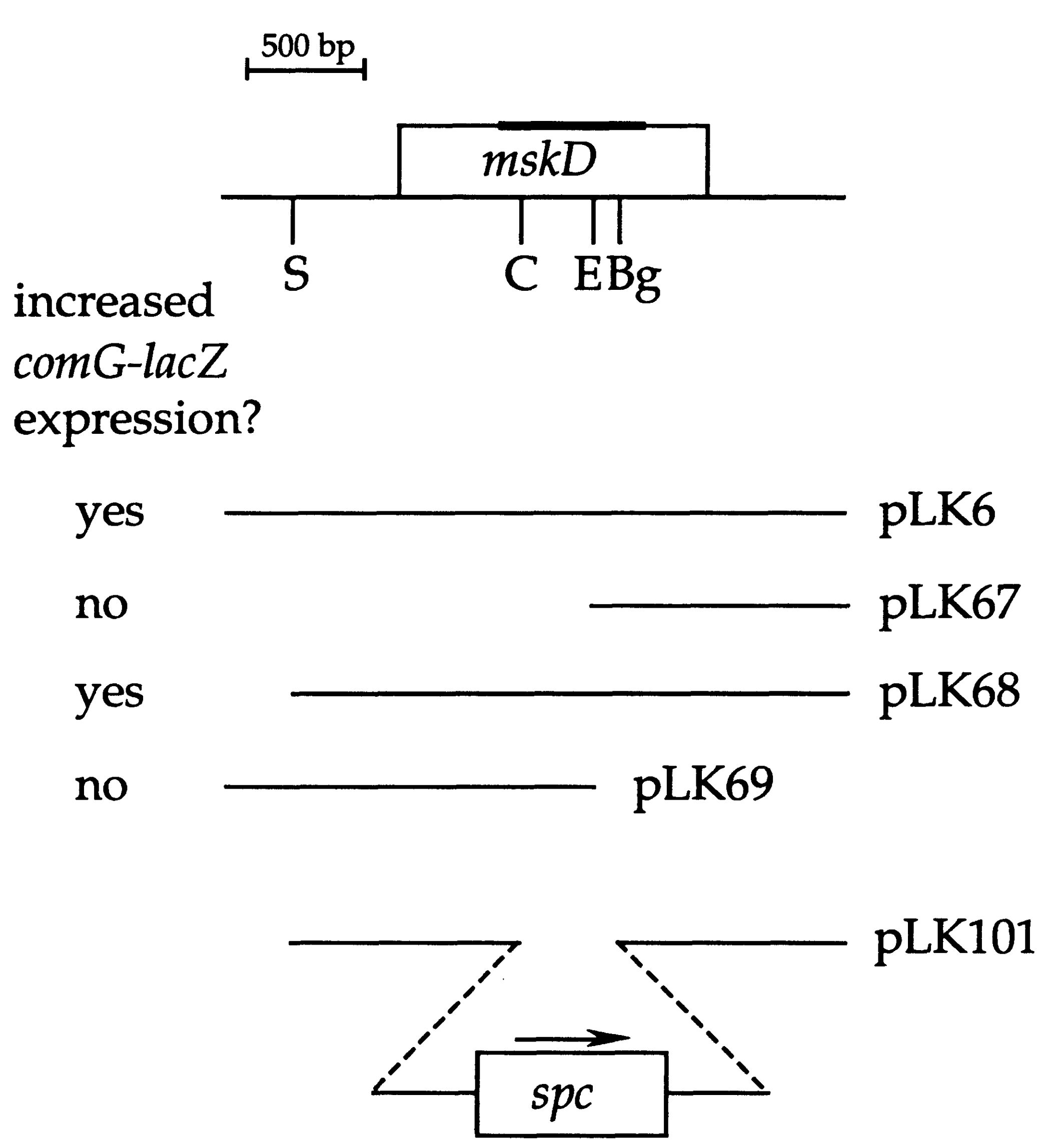

A
400
300
200
100
0
comG-lacZ
β-gal specific activity
WT(pLK6)
WT(pHP13)
Pspac-spo0K
(pLK6)
Pspac-spo0K
(pHP13)
-4
-2
0
2
4
6
B
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
β-gal specific activity
Δspo0K::erm
(pLK6)
Δspo0K::erm
(pHP13)
time (hours)

Figure 5.7. Overexpression of *mskD* has little to no effect on expression of *srfA-lacZ*. Strains were grown in S7 minimal medium with 1% glucose and 0.1% glutamate and amino acids for auxotrophic requirements as described in Chapter 7. The *srfA-lacZ* fusion is a translational fusion located in single copy at the *amyE* locus (Magnuson et al., 1994a). The *cat* marker associated with the fusion was converted to *cat::neo* (Cm^S Neo^R) using pIK105 as described (Ireton and Grossman, 1992b). pHP13 is the vector, pLK6 is the *mskD* multicopy plasmid. open circles, wild type, pHP13 (JRL740); closed circles, wild type, pLK6 (JRL741); open squares, Pspac-*spo0K*, pHP13 (JRL450); closed squares, Pspac-*spo0K*, pLK6 (JRL739); open triangles, *Δspo0K::erm*, pHP13 (JRL748); closed triangles, *Δspo0K::erm*, pLK6 (JRL749).



srfA-lacZ
WT
Pspac-spo0K
Δspo0K::erm
β-gal specific activity
1200
1000
800
600
400
200
0
-4
-2
0
2
4
6
time (hours)

Figure 5.8. Map of the *mskD* region and plasmids. Restriction site abbreviations: S, SalI; C, ClaI; E, EcoRI; Bg, BglII. Every plasmid insert shown is subcloned into the pHP13 *B. subtilis/E. coli* shuttle vector (Haima et al., 1987; Bron, 1990), except for pLK101, which is in the integrative pJH101 vector (Ferrari et al., 1983). "increased *comG-lacZ* expression?" refers to the ability of the multicopy plasmid to increase *comG-lacZ* expression as compared to the pHP13 vector in a Pspac-*spo0K* mutant (JRL454) as judged by darker blue color on a minimal plate with Xgal. The box indicating the putative extent of the *mskD* open reading frame is based on the similarity between MskD and ClpX. The thick line over the box indicates the region of *mskD* for which I have sequence data.


500 bp
mskD
S
C
E
Bg
increased
comG-lacZ
expression?
yes
pLK6
no
pLK67
yes
pLK68
no
pLK69
pLK101
spc

MskD is a *B. subtilis* homolog of ClpX of *E. coli* (Gottesman et al., 1993; Wojtkowiak et al., 1993), as there is 67% identity over 172 residues (Figure 5.9). This open reading frame was also partially sequenced by Ruth Schmidt and it was first identified by her as a ClpX homolog (R. Schmidt, personal communication; Schmidt et al., 1994). ClpX belongs to a large family of ATPases associated with the ClpP protease. Specific Clp ATPases are thought to direct the proteolytic activity of ClpP to specific substrates (Gottesman and Maurizi, 1992; Gottesman et al., 1993).

A mutation in *mskD* causes a growth defect. To characterize phenotypes caused by loss of *mskD*, I constructed a strain (JRL672) containing a deletion-insertion mutation in *mskD*, *ΔmskD::spc* (Figure 5.8, Chapter 7). This mutation caused a noticeable growth defect as the colonies that arose from the transformation were smaller, rounder, and shinier. This indicates that either *mskD* is necessary for normal growth in *B. subtilis*, unlike *clpX* of *E. coli*, or this mutation is polar on some other gene that is necessary for normal growth.

Given the pleiotropic nature of the *clpX* mutation, it is not surprising that JRL672 sporulates at a frequency of ~2% as compared to wild type cells. JRL672 gives rise to revertants of the growth defect. One of these revertants was used to make strain JRL679, and the mutation that suppresses the growth defect caused by the *ΔmskD::spc* allele is unlinked by transformation. The mutation that suppresses the growth defect also partially suppresses the sporulation defect, as JRL679 sporulates at a frequency of ~20% of wild type. It is possible that the mutation that suppresses the growth defect of *ΔmskD::spc* may be in a gene encoding a protease that interacts with MskD, or it may be in a gene that encodes a regulator of cell growth that is itself regulated by the putative MskD-protease complex.

Figure 5.9. Comparison of MskD (partial sequence) to ClpX of *E. coli*. The sequences are aligned according to the BestFit program of the UWGCG (Devereux et al., 1984). The residues in bold are highly conserved among the family of Clp ATPases (Gottesman et al., 1990; Gottesman et al., 1993).

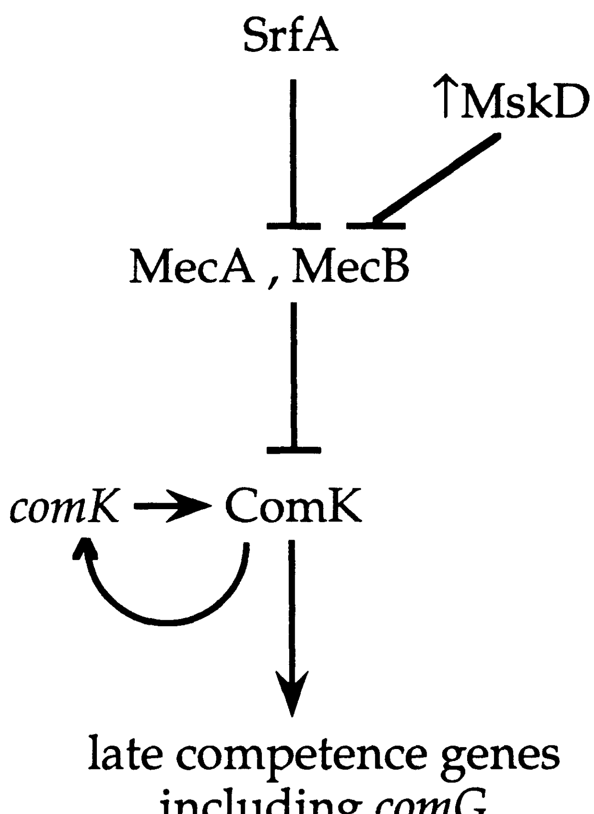
```
MskD     1 ATSLTEAGYVGEDVEIILLKLIQARCYDVGKSRKSIIYIDEIDKVARKSE
             ||.|||||||||||| |: ||:|   |||.|..::|:||||||||:.|||:
ClpX   145 ATTLTEAGYVGEDVENIIQKLLQKCDYDVQKAQRGIVYIDEIDKISRKSD

MskD    51 NPSITRDVSGEGVQQALLKILEGTVAHVPPQGGRKHPHQEFIQIDTTNIL
           |||||||||||||||||||::||||| ||||||||||:|||:|:||..||
ClpX   195 NPSITRDVSGEGVQQALLKLIEGTVAAVPPQGGRKHPQQEFLQVDTSKIL

MskD   101 FICGGRFDGIEQIIKRRLG.QKVIGFGADNKA..ADLEKEDLLSKVLPED
           ||||| |.|::.:|.:|:: ...|||||. ||  .. ..::||..| |||
ClpX   245 FICGGAFAGLDKVISHRVETGSGIGFGATVKAKSDKASEGELLAQVEPED

MskD   148 LLRFGLIPEFIGRLPVIASLEKLDE 172
           |::|||||||||||||:|.|:.|.|
ClpX   295 LIKFGLIPEFIGRLPVVATLNELSE 319
```

Overexpressed MskD may interact with the late competence genes MecA and MecB. SrfA may act negatively on MecA and MecB in the development of competence, as *mecA* and *mecB* null mutations bypass the need for *srfA* in the development of competence (Roggiani et al., 1990; Hahn et al., 1994). MecA (Kong and Dubnau, 1994) and MecB (Msadek et al., 1994) act to block activity of the competence transcription factor, ComK (Hahn et al., 1994; van Sinderen et al., 1994; van Sinderen and Venema, 1994). It has been shown that MecA binds to ComK in vitro (Kong and Dubnau, 1994). ComK positively regulates transcription of late competence genes including *comK* (van Sinderen and Venema, 1994) and *comG* (van Sinderen et al., 1994; van Sinderen and Venema, 1994).

MecB is a *B. subtilis* homolog of ClpC (Krüger et al., 1994; Msadek et al., 1994), and is 60% identical to ClpC (CD4A) from tomato (Gottesman et al., 1990). MecA shows a slight similarity to ClpP (24% identity or conservation to four or more ClpP proteins out of six (Kong and Dubnau, 1994)). However, MecA is probably not acting as a protease as it is missing the conserved serine residue (Kong and Dubnau, 1994) that is reported to be in the active site of the ClpP protease (Maurizi et al., 1990).

An attractive model is that MecA, under the direction of MecB, binds to ComK and keeps ComK from activating transcription. Consistent with this model, overexpressed MskD may displace MecB from MecA and allow activation of ComK (Figure 5.10). To test this, one might test the ability of MskD to bind directly to MecA or to interfere with the ability of MecA to bind with ComK, but it would probably be prudent to first check that overexpressing MskD had no effect on the expression of *comG* in *mecA*, *mecB* and *comK* mutant backgrounds.

Figure 5.10. A model for how increased expression of MskD leads to increased *comG* expression. See text for details.


SrfA
↑MskD
MecA , MecB
comK
ComK
late competence genes
including comG

Chapter 6:

Discussion

The Spo0K oligopeptide permease is necessary for both the initiation of sporulation (Perego et al., 1991; Rudner et al., 1991) and the development of competence and appears to act early in both of these processes (Rudner et al., 1991). To better understand how both of these developmental processes are regulated, I conducted a screen for genes which when overexpressed could bypass the need for *spo0K* in either of these developmental pathways. In this chapter, I will discuss what is known about Spo0K and how each of the three multicopy suppressors of *spo0K* that I characterized add to our understanding of the initiation of sporulation and the development of competence.

The effects of mutations in each of the genes of the *spo0K* operon. The *spo0K* operon encodes in order the ligand-binding protein, Spo0KA; the membrane pore forming proteins, Spo0KB and Spo0KC; and the two ATP-binding proteins, Spo0KD and Spo0KE (Perego et al., 1991; Rudner et al., 1991). I have used nonpolar mutations in each of the first three genes in the *spo0K* operon to show that the ligand binding protein and each of the pore proteins is required for Spo0K to fulfill its roles in the initiation of sporulation, the development of competence, and oligopeptide transport (Chapter 2). Spo0KD, one of the two ATP-binding proteins, is also required for Spo0K to fulfill its roles in the initiation of sporulation and oligopeptide transport (Perego et al., 1991) and to a slightly lesser extent in the development of competence (as judged by expression of *srfA-lacZ* and *comG-lacZ*, Chapter 2). On the other hand, the Spo0KE ATP-binding protein has only a slight effect on the initiation of sporulation, the

development of competence (as measured by expression of *srfA-lacZ* or *comG-lacZ*), and oligopeptide transport (Chapter 2).

If two ATP-binding proteins are needed for Spo0K to function under the conditions tested, and if Spo0K still functions in the absence of Spo0KE almost as well as in its presence, then either Spo0KD is acting as a homodimer in the absence of Spo0KE, or an ATP-binding protein from another ABC transporter is substituting for Spo0KE, perhaps AppF from the App oligopeptide permease (Koide and Hoch, 1994) (see Chapter 2).

The role of Spo0K in sporulation and competence. For efficient sporulation in *B. subtilis,* the cells need to be at high density, and they sense high density by the presence in the medium of at least one secreted factor that is at least in part a peptide (Grossman and Losick, 1988). I like to speculate that Spo0K is recognizing one or more such peptide factors and transducing this signal to initiate sporulation.

Two peptide factors in the medium, the ComX pheromone (Magnuson et al., 1994a) and the Competence Stimulating Factor (CSF) (Magnuson et al., 1994b), are necessary for efficient initiation of the development of competence. Recent epistasis experiments have shown that Spo0K acts on the same genetic pathway as CSF (Solomon et al., 1994), probably by interacting directly with CSF and transporting it into the cell. The mutation that activates the cryptic App oligopeptide permease bypasses the need for Spo0K for both sporulation and competence (Koide and Hoch, 1994; Solomon, 1994).

Alternatively, Spo0K (or App) may interact with the oligopeptide factors and then itself activate specific developmental pathways. Finding mutations in *spo0K* that lead to constitutive activation of sporulation or competence pathways even in the absence of transport of the peptide factors would support the latter hypothesis. A similar situation can be found in the ABC phosphate transporter

Pst in *E. coli.* Pst both transports phosphate ions into the cell and negatively regulates expression of the two-component system PhoR and PhoB (reviewed in Rao and Torriani, 1990). Since there exist mutations in *pst* that eliminate transport activity but do not affect regulatory activity, Pst must be directly involved in regulating expression of PhoR and PhoB (Cox et al., 1988; Cox et al., 1989).

The phosphorelay and sporulation. In the initiation of sporulation there are three sensor kinases, KinA (Perego et al., 1989; Antoniewski et al., 1990), KinB (Trach and Hoch, 1993), and KinC (Chapter 4 and Kobayashi et al., 1994) that donate phosphate to the response regulator Spo0F; the phosphotransferase Spo0B then transfers that phosphate to the response regulator Spo0A (Burbulys et al., 1991). This phosphorelay serves to check the state of the cell with respect to several conditions including: starvation for nutrients (Ireton et al., 1993), replication of DNA (Ireton and Grossman, 1994), lack of DNA damage (Ireton and Grossman, 1992a), a functioning Krebs cycle (Ireton et al., 1994), and high cell density (Ireton et al., 1993).

The *spo0A* mutations *rvtA11* (Sharrock et al., 1984), *sof-1* (Kawamura and Saito, 1983; Hoch et al., 1985; Spiegelman et al., 1990), and *sur0B20* (Shoji et al., 1988) cause Spo0A to more readily receive phosphate directly from KinC~P (Chapter 4 and Kobayashi et al., 1994) and bypass the need for Spo0F and Spo0B to initiate sporulation. Based on the crystal structure of the response regulator CheY, these *spo0A* mutations are all missense mutations that change residues in close physical proximity to the aspartate that receives phosphate. These mutations presumably change the structure of the phosphorylation site so that it can now receive phosphate more readily from KinC~P.

Each kinase appears to function differently under different growth conditions (Chapter 4). KinA is necessary for proper initiation of sporulation

when the cells have grown under relatively rich nutrient conditions; the requirement for KinB is greater under poorer growth conditions (Chapter 4). In a *kinA kinB* double mutant, KinC has its greatest effect in the richest growth conditions tested (Chapter 4). It is not clear whether these effects under different growth conditions are due to differences in expression or activity of the different kinases, or both.

KinC appears to be the kinase that functions earliest in donating phosphate to Spo0A through the phosphorelay, as *kinC* mutations affect expression of the *abrB* repressor as the cells enter stationary phase, but *kinA* and *kinB* mutants do not (Chapter 4). Later in stationary phase, after *kinA* and *kinB* become expressed or activated, there is enough Spo0A~P to repress transcription of *abrB* (Chapter 4).

It is not clear what signals are affecting expression or activity of the sensor kinases. *kinA* is transcribed by RNA polymerase holoenzyme containing the sporulation-specific sigma factor, sigma-H (Predich et al., 1992). *kinB* (Trach and Hoch, 1993) and *kinC* (Chapter 4) both appear to be transcribed by the major housekeeping sigma factor, sigma-A. However, based on studying expression of transcriptional *lacZ* fusions, both *kinA* (Antoniewski et al., 1990) and *kinC* (Chapter 4) are most highly expressed as the cells enter stationary phase, at least in relatively rich sporulation media. (Similar studies have not been done on *kinB* expression, at least not to my knowledge).

Based on hydropathy plots, KinB has 6 putative membrane spanning domains (Trach and Hoch, 1993) and KinC has 2 (Kobayashi et al., 1994), whereas KinA is localized to the cytoplasm (Perego et al., 1989; Antoniewski et al., 1990). Clearly, KinA cannot be responding directly to extracellular signals, but it is possible that KinB and KinC are. Perhaps KinB and KinC are responding to one or more of the aforementioned extracellular oligopeptide sporulation factors.

There is precedent for this, as recent epistasis experiments have demonstrated that the membrane sensor kinase involved in the development of competence, ComP, appears to be interacting with the extracellular competence pheromone, ComX (Solomon et al., 1994).

SinI and SinR and transcription of *spo0A*. During sporulation, *spo0A* is transcribed by holoenzyme containing sigma-H (Predich et al., 1992; Strauch et al., 1992; Siranosian and Grossman, 1994), but transcription from the sigma-H promoter is repressed by SinR (I. Smith, personal communication). This activity of SinR is blocked by expression of SinI, which binds to SinR, presumably inhibiting multimerization of SinR (Bai et al., 1993). MskB may also be capable of binding to and inhibiting SinR, since MskB is similar to SinI and overexpression of MskB leads to phenotypes similar to those seen when SinI is overexpressed or in a *sinR* mutant (Chapter 5).

One model for how depletion of functional SinR could lead to (partial) suppression of *spo0K* and *kinA* mutations is that depletion of functional SinR leads to increased expression of Spo0A, so the total amount of Spo0A increases. This leads to increased amounts of Spo0A~P, (partially) bypassing the need for *spo0K* or *kinA*.

Bai et al. point out three examples of eukaryotic DNA-binding proteins that are regulated similarly to SinR (Bai et al., 1993): Id associates with MyoD so that MyoD can no longer form hetero- or homodimers that positively regulate gene expression (Benezra et al., 1990). I-POU prevents the binding of Cf-1 to its target sequence by forming a stable heterodimer (Treacy et al., 1991). Also, IP-1 binds to AP-1 (Jun) so that it can no longer form multimers and bind to DNA (Auwerx and Sassone-Corsi, 1991).

MskD and regulation of ComK. In the development of competence, ComK is a transcription factor positively required for transcription of late competence

genes (van Sinderen et al., 1994), as well as positively regulating its own transcription (van Sinderen and Venema, 1994). MecA (Kong and Dubnau, 1994) and MecB (Msadek et al., 1994) negatively regulate ComK, and it has been shown that MecA can bind ComK (Kong and Dubnau, 1994). MecB belongs to the Clp family of ATPases, specifically ClpC. The Clp ATPases direct the ClpP protease to specific substrates (Gottesman and Maurizi, 1992; Gottesman et al., 1993), and MecA has some similarity to the ClpP protease (Kong and Dubnau, 1994). While MecA is probably not acting as a protease (it is missing the conserved serine residue thought to be in the active site of ClpP), an attractive model is that MecB directs MecA to sequester or otherwise modify ComK so ComK can not activate transcription (Kong and Dubnau, 1994; Msadek et al., 1994).

Support for this model comes from the observation that overexpression of MskD leads to increased expression of the late competence gene *comG* (Chapter 5). MskD is 67% identical to ClpX (at least over the region sequenced). A simple explanation for how overexpression of MskD leads to increased expression of *comG* is that MskD interferes with the ability of MecB and/or MecA to block activity of ComK.

An *mskD* (*clpX*) null mutation causes a growth defect, and a strain carrying this mutation gives rise to suppressors of the growth defect (Chapter 5). It will be interesting to determine what genes are normally being regulated by MskD that give rise to this growth defect and whether these genes are normally involved in development.

Multicopy suppressor screens. Screens for multicopy suppressors of mutations in regulatory genes are useful because they often lead to the isolation of genes involved in the regulatory pathways under study, or to the elucidation of mechanisms by which these pathways work. Such screens have been performed to great advantage in *Sacchoromyces cerevisiae* (Rine, 1991); however,

this is the first example of which I am aware of a screen done to isolate multicopy suppressors of a regulatory gene in *Bacillus subtilis*. (*sinR* was isolated as a multicopy inhibitor of sporulation in wild type cells (Gaur et al., 1986).) Although the screen I did was not saturating (if it were, I should have isolated *kinA* and *spo0K*), the results of this screen led to the isolation and characterization of *kinC* and to the isolation and partial characterization of *mskB* and *mskD*. As the global human population prepares to leave exponential phase growth, there is still much to learn about developmental processes associated with stationary phase in *Bacillus*.

Chapter 7:

Materials and methods

Media. Routine growth and maintenance of *E. coli* and *B. subtilis* was done in LB medium (Miller, 1972). Nutrient sporulation medium was 2xSG medium (Leighton and Doi, 1971) or DS medium (Schaeffer et al., 1965). The minimal exhaustion medium used to assay sporulation was S7 medium (Vasantha and Freese, 1980) as used previously (Jaacks et al., 1989), except that glucose was used at 0.1%. Cells were made competent in S7 minimal medium as described (Rudner et al., 1991; Magnuson et al., 1994a). Amino acids for auxotrophic requirements were added to minimal media at 40 µg/ml. Media in plates were solidified with 15 g of Agar (Difco Laboratories) per liter. Sporulation proficiency was visualized on DS or 2xSG plates. Antibiotics were used at the following concentrations: ampicillin, 100 µg/ml; chloramphenicol (Cm^R), 5 µg/ml; spectinomycin ($Spec^R$), 100 µg/ml; neomycin, 5 µg/ml; erythromycin and lincomycin together (MLS^R) at 0.5 µg/ml and 12.5 µg/ml, respectively, to select for the *erm* gene.

Strains and plasmids. Standard *E. coli* strains were used for cloning and maintaining plasmids, as previously described (Rudner et al., 1991; Ireton and Grossman, 1992b). *B. subtilis* strains used are listed in Table 7.1 and all are derived from strain 168. The JH642 (Perego et al., 1988) or PB2 (Boylan et al., 1988) strains were used as wild type, as indicated. Plasmids used are listed in Table 7.2 and some are illustrated in Figures 2.2, 4.1, 5.1, 5.8, and 7.1.

I made a null mutation in *spo0K* by deleting the first four genes of the *spo0K* operon, from *spo0KA* codon 18 (at the EspI site) to *spo0KD* codon 127 (at the BglII site) (Rudner et al., 1991), and inserting an *erm* cassette. The plasmid

TABLE 7.1 *B. subtilis* strains used.

Strains	Genotype	Comments or reference
JH642	*trpC2 pheA1*	(Perego et al., 1988)
PB2	*trpC2*	(Boylan et al., 1988)
AG522	JH642 *kinA*::Tn*917*	*kinA*::Tn*917* (Sandman et al., 1987; Antoniewski et al., 1990)
AG676	JH642 *spo0BΔPst* $pheA^{+}$	*spo0BΔ* allele cotransformed with $pheA^{+}$ (Weir et al., 1991)
JRL43	JH642 *Δspo0KD43* (*Δspo0KD*)	
JRL131	JH642 *Δspo0KA131* (*Δspo0KA*)	
JRL135	JH642 *Δspo0KDE135* (*Δspo0KDE*)	
JRL179	JH642 *Δspo0KA amyE*::P_{spo0K}-*spo0KA*	
JRL189	JH642 *Δspo0KB189* (*Δspo0KB*)	
JRL221	JH642 *Δspo0KE221::neo* (*Δspo0KE::neo*)	
JRL237	JH642 pHP13	pHP13 from BGSC (Haima et al., 1987; Bron, 1990)
JRL275	JH642 *amyE*::(*comG-lacZ neo*)	*amyE*::(*comG-lacZ neo*) (Magnuson et al., 1994a)
JRL293	JH642 *amyE*::(*srfA-lacZ cat*)	*amyE*::(*srfA-lacZ cat*) provided by D. Dubnau (Magnuson et al., 1994a)
JRL322	JH642 *Δspo0KC322* (*Δspo0KC*)	
JRL357	JH642 *Δspo0K357::neo*	
JRL358	JH642 *Δspo0K358::erm* (*Δspo0K::erm*)	
JRL407	PB2 *spo0K*::pJL58	
JRL408	PB2 Pspac-*spo0K*	
JRL417	PB2 *Δspo0K::erm*	

JRL450	PB2 Pspac-*spo0K amyE*::(*srfA-lacZ neo*) pHP13	
JRL454	PB2 Pspac-*spo0K amyE*::(*comG-lacZ neo*) pJL52	
JRL455	PB2 Pspac-*spo0K amyE*::(*comG-lacZ neo*) pHP13	
JRL459	PB2 Pspac-*spo0K spoIIA*$^+$::(*spoIIA-lacZ neo*) pJL52	*spoIIA-lacZ* (Wu et al., 1989; Ireton and Grossman, 1994)
JRL485	JH642 *Δspo0KA amyE*::(*comG-lacZ neo*)	
JRL486	JH642 *Δspo0KA amyE*::(*srfA-lacZ spc*)	
JRL488	JH642 *Δspo0KD amyE*::(*comG-lacZ neo*)	
JRL489	JH642 *Δspo0KD amyE*::(*srfA-lacZ spc*)	
JRL492	JH642 *Δspo0KDE amyE*::(*srfA-lacZ spc*)	
JRL493	JH642 *Δspo0K*::*erm amyE*::(*comG-lacZ neo*)	
JRL494	JH642 *Δspo0K*::*erm amyE*::(*srfA-lacZ spc*)	
JRL515	PB2 *amyE*::(*comG-lacZ neo*) pHP13	
JRL523	PB2 *Δspo0K*::*erm amyE*::(*comG-lacZ neo*) pHP13	
JRL530	JH642 *spo0A9V* pLK2	*spo0A9V* (Piggot and Coote, 1976)
JRL532	JH642 *spo0A9V* pHP13	
JRL539	JH642 *spo0E11* pLK10	*spo0E* (Perego and Hoch, 1987; Perego and Hoch, 1991; Ohlsen et al., 1994)
JRL540	JH642 *spo0E11* pHP13	
JRL550	JH642 *spo0J93* pLK2	*spo0J93* (Piggot and Coote, 1976)
JRL551	JH642 *spo0J93* pLK10	

JRL552	JH642 *spo0J93* pHP13	
JRL555	JH642 *Δspo0K::erm* pLK2	
JRL557	JH642 *Δspo0K::erm* pLK10	
JRL558	JH642 *Δspo0K::erm* pHP13	
JRL595	JH642 pLK2	
JRL645	PB2 *ΔkinC645::spc* (*ΔkinC::spc*)	
JRL660	JH642 *ΔkinC::spc*	
JRL672	JH642 *ΔmskD672::spc* (*ΔmskD::spc*)	
JRL679	JH642 *ΔmskD::spc suppressor*	
JRL682	JH642 *Δspo0KE::spc amyE::(comG-lacZ neo)*	
JRL685	JH642 *Δspo0KE::spc amyE::(srfA-lacZ cat)*	
JRL739	PB2 Pspac-*spo0K amyE::(srfA-lacZ neo)* pLK6	
JRL740	PB2 *amyE::(srfA-lacZ neo)* pHP13	
JRL741	PB2 *amyE::(srfA-lacZ neo)* pLK6	
JRL748	PB2 *Δspo0K::erm amyE::(srfA-lacZ neo)* pHP13	
JRL749	PB2 *Δspo0K::erm amyE::(srfA-lacZ neo)* pLK6	
JRL753	JH642 *ΔkinC::spc spo0A⁺-cat*	
JRL763	JH642 *spo0BΔPst pheA⁺ sof1-cat*	*sof-1* (Kawamura and Saito, 1983; Hoch et al., 1985) ~90% linked to *cat* by transformation (Grossman et al., 1992)
JRL764	JH642 *spo0BΔPst pheA⁺ rvtA11-cat*	*rvtA11* (Sharrock et al., 1984)
JRL766	JH642 *spo0BΔPst pheA⁺ spo0A⁺-cat*	

JRL767	JH642 *spo0BΔPst pheA*$^+$ *sof1-cat ΔkinC::spc*	
JRL768	JH642 *spo0BΔPst pheA*$^+$ *rvtA11-cat ΔkinC::spc*	
JRL770	JH642 *spo0BΔPst pheA*$^+$ *rvtA11-cat kinA*::Tn*917*	
JRL783	JH642 *spo0FΔS spo0BΔPst pheA*$^+$ *rvtA11-cat*	*spo0FΔS* described in (Kawamura and Saito, 1983)
JRL790	JH642 *ΔkinC::spc sof1-cat*	
JRL791	JH642 *ΔkinC::spc rvtA11-cat*	
JRL792	JH642 *spo0FΔS spo0BΔPst pheA*$^+$ *rvtA11-cat ΔkinC::spc*	
JRL794	JH642 *spo0FΔS spo0BΔPst pheA*$^+$ *rvtA11-cat kinA*::Tn*917*	
JRL796	JH642 *spo0FΔS spo0BΔPst pheA*$^+$ pLK2	
JRL797	JH642 *spo0FΔS spo0BΔPst pheA*$^+$ pHP13	
JRL812	JH642 *thr*::(*kinC-lacZ erm*)	
JRL871	JH642 *ΔmskB871::spc* (*ΔmskB::spc*)	
JRL903	PB2 *Δspo0K::erm* pLK64	
JRL920	JH642 *kinC*::pLK124	
JRL923	JH642 *spo0FΔS* pHP13	
JRL925	JH642 *spo0FΔS* pLK2	
JRL951	PB2 *Δspo0K::erm* pLK2	
JRL990	JH642 *kinA*::Tn*917* pLK11	
JRL991	JH642 *kinA*::Tn917 pHP13	
JRL992	JH642 *spo0E11* pLK2	

JRL993	JH642 *spo0E11* pHP13	
JRL995	JH642 *spo0BΔPst pheA*+ pLK2	
JRL996	JH642 *spo0BΔPst pheA*+ pHP13	
JRL1004	JH642 *ΔkinBkapB*::*spc kinC*::pLK124	*ΔkinBkapB*::*spc* (LeDeaux et al., 1994)
JRL1007	JH642 *kinA*::Tn*917 ΔkinBkapB*::*spc kinC*::pLK124	
JRL1010	JH642 *kinA*::Tn*917* pHP13	
JRL1011	JH642 *kinA*::Tn*917* pLK2	
JRL1018	JH642 *amyE*::*(abrB-lacZ neo)*	
JRL1035	JH642 *kinC*::pLK124 *amyE*::*(abrB-lacZ neo)*	
JRL1036	JH642 *kinA*::Tn*917 ΔkinBkapB*::*spc amyE*::*(abrB-lacZ neo)*	
JRL1039	JH642 *kinA*::Tn*917 ΔkinBkapB*::*spc kinC*::pLK124 *amyE*::*(abrB-lacZ neo)*	
JRL1046	JH642 *kinA*::Tn*917 kinC*::pLK124	
JRL1058	JH642 *spo0BΔPst pheA*+ *amyE*::*(abrB-lacZ neo)*	
JRL1081	JH642 *kinA*::Tn*917 ΔkinBkapB*::*spc orf277*::pLK126	
JRL1082	JH642 *Δspo0KC amyE*::*(srfA-lacZ cat)*	
JRL1102	JH642 *Δspo0KB amyE*::*(comG-lacZ neo)*	
JRL1103	JH642 *Δspo0KC amyE*::*(comG-lacZ neo)*	
JRL1104	JH642 *Δspo0KDE amyE*::*(comG-lacZ neo)*	
JRL1105	JH642 *Δspo0KB amyE*::*(srfA-lacZ cat)*	
JRL1114	JH642 *Δspo0KB amyE*::Pspac-*spo0KB*	
JRL1115	JH642 *Δspo0KB amyE*::Pspac-*spo0KBC*	

JRL1117	JH642 *ΔspoOKC amyE*::Pspac-*spoOKB*	
JRL1118	JH642 *ΔspoOKB amyE*::Pspac-*spoOKBC*	
JRL1124	JH642 *ΔspoOE::spc spoOFΔS spoOBΔPst* *pheA*+ pHP13	
JRL1125	JH642 *ΔspoOE::spc spoOFΔS spoOBΔPst* *pheA*+ pLK2	
JRL1131	PB2 *amyE*::(*comG-lacZ neo*) pLK6	
JRL1132	PB2 Pspac-*spoOK amyE*::(*comG-lacZ neo*) pLK6	
JRL1133	PB2 *ΔspoOK::erm amyE*::(*comG-lacZ neo*) pLK6	
KI418	JH642 *spoOKA*::Tn*917*	(Rudner et al., 1991)
KI644	JH642 *spoOE11 kinA*::Tn*917*	
KI1521	JH642 *spoOF221 rvtA11-cat::spc*	*spoOF221* (Piggot and Coote, 1976)
NY120	JH642 *ΔkinBkapB::spc*	
NY121	JH642 *kinA*::Tn*917* *ΔkinBkapB::spc*	

TABLE 7.2 Plasmids used.

Plasmid	Comments, source, or reference[a]
Vectors	
pBluescriptII KS+	Ap; used for subcloning and sequencing (Stratagene)
pAG58	Ap Cm; (Jaacks et al., 1989)
pJH101	Ap Tc Cm; integrative vector (Ferrari et al., 1983)
pGEM3Zf(+)::*cat-1* (pGEM::*cat*)	Ap Cm; integrative vector (Youngman et al., 1989)
pUC18::*erm*	Ap MLS; source of *erm* cassette (Kenney and Moran, 1987)
pBEST501	Ap Neo; source of *neo* cassette (Itaya et al., 1989)
pUS19	Ap Spec; integrative vector, source of *spc* cassette (Benson and Haldenwang, 1993)
pJL62	Ap Spec; 1.1 kb BglI (blunted)-NdeI (blunted) *spc* cassette from pUS19 cloned into NcoI (blunted) of *cat* gene in pJH101; used to convert Cm^R $Spec^S$ strains to Cm^S $Spec^R$
pIK105	Ap Neo; used to convert Cm^R Neo^S strains to Cm^S Neo^R (Ireton and Grossman, 1992b)
pJL73	Ap Spec; 1.1 kb BglI (blunted)-NdeI (blunted) *spc* cassette from pUS19 cloned into SmaI site of pBluescript SK+
pJL74	Ap Spec; same as pJL73 except the *spc* cassette is cloned in the opposite orientation
pHP13	MLS Cm; *B. subtilis E. coli* shuttle vector (Haima et al., 1987; Bron, 1990)
pJL52	MLS; 0.5 kb BamHI (blunted)-NcoI (blunted) deletion of pHP13; helper plasmid used in conjunction with pHP13 for homology assistance

pDR63	Ap Cm; vector used to recombine fragments into the chromosome at *amyE* by double crossover, derived from pDH32 (Shimotsu and Henner, 1986)
pDR67	Ap Cm; vector used to place fragments under control of the IPTG-inducible promoter Pspac and recombine into the chromosome at *amyE* by double crossover (Ireton et al., 1993)
pDG793	Ap MLS; vector used to construct *lacZ* transcriptional fusions and recombine into the chromosome at *thrC* by double crossover (a gift from P. Stragier)
<u>Others</u>	
pDR9	Ap Cm; clone of *spo0KB-E* in pJH101 (Rudner et al., 1991)
pDR18	Ap Cm; clone of *spo0K* promoter region from PvuII site upstream of the promoter to EcoRI site in *spo0KA* in pJH101 (Rudner et al., 1991)
pDR21	Ap; clone of the *spo0K* promoter region (as in pDR18) in pBluescript II SK+ (Rudner et al., 1991)
pJL9	Ap Cm; 408 bp BglII (blunted)-XcmI (blunted) deletion of pDR9, used to make *Δspo0KD*
pJL10	Ap Cm; 3.6 kb EcoRI fragment from pDR9 cloned into EcoRI of pDR18, putting all of *spo0KA-D* on a single integrational plasmid
pJL12	Ap Cm; 1 kb BglII (blunted)-AatII (blunted) deletion of pJL10
pJL18	Ap Cm; 1371 bp EspI (blunted)-EcoRI (blunted) deletion of pJL12; used to make *Δspo0KA*
pJL19	Ap Cm; 1341 bp BglII (blunted)-ClaI (blunted) deletion of pDR9; used to make *Δspo0KDE*

pJL28	Ap Cm; 2.9 kb BamHI-NruI (blunted) fragment of pJL10 cloned into BamHI-EcoRI (blunted) of pDR63; used to make *amyE*::P_{spo0K}-*spo0KA*
pJL29	Ap Cm; 447 bp RsrII (blunted)-ApaI (blunted) deletion of pJL10; used to make *Δspo0KB*
pJL30	Ap Cm; 1.3 kb SphI (blunted)-SplI (blunted) deletion of pDR9
pJL32	Ap Cm; 2.3 kb NcoI (blunted)-BglII fragment of pJL10 cloned into SmaI-BglII of pDR67; used to make *amyE*::Pspac-*spo0KBC*
pJL34	Ap Cm; 573 bp PvuII-SnaBI deletion of pJL30; used to make *Δspo0KC*
pJL36	Ap Cm; 1.8 kb PvuII-AatII (blunted) deletion of pJL30
pJL42	Ap Cm Neo; 1.3 kb *neo* cassette from pBEST501 cloned into EcoRV-EcoRI of pJL36; used to make *Δspo0KE*::*neo*
pJL45	Ap Cm; 311 bp EcoRI (blunted)-SalI fragment, containing Pspac, from pAG58 cloned into EcoNI (blunted)-SalI of pJH101
pJL47	Ap Cm; Pspac replacement vector (Figure 7.1); 1.3 kb BamHI-SphI fragment containing *lacI* from pAG58 cloned into pJL45 so transcription of *lacI* is opposite that from Pspac
pJL49	Ap Cm Neo; 1.3 kb *neo* cassette from pBEST501 cloned into EspI-BglII (sites lost) of pJL10; used to make *Δspo0K*::*neo*
pJL50	Ap Cm MLS; 2.3 kb *erm* cassette from pUC18::*erm* cloned into EspI-BglII (sites lost) of pJL10; used to make *Δspo0K*::*erm*
pJL51	Ap Cm; 0.9 kb XmnI (blunted)-ClaI (blunted) fragment from pDR21 5 bp downstream from *spo0K* promoter cloned into SalI (blunted) site of pJL47

pJL58	Ap Cm; 0.6 kb BamHI-SspI (blunted) fragment from pDR21 containing a 368 bp fragment upstream (23 bp) of the *spo0K* promoter cloned into ClaI (blunted)-BamHI (blunted) of pJL51; used to make Pspac-*spo0K*
pJL75	Ap Cm Spec; 1.1 kb *spc* cassette from pUS19 cloned into EcoRV-EcoRI of pJL36; used to make *Δspo0KE::spc*
pJL79	Ap Cm; 1.3 kb NcoI (blunted)-PvuII of pJL12 cloned into SmaI of pDR67; used to make *amyE*::Pspac-*spo0KB*
pLK2	MLS Cm; original *kinC* clone, 3.5 kb Sau3A partial digest of JH642 chromosomal DNA cloned into BamHI of pHP13
pLK6	MLS Cm; original *mskD* clone, 2.7 kb Sau3A partial digest of JH642 chromosomal DNA cloned into BamHI of pHP13
pLK10	MLS Cm; an original *mskB* clone, 2.8 kb Sau3A partial digest of JH642 chromosomal DNA cloned into BamHI of pHP13, contains 135 bp deletion relative to pLK11
pLK11	MLS Cm; an original *mskB* clone, 3.0 kb Sau3A partial digest of JH642 chromosomal DNA cloned into BamHI of pHP13
pLK21	MLS Cm; 2.1 kb PstI fragment from pLK2 cloned into PstI site of pHP13
pLK22	MLS Cm; 1.4 kb ClaI (blunted)-HindIII (blunted) deletion of pLK2
pLK23	MLS Cm; 1.4 kb PstI deletion of pLK2
pLK24	Ap Cm; 3.5 kb EcoRI (blunted) fragment of pLK2 cloned into PstI (blunted) of pGEM*cat*
pLK25	Ap Cm Spec; 1.1 kb PstI-BamHI (blunted) *spc* cassette of pJL74 cloned into PstI-ClaI (blunted) of pLK24, used to make *ΔkinC::spc*

pLK56	Ap; 3.5 kb EcoRI fragment from pLK2 cloned into EcoRI of pBluescriptII KS+
pLK60	Ap Cm; 1.3 HindIII fragment of pLK11 cloned into HindIII of pJH101
pLK62	Ap Cm; 2.5 kb SalI (blunted) fragment of pLK6 cloned into SalI (blunted)-EcoRI (blunted) of pJH101
pLK63	MLS Cm; 1.3 kb HindIII deletion of pLK11
pLK64	MLS Cm; 1.3 kb HindIII fragment of pLK11 cloned into HindIII of pHP13
pLK65	MLS Cm; 1.1 kb HindIII deletion of pLK10
pLK66	MLS Cm; 1.1 kb HindIII fragment of pLK10 cloned into HindIII of pHP13
pLK67	MLS Cm; 1.4 kb EcoRI deletion of pLK6
pLK68	MLS Cm; 2.5 kb SalI fragment of pLK6 cloned into SalI of pHP13
pLK69	MLS Cm; 1.4 kb EcoRI fragment from pLK6 cloned into EcoRI of pHP13
pLK101	Ap Cm Spec; 1.1 kb BamHI-ClaI *spc* cassette from pJL74 cloned into BglII-ClaI of pLK62; used to make *ΔmskD::spc*
pLK102	Ap; 2.2 kb ClaI deletion of pLK56
pLK104	Ap; 0.4 kb EcoRV-SmaI deletion of pLK102
pLK114	Ap MLS; *kinC-lacZ*; 2.3 kb EcoRI-ClaI (blunted) fragment of pLK2 cloned into EcoRI-BamHI (blunted) of pDG793; used to make *thrC::kinC-lacZ*
pLK115	Ap Cm Spec; 1.1 kb SpeI-EcoRV *spc* cassette from pJL74 cloned into SpeI-XcmI (blunted) of pLK60; used to make *ΔmskB::spc*
pLK116	MLS Cm; 0.4 kb EcoRV-XcmI (blunted) deletion of pLK64
pLK117	MLS Cm; 0.8 kb XcmI (blunted)-SmaI deletion of pLK64

pLK118	MLS Cm; 0.5 kb EcoRV-NdeI (blunted) deletion of pLK64
pLK119	MLS Cm; 0.8 kb NdeI (blunted)-SmaI deletion of pLK64
pLK120	Ap Cm; 0.7 kb EcoRV fragment from pLK2 into SmaI of pGEM::*cat*, contains *kinC* promoter
pLK121	Ap Cm; 0.2 kb NdeI (blunted)-EcoRI (blunted) deletion of pLK120
pLK122	MLS Cm; pLK64 digested with XcmI, blunted, and religated
pLK123	MLS Cm; pLK64 digested with NdeI, blunted, and religated
pLK124	Ap Cm; 0.4 kb BamHI-PstI fragment of pLK120 cloned into pGEM*cat*
pLK125	MLS Cm; 0.6 kb ApaI (blunted)-SalI (blunted) deletion of pLK2
pLK126	Ap Cm; 102 bp ApaI (blunted)-BamHI fragment of pLK104 cloned into SalI (blunted)-BamHI of pGEM::*cat*, used to disrupt *orf277*

[a]Ap, Tc, Cm, Neo, Spec, MLS refer to resistance to ampicillin, tetracycline, chloramphenicol, neomycin, spectinomycin, and erythromycin + lincomycin, respectively. All sizes in kb are approximate. Klenow fragment of DNA polymerase I was used for all blunting reactions, as described (Ausubel et al., 1990).

that contains this mutation is pJL50 (Figure 2.2, Table 7.2), which has the *cat* gene in the plasmid backbone. I introduced this mutation into the chromosome by transforming wild type cells with linearized pJL50 and selecting for MLS^R. One of the MLS^R Cm^S transformants, resulting from a double crossover, was chosen as the *ΔspoOK::erm* mutant. A similar deletion/insertion mutation was made inserting a *neo* cassette (*ΔspoOK::neo*) instead of *erm*. Deletion/insertion mutations in *spoOKE* were made similarly using linearized pJL42, which inserts a *neo* cassette, and pJL75, which inserts a *spc* cassette (see Table 2.1, Figure 2.2). Construction of the deletion alleles *ΔspoOKA*, *ΔspoOKB*, *ΔspoOKC*, and *ΔspoOKD*, and *ΔspoOKDE* is described in Chapter 2.

The *ΔspoOE::spc* allele used was constructed by Keith Ireton and contains the *spc* cassette from pUS19 inserted into a deletion of *spoOE* that starts from 13 bp upstream of the *spoOE* start codon and ends at the G in the TAG stop codon (Ireton, 1994).

A null mutation in *kinC* was made by deleting from 25 bp upstream of the putative *kinC* start codon (the PstI site in Figure 4.1) to codon 211 (the ClaI site in Figure 4.1) and inserting a *spc* cassette. This mutation, contained on pLK25 (see Figure 4.1), was incorporated into the chromosome by transforming linearized plasmid into PB2 and selecting for $Spec^R$. pLK25 has the *cat* gene in the plasmid backbone. I screened for a double crossover event by screening for transformants that were $Spec^R$ and Cm^S.

The *rvtA11* (Sharrock et al., 1984) and *sof-1* (Kawamura and Saito, 1983) alleles of *spoOA* that were used are ~90% linked by transformation to *cat* or to *spc* (Grossman et al., 1992; Ireton and Grossman, 1992a; Ireton and Grossman, 1994), where the *spc* gene was inserted at the NcoI site of the *cat* gene using pJL62 (Table 7.2).

I constructed a *kinC-lacZ* transcriptional fusion that contains sequence from ~1.5 kb upstream of the putative *kinC* start codon to codon 211 and introduced it into the *thrC* locus of the chromosome, using the *lacZ* fusion vector pDG793 (provided by Patrick Stragier). The plasmid containing this fusion is pLK114 (Table 7.2, Figure 4.1). pLK114 was linearized and transformed into wild type cells selecting for MLS^R. Double crossover events that resulted in the introduction of the *kinC-lacZ* fusion at *thrC* caused a Thr^- phenotype.

The *ΔkinBkapB::spc* (*kinB*) allele used was constructed by Namyi Yu (LeDeaux et al., 1994) and is a deletion/insertion mutation that contains the *spc* cassette from pJL73 inserted between the SacII site at codon 232 (of 428) of *kinB* and the BssHII site downstream of *kapB,* the gene downstream of *kinB* (Trach and Hoch, 1993).

I made a null mutation in *mskB* by deleting from 64 bases upstream of the putative *mskB* start codon (at the XcmI site in *orf45*) to the last sense codon in *mskB* (at the SpeI site in *mskB*) and inserting a *spc* cassette (see Figure 4.1). This mutation is contained in pLK115 (see Figure 4.1; Table 7.2), and was introduced into the chromosome by double crossover in the same manner as the *kinC* null mutation described above.

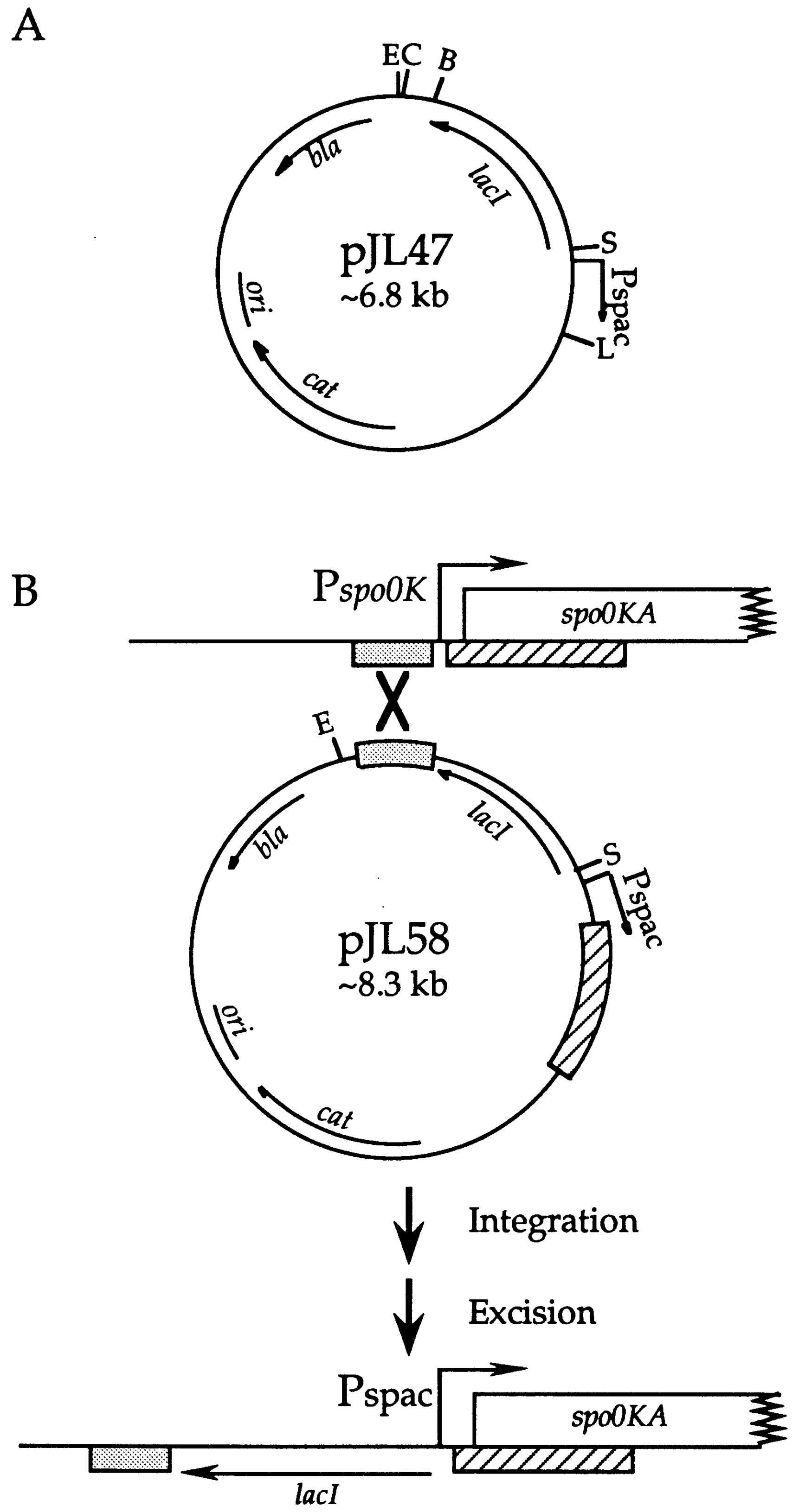
The *mskD* null mutation was made by deleting from the ClaI to the BglII sites in *mskD* and inserting a *spc* cassette. This mutation is contained in pLK101 (see Figure 5.8; Table 7.2) and was introduced into the chromosome as described above.

Construction of a conditional *spo0K* mutant (Pspac-*spo0K*). I constructed a conditional mutation of *spo0K* by replacing the normal *spo0K* promoter with the LacI-repressible/IPTG-inducible promoter Pspac (Yansura and Henner, 1984; Jaacks et al., 1989; Henner, 1990), using the vector pJL47 (Figure 7.1A). pJL47 can

be used to replace the promoter of a gene with the *lacI* cassette and Pspac in a two-step process without leaving behind any drug resistance marker.

pJL58 contains DNA from immediately upstream and downstream of the *spo0K* promoter cloned into pJL47 (Figure 7.1B) and was used to replace the normal *spo0K* promoter with Pspac in two steps. Transformation of pJL58 into wild type cells gave two types of transformants. One class was Spo^+, and most likely resulted from recombination with sequences upstream of the promoter. The other class was Spo^- in the absence of IPTG and Spo^+ in the presence of IPTG. This class most likely resulted from recombination with sequences downstream of the promoter. Three Spo^+ transformants were chosen and used to screen for a second recombination event that would leave behind the *lacI*/Pspac cassette in place of the *spo0K* promoter (Figure 7.1B). The Spo^+ transformants were grown to stationary phase in LB medium in the absence of selection for the integrated plasmid, that is, in the absence of chloramphenicol. I screened ~13,000 colonies on sporulation plates (lacking IPTG and Cm) and identified two Spo^- colonies that were also Cm^S. The sporulation phenotype of both of these colonies was completely dependent on IPTG; they were Spo^+ in the presence of 1 mM IPTG, and Spo^- in the absence of IPTG (data not shown). The sporulation and competence phenotypes in the absence of IPTG were indistinguishable from that of a *spo0K* mutant. One isolate, JRL408 (Pspac-*spo0K*), was chosen for further experiments.

Figure 7.1. Making a LacI-repressible, IPTG-inducible allele of *spo0K*. A. pJL47 contains *lacI* under control of the constitutive P_{pen} promoter and the LacI-repressible, IPTG-inducible promoter P_{spac} (Yansura and Henner, 1984; Henner, 1990). B. pJL58 contains sequence from both upstream (stippled box) and downstream (striped box) of the *spo0K* promoter cloned upstream and downstream, respectively, of the *lacI*-P_{spac} cassette of pJL47. When transformed into wild type cells selecting for Cm^R (integration step), pJL58 can recombine either with the upstream *spo0K* sequence (shown), which would result in a phenotypically Spo^+ transformant, or with the downstream *spo0K* sequence (not shown), which would result in a transformant which was Spo^+ only in the presence of IPTG. Cells that had undergone a second recombination event that left behind the *lacI*-P_{spac} cassette in place of P_{spo0K} (excision step) were isolated as described in Chapter 7. These cells were Cm^S and Spo^- in the absence of IPTG and Spo^+ in the presence of IPTG. Restriction site abbreviations: E, EcoRI; C, ClaI; B, BamHI; S, SphI; L, SalI.


A
EC
B
bla
lacI
S
Pspac
L
pJL47
~6.8 kb
ori
cat
B
Pspo0K
spo0KA
E
lacI
bla
S
Pspac
pJL58
~8.3 kb
ori
cat
Integration
Excision
Pspac
spo0KA
lacI

Sporulation assays. Cells were grown in DS, 2xSG, or minimal exhaustion medium at 37°C unless otherwise indicated and spores were assayed approximately 20 hours after the end of exponential growth. The number of viable cells/ml culture was determined as the total number of CFU on LB plates. The number of spores/ml of culture was determined as the number of CFU after heat treatment (80°C for 20 min). Sporulation frequency is the ratio of spores/ml to viable cells/ml.

β-galactosidase assays. For determination of β-galactosidase specific activity, cells were grown in the indicated medium and samples were taken at the indicated times. Prior to the enzyme assay, cells were removed by centrifugation and resuspended in Spizizen salts (Spizizen, 1958). β-galactosidase specific activity is expressed as (ΔA_{420} per minute per ml of culture per unit of optical density at 600 nm) X 1000 (Miller, 1972).

Sorting the multicopy suppressing plasmid isolates. The 12 plasmid isolates listed in Table 3.1 were originally divided into four classes based on the morphology that they imparted to colonies. Plasmids in the pLK11 class did not suppress the sporulation phenotype of *spo0K* mutants to as great an extent as plasmids in the pLK2 class and caused the colonies to have a slightly rougher appearance. Plasmids in the pLK6 class gave rise to darker blue colonies on minimal Xgal plates in a Pspac-*spo0K* background than did plasmids in the pLK4 class.

To verify that only four (essentially) different plasmid inserts were represented by these 12 isolates, each plasmid and also the parent vector pHP13 was digested with each of three restriction enzymes that cut at sites of 5 bases: HphI, HgaI, and AvaII. Single restriction enzyme digests of plasmids belonging to a suspected class were electrophoresed on an agarose gel, and except for pLK10, each plasmid belonging to a suspected class gave the same restriction

pattern. The only difference between pLK10 and the other two plasmids in its class were two bands in each digest that were shifted ~0.15 kb relative to corresponding bands in the digests of the other two plasmids, pLK11 and pLK15; otherwise the digests of the three plasmids gave the same pattern. Only representative plasmids in each class were chosen for further study.

DNA sequencing. The Sequenase v. 2.0 kit (U.S. Biochemical Corp.) was used to sequence double stranded plasmid DNA. To sequence *kinC*, sequence of one or the other strand was determined from different subclones of pLK2 into pBluescript II KS using either the universal or reverse primer. To determine the sequence of the opposite strand for the *kinC* region in pLK2, primers were made complementary to the sequence determined using the pBluescript subclones, and these primers were used to sequence pLK2.

The sequence I have for the *mskB* region covers the entire pLK64 insert. Some of this sequence data comes from sequencing pLK64 and pLK66 (see Figure 5.1) using the universal and reverse primers. (Because the polylinker in pHP13 is in sequence derived in pUC9, one can use the universal and reverse primers to sequence the ends of inserts in pHP13.) To sequence across the middle of the pLK64 insert, primers were made complementary to the determined sequence and were used to sequence the pLK64 insert that had been subcloned into pBluescript II KS. However, this sequence is derived mainly from only one strand or the other. This is why I am more confident of the data from the chromosome sequencing project, which comes mainly from both strands (Glaser et al., 1993), and cite that data in Figure 5.2.

Sequence for *mskD* comes only from sequencing the ends of the inserts in pLK67 and pLK69 away from the EcoRI site in pLK6 (see Figure 5.8) using the universal primer.

Database searching. The KinC and Orf277 sequences were compared to the combined nonredundant protein sequence database using the BLAST software package (Altschul et al., 1990) at the National Center for Biotechnology Information. To compensate for the fact that MskB and Orf45 are small enough that their sequences might not have been identified and deposited in a protein database, they were compared to the combined nonredundant nucleotide sequence database translated in all six reading frames using BLAST (Altschul et al., 1990). To compensate for possible frameshifts in the *mskD* sequence data, this sequence was translated in all six reading frames and compared to the combined nonredundant protein sequence database translated in all six frames, again using the BLAST software package (Altschul et al., 1990).

Transduction. I grew a PBS1 transducing lysate on JRL660 (*ΔkinC::spc, trpC2, pheA1*) and used it to transduce some of the mapping kit strains (Dedonder et al., 1977). The protocols used were essentially as described (Cutting and Vander Horn, 1990). In the three factor cross between *ΔkinC::spc, spo0E11*, and *kinA*::Tn*917*, transductants were tested for MLS^R and the colony morphology was analyzed to distinguish between the different classes of recombinants. It was relatively easy to distinguish *spo0E11* from *kinA*::Tn*917* from the *spo0E11 kinA*::Tn*917* double mutant.

Primer extension analysis. To determine the 5' end of the *kinC* and *mskB* transcripts, strains JRL951 (*Δspo0K::erm* pLK2) and JRL903 (*Δspo0K::erm* pLK64) respectively were grown in 2xSG medium with chloramphenicol. 50 ml samples were taken at various times for preparation of RNA essentially as described (Ausubel et al., 1990; Ireton and Grossman, 1992b).
The sequence of the primer used to map the 5' end of the *kinC* transcript, LKP16, was 5'-TTCTTCAGAAAGCTGTTTATACTTCCATTC-3', and its complement is underlined in Figure 4.2. The sequence of the primer used to map the 5' end of

the *mskB* transcript, LKP14, was 5'-CTAAAAACTGCCTCACATCATGAATGCCTA-3', and its complement is underlined in Figure 5.2. The primers were end-labeled with ^{32}P, essentially as described (Ausubel et al., 1990), and purified with a NICK™Spin column using the protocol supplied (Pharmacia). The protocol for hybridization extension reactions was essentially as described (Ausubel et al., 1990; Ireton and Grossman, 1992b) except I used 200 μg of RNA in each multicopy *kinC* (pLK2) sample and 100 μg of RNA in each multicopy *mskB* (pLK64) sample, instead of 50 μg.

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