DETERMINANTS OF COMPARTMENTALIZATION OF GENE EXPRESSION DURING SPORULATION IN *BACILLUS SUBTILIS*

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ABSTRACT

Determinants of compartmentalization of gene expression during sporulation in *Bacillus subtilis*
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Bacillus subtilis, a benign gram-positive bacterium, utilizes the strategy of sporulation, which enables it to survive stresses such as starvation, desiccation, and UV irradiation. The spore provides greatly heightened safety to heat and noxious chemicals and remains dormant until conditions become favorable to growth. Sporulation by *Bacillus subtilis* is a primitive example of cell differentiation. The study of sporulation by *Bacillus subtilis* has become a paradigm for the study of differentiation in prokaryotes. Central to this process is the establishment of distinct patterns of gene expression in the cell types involved.

Our laboratory has developed a two-part *sacB*/SacY probe to study the temporal and spatial compartmentalization of gene expression. It utilizes the anti-terminator protein SacY to control the transcription of reporter *lacZ*, (cloned downstream of the *sacB* gene,) which is regulated by anti-termination. Expression of *sacB* and SacY is regulated by a pair of promoters specific for σ^F (prespore specific) and σ^E (mother cell specific.) Both SacY and *sacB* must be in the same compartment of the sporulating cell in order to obtain β-galactosidase activity.

Mutagenesis of *Bacillus subtilis* was employed to identify determinants of compartmentalization of gene expression during sporulation. Mutants were screened for loss of compartmentalization using the two-part probe.

In addition to the two-part *sacB*/SacY probe, a second method was developed; transposon mutagenesis was performed on strains where expression of *gfp* was regulated by promoters recognized by either σ^F or σ^E. Cells deficient in sporulation were isolated and evaluated by fluorescence microscopy for uncompartmentalized *gfp* expression.

A rescue vector was developed that allowed for efficient cloning of Tn*10* insertions. This plamid, pJP17, proved to be an essential tool. Mutations causing uncompartmentalized σ^F activity were identified in *spoIIIE*, *spoIIIAA*, *spoIIIAB, spoIIIJ, spoIIE*, *spoIIAA*, *spoIID, spoIIM, kinA* and *ald.* The *spoIIIE* mutation provides the most dramatic phenotype, and was the only mutation, that resulted in 100% loss of compartmentalization during stage II of sporulation. In contrast to all other mutants, the dramatic stage II loss of compartmentalized activity of σ^F indicates a regulatory role for SpoIIIE, which has yet to be elucidated. Taken together, these results indicate a central role for SpoIIIE in preventing activation of σ^F in the mother cell in addition to its DNA translocation activity.

ACKNOWLEDGEMENTS AND DEDICATION

This work is dedicated with great affection and admiration to my friend and mentor,

Patrick Piggot, Ph.D.

I would also like to acknowledge Andi, Max and Luna, my dear family.

I believe a leaf of grass is no less

than the journey-work of the stars.

-Walt Whitman

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bp	Base pairs
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraactic acid
IPTG	isopropyl-β-D-thiogalactoside
Kb	kilobase pairs
LA	Luria-Bertani agar
LB	Luria-Bertani broth
M	Moles
mg	milligrams
ml	milliliter
mm	millimolar
MSSM	modified Schaffer's sporulation medium
PCR	polymerase chain reaction
rpm	revolutions per minute
SDS	Sodium dodecyl sulfate
SSA	Schaeffer's Sporulation Agar
μg	micrograms
μl	microliters
μM	micromolar
X-Gal	5-bromo-4-chloro-2-indolyl-β- galactoside

CHAPTER 1

INTRODUCTION

A Paradigm for Study of Differentiation

Bacillus subtilis is a benign Gram-positive bacterium commonly found in soil. Originally named *Vibrio subtilis* in 1835, it is one of the first bacterial species to be studied. When nutrients become scarce or in response to high cell-density, *B. subtilis* can divide asymmetrically, producing a tough, protective endospore that can protect it against extreme environmental conditions such as heat, salt, radiation and desiccation. This allows *B. subtilis* to survive until environmental conditions become favorable to growth. Endospore formation by *B. subtilis* is considered a very early example of differentiation, and has become a paradigm for the study of cellular differentiation.

B. subtilis has gained popularity as a model organism for the laboratory study of gram positive bacteria. There are several characteristics which make working with *B. subtilis* attractive at the bench, and in particular for the study of sporulation. Laboratory strains of *B. subtilis* are easy to manipulate genetically due to a natural competence to the uptake of foreign DNA along with high rates of homologous recombination. (Dubnau and Lovett, 2002). *B. subtilis* viability does not depend on its ability to sporulate, and mutant strains deficient in the ability to sporulate are easy to generate and identify (Piggot and Coote, 1976). *B. subtilis* is not considered a human pathogen, and was used for the study of biological warfare in 1966, when the U.S. Army dispensed *B. subtilis* variant *niger* throughout the New York City subway system (Cole, 1995; Carlton, 2001). More than a million civilians where harmlessly exposed when army scientists dropped light bulbs

filled with the bacteria onto ventilation grates (Cole, 1995; Carlton, 2001). Similar to *Escherichia coli*, the prevailing laboratory model organism for the study of gram negative bacterium, many tools used by molecular biologists work well in *B. subtilis,* including inducible plasmids and transposons (Hoch, 1991). The ease with which one can add or knock out genes allows for relatively quick answers as compared to eukaryotic systems where knocking out genes can take years. Genetic screens have evolved along with advances in molecular biology, and have incorporated the use of green fluorescent proteins and reporter systems using inducible promoters. *B. subtilis* is one of the most well characterized bacteria. The entire *B. subtilis* genome has been sequenced, and over 125 genes essential for sporulation have been identified, most of which were identified using classical genetics methodologies (Piggot and Losick, 2002). Microarray analysis of genes responding to the RNA polymerase sigma factors governing sporulation has provided insights into global changes in gene expression during sporulation and identified many uncharacterized genes involved in the sporulation process (Silvaggi and Losick, 2004). Recently, in-silico methods employing mathematical simulations been applied to the study of the regulation of σ^F, an early sporulation sigma factor (Yudkin and Clarkson, 2005). The intense study of sporulation in *B. subtilis* is complemented by the genetic, biochemical and histological research in other related areas of its growth and development, such as stress responses. The vast amount of research conducted on the biology of *B. subtilis* provides a great wealth of information and along with its amenability to laboratory technique, makes it a clear choice for investigators interested in studying early development biology.

The sporulation process is described in eight morphological stages. The following introduction will begin with a description of the morphological changes in the sporulating cell, followed by a discussion of the cell processes that account for these morphological changes. A particular emphasis will be placed on the regulation of early RNA polymerase sigma factors, the gate keepers for expression of genes responsible for differentiating *B. subtilis.*

Morphological Stages of Sporulation

Stage 0 refers to the vegetative cell which is rod shaped and flagellated. When a cell enters into sporulation, the two chromosomes condense and form a serpentine filament of chromatin that stretches across the length of the cell (Ryter, 1965) (Fig. 1). This reorganization of chromosomes is the first visible step in sporulation and is designated Stage I. The axial filament formation is facilitated by the anchoring of each chromosome to a cell pole (Thomaides et al., 2001). It is also during stage I that the cell determines its divisional site. In contrast to the vegetative division at midcell, the sporulation septum is assembled approximately one quarter of the way in from the cell pole. Stage II of sporulation indicates the completion of the asymmetric septum. Although the cell uses the same machinery to synthesize both divisional septa, they are not the same, for example the sporulation septum is thinner. At stage II the cell is divided into the smaller prespore, and the larger mother cell. The prespore contains only one third of the chromosome targeted for the developing spore, however the remaining two thirds is pumped in by the DNA translocase activity of SpoIIIE (Bath et al. 2000; Wu and Errington, 1994.) What follows the assembly of the septum is a phagocytosis-like

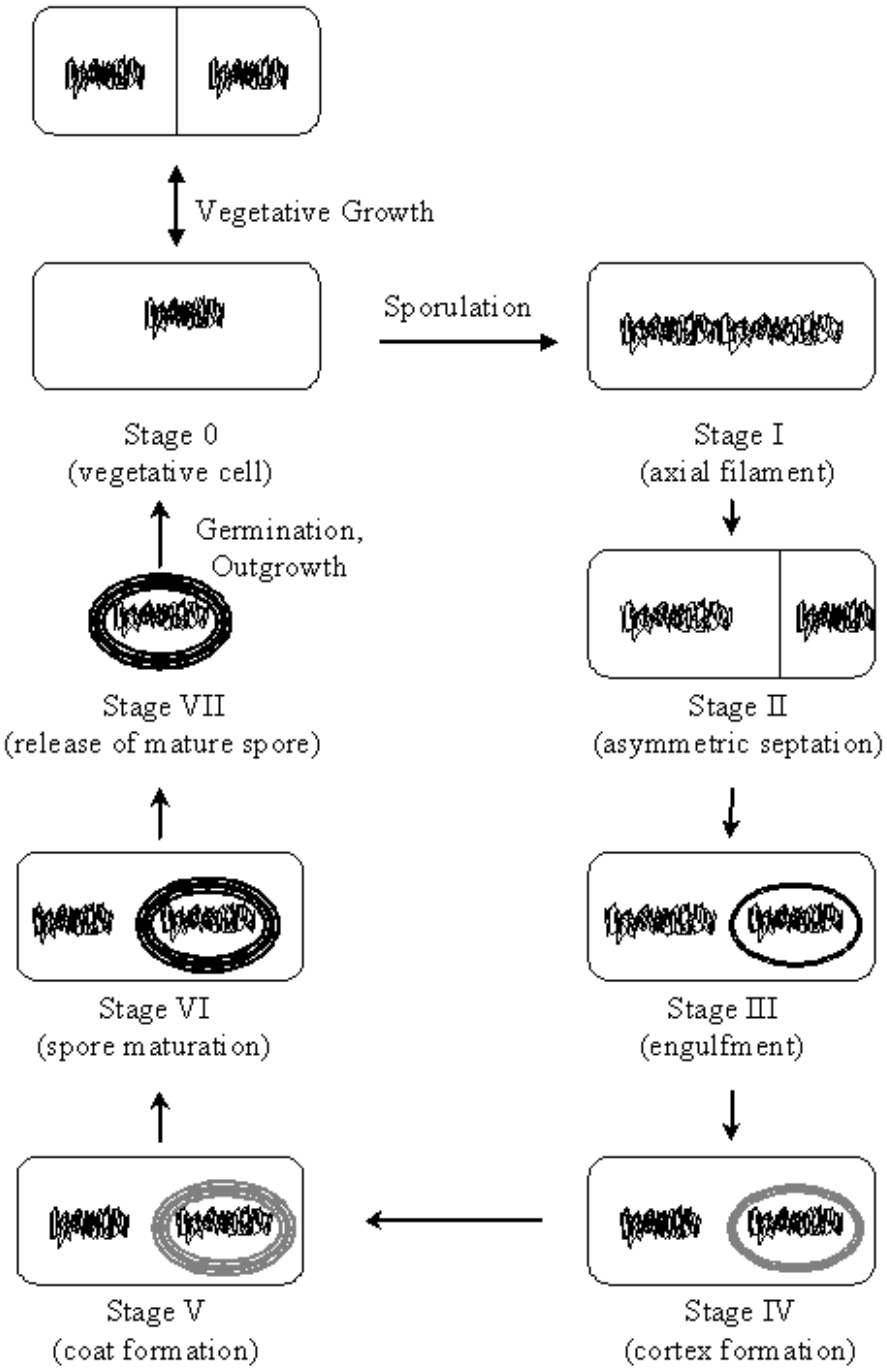


Figure 1. Schematic representation of the stages of sporulation.

process wherein the mother cell engulfs the prespore, (stage III). It begins with a thinning of the septal wall, followed by the bulging of the prespore into the mother cell. The septal membrane migrates along the sides of the prespore until they fuse at the cell pole releasing it within the mother cell. The completion of this process results in a free-floating protoplast, which designates stage III of sporulation (Piggot et al., 1994). The protoplast derived from stage III contains two membranes, one from the mother cell and one from the prespore. The final four stages of sporulation involve the development of the spore inside the mother cell. During stage IV, two layers of peptidoglycan are deposited between these membranes: the cortex and the primordial germ cell wall. At this point the prespores become phase bright (Foster and Popham, 2002). Stage V of sporulation indicates construction of the spore coat, which is made of a complex matrix of proteins deposited on the outer membrane of the prespore, (Driks, 2002).
The spore matures and gains its ability to withstand high temperatures and UV radiation during stage VI (Nicholson, et al., 2000). Finally, Stage VII occurs when the mother cell lyses, releasing the mature spore into the environment. The spore will remain dormant until conditions favorable to germination arise. Following germination and outgrowth, the cell will resume the vegetative growth cycle for as long as conditions allow, (Paidhungat and Setlow, 2002). The nomenclature for sporulation genes is linked to its mutant's effect on the developing spore. A mutant gene whose phenotype displays a blockage in stage two sporulation would be identified as *spoII* plus a letter, for example s*poIIA,* s*poIIAB,* etc.

Sporulation in *B. subtilis* is an early example of prokaryotic differentiation, which requires an ability to regulate global changes in gene expression. These changes in gene

expression are directed by the activation of alternative RNA polymerase σ factors (Piggot and Losick, 2002). However, the overall regulation of sporulation is a complex cascade of biochemical signaling and genetic regulation; for example, inactive regulatory proteins can become active through the physiological signals generated by conditions such as nutrient deprivation, high cell density, and DNA damage. The next section will highlight examples of this interplay between biochemical and genetic regulation that can result in the initiation of sporulation.

Initiation of Sporulation

There are a variety of integrative signals, which culminate into the activation of Spo0A, the master regulator for entry into spore formation. These signals include those derived from cell density, nutrient starvation and cell cycle (Perego and Hoch, 2002). The phosphorylated form of Spo0A is a DNA binding protein whose ability to alter transcription is based in part on its accumulation in the cell. Spo0A-PO_4 is both a positive and negative regulator of gene expression with a large number of genetic loci under its control. Phosphorylation of Spo0A is controlled by a multi-component phosphorelay which is a more complex version of the two-component system (Burbulys et al., 1991) (fig. 2). The series of reactions collectively referred to as the phosphorelay begins with autophosphorylation of histidine kinase KinA or KinB (Trach and Hoch, 1993) and may involve three other kinases; KinC (LedDeaux et al., 1995), KinD (Jaing et al., 1999) and KinE (Fabret et al., 1999). This phosphate is donated to the response regulator Spo0F. Phosphotransferase Spo0B then transfers the phosphate group from Spo0F to Spo0A. Activated Spo0A-PO_4 can then bind to both RNA polymerase at a

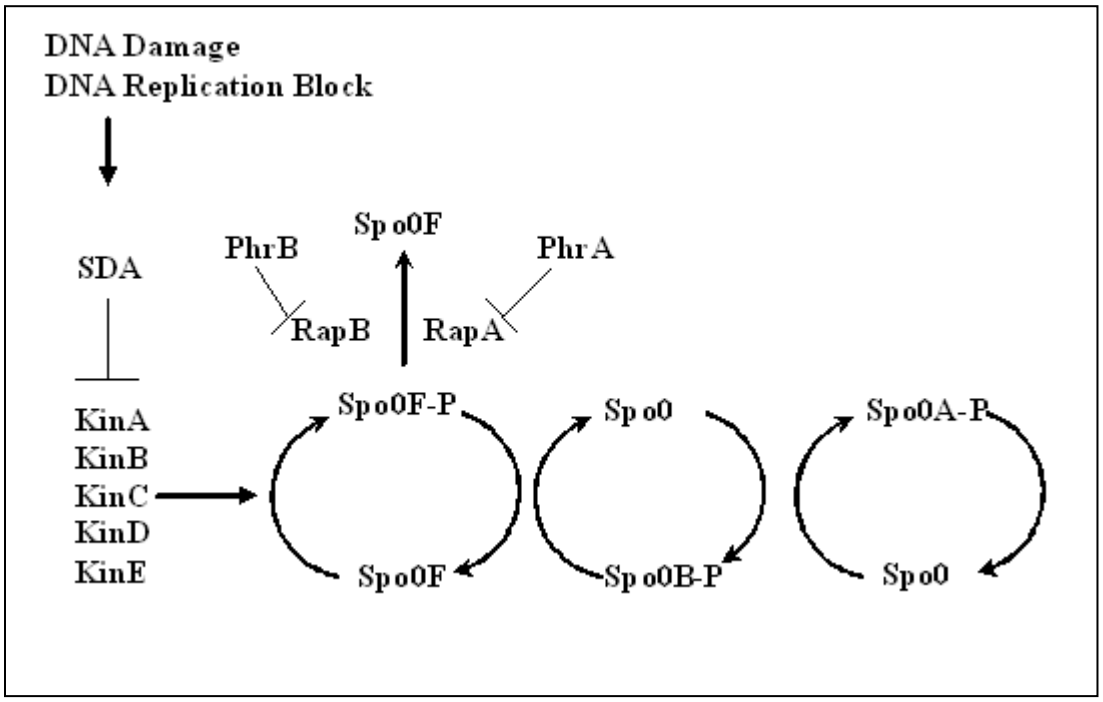


Figure 2. Phosophorelay activation of SpoOA. A representation of phosphorelay activation of spoOA which balances positive and negative signals in its decision to sporulate. Adapted from Piggot, 2000.

seven base sequence in or near σ^A (the main vegetative sigma factor) and σ^H (a transition-state sigma factor) dependent promoters (Perego and Hoch, 2002). The signals to which KinA and KinB respond have yet to be described. Countering the series of phosphorylations that lead to activation of Spo0A are several phosphatases: RapA and RapB specifically dephosphorylate Spo0F-PO_4; Spo0E, YisI and YnzD specifically dephosphorylate Spo0A-PO_4 (Perego, 2001). The balance of these reactions determine whether there is enough Spo0A-PO_4 to sufficiently activate the series of genes necessary for initiation of sporulation. The phosphorelay serves as a conduit for the internal and external signals that govern the cell's commitment to entry into sporulation. These mechanisms for controlling when the cell enters into sporulation are described below. High cell density is recognized by the cell as a signal for sporulation. The mechanism for high cell density signaling lies in the inhibition of Rap (response regulator aspartyl phosphatase) proteins RapA, RapB (Perego et al., 1994) and RapE (Jiang et al., 2000), which thus increases the intracellular concentration of Spo0A-PO_4. RapA and RapE are cotranscribed with signaling peptide precursors PhrA and PhrE, which are processed and secreted as 19-residue peptides. When cell density is high they are processed to pentapeptides and reimported by an oligopeptide permease (Opp) where the peptide from PhrA inhibits RapA, from PhrE inhibits RapE; and from PhrC inhibits RapB (Perego, 1997).

Nutrient starvation is required along with high cell density for the onset of sporulation. The starvation signal is thought to be mediated by depletion of GTP and GDP, which always correlates with the onset sporulation. Sporulation can be induced by decoyinine treatment, which inhibits the synthesis of GMP (Mitani et al., 1977). GTP

levels influence the ability of CodY (a repressor of *phrA*. *phrE* and *kinB*), to bind DNA. Thus, decreased levels of GTP inhibit CodY's ability to repress these positive regulators of the phosphorelay (Molle et al., 2003). Additionally, the onset of sporulation is dependent on a functioning Krebs cycle (Ireton et al., 1995), however the molecular basis of this pathway is not yet understood.

The decision to sporulate takes into consideration intracellular signals as well as external signals. Autophosphorylation of KinA is impaired by Sda (suppressor of DnaA), which is expressed in response to DNA damage or blockage of DNA replication (Ireton and Grossman, 1994). Therefore the cell is able to evaluate the integrity of its DNA prior to entry into sporulation. Recently, a DNA binding protein DisA (DNA integrity scanning protein) has been identified as being required to delay the start of sporulation in response to damaged DNA. It has been proposed that DisA scans the chromosome in search of lesions (Bejerano-Sagie et. al., 2006).

The phosphorelay also integrates signals that monitor for the presence of glucose in growth medium (Hoch et al., 1985). Additionally, mutations in several TCA cycle genes have been demonstrated to block Spo0A activation (Craig et al., 1997, Ireton et al., 1995). The redundancies in both positive and negative regulators of the sporulation phosphorelay ensure that the irreversible decision to sporulate is only undertaken when beneficial. When enough Spo0A-PO_4 has accumulated, sporulation is initiated and the expression of over 500 genes is altered, representing approximately one eighth of the entire genome (Fawcett and Youngman, 2000).

Spo0A Regulon

At least 121 genes have been identified as being under the direct control of SpoOA. Two thirds of these genes are negatively regulated, and 25 are transcription factors themselves (Molle et al., 2003). Spo0A blocks expression of the *abrB* gene, which encodes the "transition state" protein AbrB, which is itself a repressor protein (Perego et al., 1988). Depletion of AbrB results in the activation of genes under its negative control (Strauch and Hoch, 1993). AbrB represses expression of *spo0H*, which encodes σ^H. Since transcription of Spo0A is mediated by σ^H, repression of the *abrB* gene thereby enhances transcription of *spo0A,* setting up a self-reinforcing cycle (Strauch and Hoch, 1993). Among the most important targets for positive regulation by Spo0A-PO_4 are *spoIIE* and the *spoIIA* and *spoIIG* operons, which encode cell specific sigma factors σ^E and σ^F, and other proteins involved in their activation following the asymmetric division (Piggot and Losick. 2002). Another example of Spo0A directed positive regulation is the transcription of *racA*, which encodes for an effecter of axial filament formation (Ben-Yehuda et al., 2003, Wu and Errington, 2003). In sum, activation of Spo0A leads to global changes in gene expression, which allows for the expression of all genes required for chromosome partitioning, asymmetric division and the separation into two distinct cells with different genetic programs.

Transition-State Sigma Factor σ^H

σ^H is the first of five alternative sigma factors to become active during the process of sporulation. Each of these sigma factors is subject to spatial and temporal control, and together they direct genetic expression during sporulation (Errington, 1996). σ^H directs

the transcription of several genes, which transition the cell from exponential growth to stationary phase, including the development of genetic competence and initiation of sporulation (Siranosian and Grossman, 1994). σ^H directly activates the expression of genes essential for early sporulation including *spo0A, spo0F, kinA*, and several members of the *phr* family of genes, (Predich et al., 1992). Some of the genes containing promoter sequences for σ^H have additional promoter sequences that are recognized by other sigma factors. The genes for *spo0A, ftsA* (cell division), *dnaG* (DNA replication), and *sigA* (σ^A) all contain promoter sequence for both σ^H and σ^A (Britton and Grossman, 2002). A combination of DNA microarray analysis and bioinformatics has identified many new genes under the direct control of σ^H which help in adapting the cell to conditions of nutrient depletion (Britton and Grossman, 2002). In *Bacillus anthracis* σ^H is required for toxin gene expression (Hadjifrangiskou et al., 2006).

Sporulation (Spo$^-$) Mutants and *spo* Loci

Sporulation is not an essential part of the *B. subtilis* life cycle, which allows for the generation of mutants that grow perfectly well under vegetative conditions but are unable to sporulate. These Spo$^-$ mutants continue to be extensively studied in *B. subtilis*. Colonies of Spo$^-$ strains are easily discernable by their relative lack of pigmentation, and can be positively identified by phase-contrast microscopy through their absence of phase bright spores (Piggot and Losick, 2002). Spo$^-$ mutants can also be identified by their sensitivity to extreme heat, (spores can typically survive 20 minutes at 80C, a condition which is lethal to Spo cells). The stage at which a Spo$^-$ mutant is blocked is determinable histologically, and mutants are designated accordingly. SpoII mutants are blocked at

stage II and cannot proceed beyond asymmetric septation. SpoIII mutants are blocked at stage III and do not proceed beyond engulfment, and so on. Spo mutations map by definition to *spo* loci and over 40 *spo* loci have been identified (Piggot and Losick, 2002). Thus far no *spoI* mutants have been identified, which has made the study of stage I axial filament formation difficult. However alternatives to classical genetic techniques have provided some insights into the mechanism of axial filament formation and will be discussed below.

Axial Filament Formation

The *B. subtilis* chromosome is a circular molecule with a single origin of replication (*oriC*). The nucleoid structure was originally thought to be diffuse and disorganized based on electron and fluorescence microscopy, however recent studies have shown it to be highly organized and compact. The *oriC* region is located $0^{o}/360^{o}$ on the chromosome and medially within the cell.

Following initiation of sporulation, the sister chromosomes are remodeled into a single elongated DNA mass, which extends from pole to pole (Ryter et al., 1966). The replication of origin locates to the extreme ends of the cell. During Stage I, the cell division machinery is also relocated to cell poles (Levin and Losick. 1996). The process of axial filament formation is poorly understood however it believed that SMC (structural maintenance of chromosomes) proteins are required. Interestingly, SMC mutants are able to activate spo0A but are unable to form axial filaments nor proceed to asymmetric division (Grumman and Losick, 2001). Additional work in this field has been dedicated to explaining the anucleate prespore phenotype identified in the *divIVA* (a functional

homology of the *E. coli* MinE protein) and *racA* (remodeling and anchoring of the chromosome) mutants (Thomaides et al., 2004, Ben-Yahuda et al., 2003).

It will be interesting to see what if any epigenetic controls are involved at this stage. Epigenetic controls are not restricted to eukaryotes and have been identified in *B. subtilis* (Grandjean et al., 1998). It is tempting to speculate that a very early example of the epigenetic control seen in differentiating eukaryotic cells has some of its foundations in axial filament formation. Axial filament formation is immediately followed by the asymmetric sporulation division and will be discussed next.

Asymmetric Division

One of the distinctive morphological features of vegetatively growing *B. subtilis* is the medial assembly of the divisional septum with respect to the cell poles. This lies in contrast to the sporulation septum, which is asymmetrically located. The mechanism that ensures the correct placement of the division septum, is only partly understood. As in *E. coli*, formation of the division septum is catalyzed by a number of proteins that assemble into a ring structure at the future division site. Assembly of these proteins into a ring (Z ring) occurs in a hierarchical order that is initiated by the FtsZ protein, a structural and functional analog of tubulins found in virtually all prokaryotes (Löwe and Amos, 1998). FtsZ is the major cytoskeletal component of the bacterial cell division machinery. Two mechanisms have been implicated in the proper placement of the Z ring at mid cell: nucleoid occlusion and the inhibition of polar Ftsz assembly by min system components MinCD and DivIVA (Errington et al., 2003). However during sporulation, two Z rings are deployed, one near each pole of the cell (Levin and Losick, 1996). Only one of these

rings will be utilized however, resulting in two unequally sized cells each with differing fates. Initially a single Z ring forms at mid-cell as in exponentially growing cells, however FtsZ then relocates to the cell poles via a dynamic helical structure (Ben-Yehuda and Losick, 2002), which resembles the helical structure formed by bacterial proteins Mbl and MreB (Jones et al., 2001).

Expression of the *ftsAZ* operon is governed by three promoters, one being recognized by σ^H (Gholamhoseinian et al., 1992). However, asymmetric septation is only moderately impaired by removal of the σ^H promoter (Gonzy-Tréboul et al., 1992). A similar effect has also been demonstrated in *spoIIE* mutants (*spoIIE* encodes an activator of σ^F in the prespore), which are only moderately deficient in polar Z ring formation (Khvorova et al., 1998) and asymmetric division (Piggot and Coote, 1976; Barák and Youngman**,** 1996). The combined effect of a *SpoIIE* mutation and inactivation of the σ^H promoter for the *ftsAZ* operon, is a severe disruption of polar Z ring formation and asymmetric division. Conversely, when both *spoIIE* and *ftsAZ* are over expressed in exponentially growing cells, asymmetric division occurs (Ben-Yehuda and Losick, 2002). Taken together, these results indicate overlapping roles for *spoIIE* and *ftsAZ* in polar Z ring formation and asymmetric division. It has been also shown that FtsZ and SpoIIE interact (Lucet et al., 2000), and that SpoIIE localization to the asymmetric division sites depends on this interaction (Levin et al., 1997). However, the mechanism by which these proteins assist in polar Z ring formation and asymmetric division remains undetermined. The mechanism for choosing which of the two polar Z rings will become the sporulation septum is also unknown. FtsA is a potential candidate for playing a role in determining which of the two Z rings are chosen for two reasons: 1) it is the first protein known to be

recruited by the polar Z ring sites; and 2) it localizes to one divisional site and not the other (Feucht et al., 2001). FtsZ interacts with FtsA, as well as negative regulator EzrA (Levin et al., 1999) and positive regulator ZapA (Gueiros-Filho and Losick, 2002). Although it is clear that Z-ring formation is subject to tight spatial and temporal regulation, the mechanism of Z-ring formation and its regulation remain unresolved in both the vegetative and sporulation division in *B. subtilis*.

Chromosome Segregation

In vegetatively growing cells, bi-directional replication begins at the *oriC* region and the two chromosomes are pulled towards the cell quarters from this site. The remaining region between the *oriC* region and the terminus are positioned near midcell. Spo0J is a ParB family member, which binds proximal to the *oriC* region at parS nucleation sites (Murray and Errington, 2006). Spo0J interacts with Soj, the *B. subtilis* ParA homolog which is also a negative regulator of transcription. Although Spo0J/Soj are implicated in segregation of the chromosomes, a yet to be elucidated mechanism(s) must also exist since mutations in Spo0J or Soj do not completely abolish the cells ability to separate the chromosomes (Ireton and Grossman, 1994, Autret and Errington, 2001). Recently, it has been reported that the subcell positioning of the 0^{o} region during replication is independent on the *oriC* site (Berkmen and Grossman, 2006).

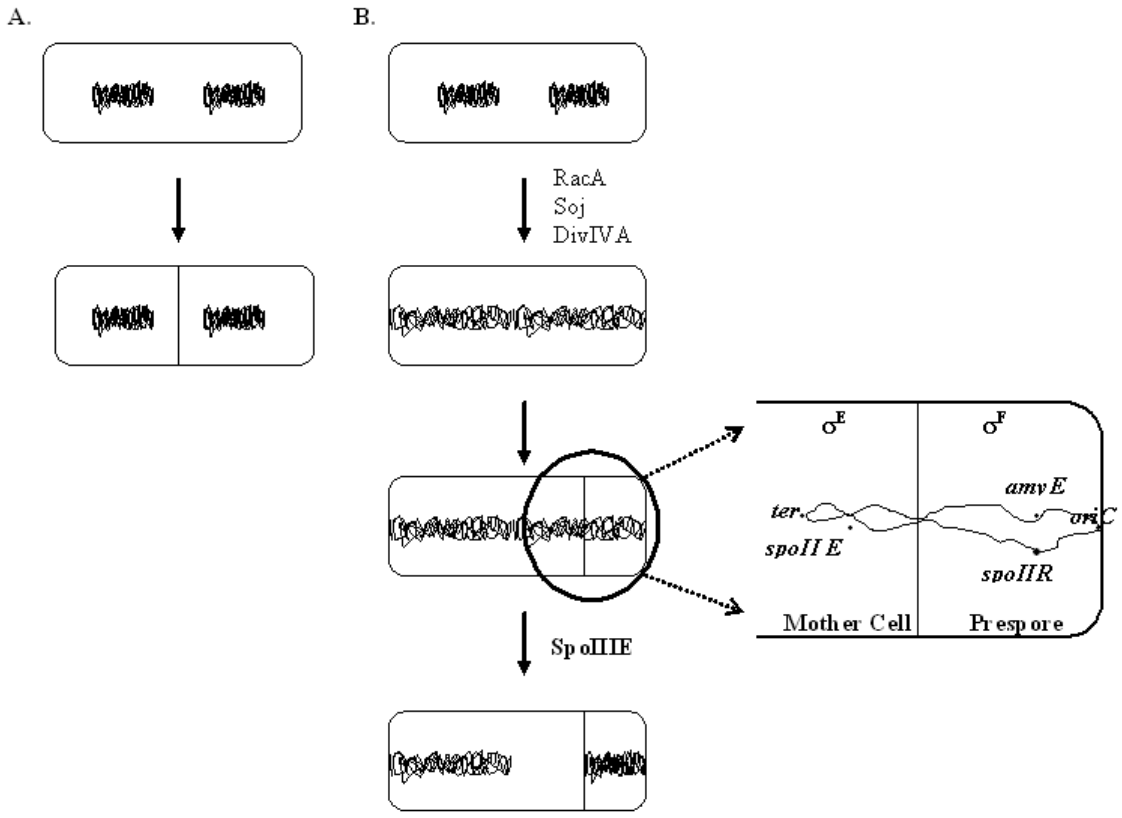


Figure 3. *B. subtilis* division. A vegetatively growing *B. subtilis* divide medially. B. Sporulating *B. subtilis* divide asymmetrically. The sporulation septum thereby traps one third of the chromosome in the prespore. The remaining two thirds of the chromosome is then pumped into the prespore through the DNA translocase activity of SpoIIIE. The terminus region of the chromosome is labeled *ter* and the origin of replication is labeled *O*. The *spoIIR* and *amyE* loci are shown in the prespore near the origin of replication, accessible to expression by σ^F.

During sporulation, chromosome segregation proceeds directly after the formation of the asymmetric septum by what seems to be an entirely different mechanism. One dramatic difference between the two mechanisms is the completion of the sporulation septum prior to the translocation of the chromosome (Fig. 3). The sporulation septum thereby traps one third of the chromosome in the prespore (Wu and Errington, 1998). The remaining two thirds of the chromosome is then pumped into the prespore through the DNA translocase activity of SpoIIIE. Evidence to support a role for SpoIIIE in chromosome transfer follows: 1) *spoIII* mutants are unable to transfer the chromosome from the mother cell to the prespore (Wu and Errington, 1998); 2) SpoIIIE localizes to the center of the sporulation septum (Wu and Errington, 1997); and 3) SpoIIIE is a DNA translocase, which uses ATP to transport the chromosome into the prespore (Bath et al., 2000). SpoIIIE is a multifunctional protein that is also required for compartmentalization of cell specific sigma factor activity and for membrane fusion after engulfment (Liu et el., 2006). A more in depth discussion of SpoIIIE and its activities will follow later in this introduction.

Compartmentalization of Gene Expression

The completion of septum formation heralds the beginning of two different programs of gene expression in the prespore and the mother cell. This compartmentalized gene expression results in the activation of σ^F in the prespore followed by σ^E in the mother cell. RNA polymerase sigma factors direct the transcription of genes via recognition of specific promoter sequences. Therefore activation of σ^F will result in the transcription of different genes in the prespore than those transcribed via σ^E

in the mother cell (Errington, 1993, Losick and Straiger 1992). Furthermore, σ^F will remain inactive in the mother cell, and σ^E will remain inactive in the prespore. A central question in our understanding of compartmentalization is whether σ^F is selectively activated in the prespore, blocked from activation in the mother cell, or both. Several models have been posed over the years to explain the mechanism of compartmentalized gene activity and will be discussed following a summary of the evidence that gene expression is compartmentalized.

Evidence for Compartmentalized Gene Expression

Evidence for differences in the protein content of the mother cell versus the prespore came from biochemical comparison of extracts enriched for the contents of either the mother cell or the prespore (Andreoli et al., 1973; Eaton and Ellar, 1974; Fujita et al., 1977; Singh et al., 1977). This strongly supported the idea that spore formation gave rise to two distinct genetic programs. The first evidence for compartmentalized gene expression of *spo* loci came through the study of bacterial mosaics (de Lencastre and Piggot, 1979). Transformation of *B. subtilis* was carried out such that only one of the two chromosomes was *spo*$^+$. Since the chromosomes are divided randomly in the sporulating cell and the mother cell dies, it is possible to infer whether a *spo* locus was expressed in the mother cell or prespore. Several mutants gave rise to a mixed spo progeny (Spo$^-$ and Spo$^+$ transformants) which indicates that the mother cell required a spo$^+$ allele for normal sporulation, and other mutants gave rise only to spo$^+$ transformant, indicating that the allele in the prespore needed to be spo$^+$ (de Lencastre and Piggot, 1979; Piggot, 1978; Piggot and de Lencastre 1978). Eventually it became possible to

visualize compartmentalization histologically using immunoelectron microscopy in the form of prespore localization of small acid-soluble proteins (SASP's) (Francesconi et al., 1988). However, immunofluorescence techniques followed by fluorescence microscopy of cells expressing GFP (green fluorescent protein) fusions proved to be extremely robust technologies for the study of compartmentalization of gene expression. These methods demonstrated that in normal sporulating cells the activities of σ^F and σ^G are restricted to the prespore, that the activities of σ^E and σ^K are restricted to the mother cell and furthermore, that σ^F and σ^E are active following the completion of septation, and σ^G and σ^K do not become active until the completion of engulfment (Harry et al., 1995; Lewis and Errington, 1996; Pogliano and Losick, 1995; Webb et al., 1995; Zheng et al., 1996). Evidence for the completeness of temporal and spatial compartmentalization of gene expression was recently provided by the use of a novel two part transcription probe. This technique confirmed the spatial compartmentalization of sigma factors, and importantly, this technique made it possible for the first time to show that there is also sharp temporal compartmentalization, with little or no overlap in the activities of σ^F and σ^G or of σ^E and σ^K, at least with respect to the promoters tested (Li and Piggot. 2001).

Activation of σ^F

spoIIAC encodes RNA polymerase sigma factor σ^F and is part of the tricistronic operon *spoIIA* (Fort and Piggot1984). *spoIIA* is transcribed prior to asymmetric septation (Gholamhoseinian and Piggot, 1989) in a Spo0A and σ^H dependent manner (Trach et al., 1991; Wu et al., 1989; Wu et al., 1991). However, σ^F does not become active until after septation is complete (Gholamhoseinian and Piggot,1989; Karmazyn-Campelli et al.,

1989), indicating posttranslational regulation. The other two products of the *spoIIA* operon (SpoIIAA and SpoIIAB) were shown by genetic analysis to regulate the activity of σ^F as follows; over-expression of SpoIIAB inhibited activity of σ^F; and *spoIIAB* mutants displayed increased activity of σ^F (Schmidt et al., 1990). Conversely, a mutation in *spoIIAA* abolished σ^F activity, but activity of σ^F could be restored by mutating *spoIIAB*. Taken together, these results suggested that σ^F activity is negatively regulated by SpoIIAB, which is in turn negatively regulated by SpoIIAA (Schmidt, 1990). Deletion of *spoIIAB* resulted in hyperactivity of σ^F, a blockage of sporulation prior to asymmetric division and extensive lysis (Coppolecchia et al., 1991). A mechanism for the activation of σ^F was determined by biochemical analysis; SpoIIAB was shown to bind directly to σ^F and SpoIIAA was shown to inhibit the binding of SpoIIAB (Duncan et al., 1993; Min et al., 1993). The phosphorylation state of SpoIIAA ultimately determines the activity of σ^F. Based on its similarity to other protein kinases, SpoIIAB was found to phosphorylate SpoIIAA on a serine residue at position 58 (Min et al. 1993, Najafi et al., 1995). Conversion of this serine residue to an alanine (mimicking dephosphorylation) resulted in constitutive σ^F activity, and conversion of this serine to an aspartic acid (mimicking phosphorylation) repressed σ^F activity (Diederich et al., 1994). In sum, σ^F is held inactive by a dimer of SpoIIAB unless SpoIIAA becomes dephosphorylated and sequesters SpoIIAB, thus freeing σ^F from SpoIIAB mediated inactivation. Recently, the crystal structures of both the pre-phosphorylation complex and the inhibitory complex, SpoIIAB(ATP) and SpoIIAB(ADP) bound to SpoIIAA, respectively were reported (Masuda et al., 2004), which provided strong support to the prevailing model of SpoIIAA function as an anti-anti-sigma in releasing σ^F from SpoIIAB. Although it is generally

believed that phosphorylation of SpoIIAA is a consequence of the demonstrated protein kinase activity of SpoIIAB, it does not explain compartmentalization of σ^F activity to the prespore. One could speculate the existence of a SpoIIAA specific phosphatase that is only active in the prespore to explain compartmentalization of σ^F activity. This turned out to be partially true: identification of SpoIIE as a membrane bound serine phosphatase which dephosphorylates SpoIIAA at a serine residue at position 58 provided clues but no solution (Duncan et al., 1995, Arigoni et al., 1996). Although SpoIIE was shown to localize to the asymmetric division sites (Arigoni et al., 1995, Barák et al., 1996), this in itself is not enough to completely explain compartmentalization, which will be discussed in the following section. A model for activation of σ^F is presented in figure 4.

Compartmentalization of σ^F Activity

Attempts to explain compartmentalization of σ^F activity have resulted in more hand waving than the average swing dance contest. However, out of this fertile compost heap of dead and decaying hypotheses will certainly emerge an accurate model. This section will briefly review models of compartmentalization, more or less in the order in which they were proposed. Some of the models presented here are now defunct, however these dead ends are worth mentioning in that they eliminate plausible mechanisms from further consideration.

ATP/ADP ratio

Early attempts at explaining activation of σ^F attempted to link signals for nutrient deprivation with SpoIIAA. It was hypothesized that SpoIIAA could function as a sensor

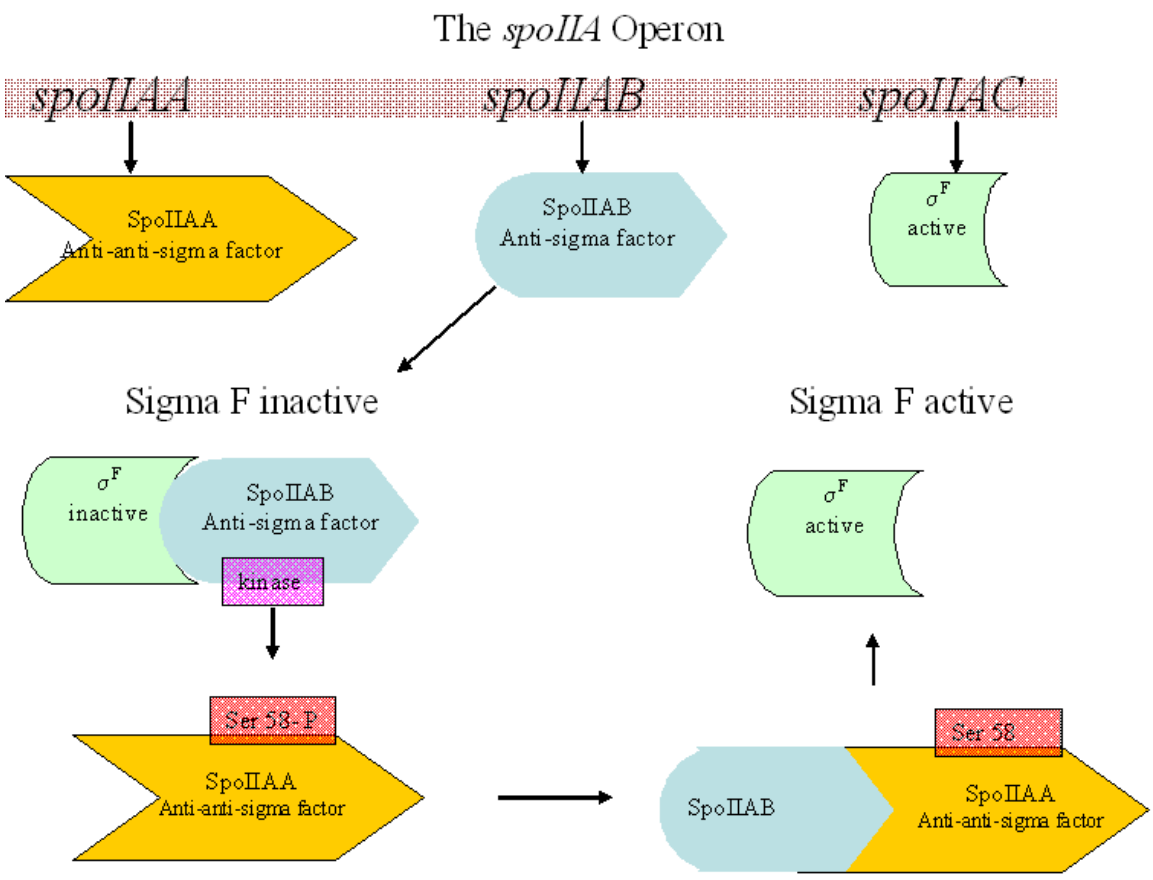


Figure 4. The *spoIIA* operon. The *spoIIA* operon is comprised of three genes: *spoIIAA, spoIIA and spoIIAC*. *spoIIAC* encodes sigma factor F. SpoIIAA is phosphorylated by protein kinase SpoIIAB on a serine residue at position 58 (Ser58-P). SpoIIE is a serine phosphatase which dephosphorylates SpoIIAA at serine residue at position 58 (Ser58). SpoIIAB binds σ^F, holding it inactive. Dephosophorylated SpoIIAA binds SpoIIAB, freeing σ^F from SpoIIAB, and thus activating σ^F.

that blocks σ^F repression by SpoIIAB in response to certain nutritional signals (Rather et al., 1990). Binding of SpoIIAB was shown to be influenced by concentrations of ATP and ADP whereby SpoIIAB bound σ^F in the presence of ATP, and SpoIIAA in the presence of ADP (Alper et al., 1994, Diederich et al., 1994, Duncan et al., 1996). It was hypothesized that a difference in the ATP/ADP ratio between the two compartments could account for compartmentalization. However, further in vitro studies eventually showed that the ATP/ADP ratio was not responsible for regulation of whether SpoIIAB preferentially binds σ^F or SpoIIAA (Magnin et al., 1997). Furthermore, neither a difference in the ATP/ADP ratio between the two compartments, nor a regulatory role for these nucleotides has been demonstrated.

SpoIIE Localization

If SpoIIE molecules were preferentially inherited by the prespore, it could provide a convenient explanation for compartmentalization. Early histological analysis of SpoIIE-GFP seemed to support this line of research (Wu et al., 1998), however further studies focusing on more precise quantification indicated that the total fluorescence in the prespore was not elevated relative to that in the mother cell. Furthermore, SpoIIE-GFP was shown to be present in both the mother cell and the prespore when first translated (King et al., 1999). Taken together these results disproved the preferential inheritance model for SpoIIE. However, the localization of SpoIIE to the asymmetric septum is still considered an important factor in compartmentalization. Removal of the transmembrane domain of SpoIIE reduces sporulation by 50% (Arigoni et al., 1999), as compared with the 10^7 reduction in sporulation seen when inactivating *spoIIE* (Piggot and Coote, 1976).

It was therefore proposed that spoIIE activity was regulated by a cytoplasmic inhibitor, which might preferentially be inherited to the mother cell. However, evidence to support the existence of such a SpoIIE inhibitor is still lacking.

Transient Genetic Asymmetry

A new model predicting differential expression of the regulatory proteins governing compartmentalization was based on the observation that the sporulation septum traps only one third of the chromosome destined for the spore into the prespore (Wu and Errington, 1995). During the 15 minutes it takes to translocate the remaining two thirds of this chromosome into the prespore, the mother cell and prespore have different sets of genes present. If regulatory genes are expressed in one compartment and not the other, could this transient genetic asymmetry explain compartmentalization? To test this theory, the efficiency of sporulation was measured in strains engineered such that the gene for σ^F (*spoIIAC*) was moved from its original position to various chromosomal loci in a Δ*spoIIAA* and Δ*spoIIE* background (Frandsen et al., 1999). When *spoIIAC* was left in its wild-type position, which is proximal to *spoIIAB* and near the terminus, the expected severe decrease in sporulation efficiency was observed because of the Δ*spoIIAA* background. However, in strains where *spoIIAC* was moved near the origin (which is trapped in the prespore during polar septation), a modest level of sporulation was restored. Although the authors acknowledge that this is an artificial system, it does demonstrate that if a gene for an inhibitor of σ^F existed near the terminus, it could possibly be expressed only in the mother cell for a period of time.

Another series of experiments asked what would be the effect on σ^F activation of moving the *spoIIAB* gene (Dworkin and Losick, 2001). Similar to the previous set of experiments, strains carrying the gene for *spoIIAB* at different chromosomal positions were constructed. Not surprisingly, this had little effect on sporulation or σ^F activity. As discussed previously, strains expressing a mutant form of SpoIIE, which fails to localize to the polar septum, only exhibits a modest decrease in efficiency of sporulation. However, the effect of this mutation was dramatic when the position of the gene for *spoIIAB* was altered. When *spoIIAB* was moved to positions near the origin and sporulation was significantly reduced in the strain expressing the mutant form of SpoIIE which failed to localize to the polar septum (Dworkin and Losick, 2001).

Free SpoIIAB has a half-life of approximately 25 minutes (Pan et al., 2001), which is short compared to that of SpoIIAA and σ^F. Furthermore, a strain carrying a stable mutant form of SpoIIAB was constructed and was shown to exhibit a reduction in sporulation and σ^F activation (Pan et al., 2001). Therefore, SpoIIAB instability might contribute to the effects of transient genetic asymmetry by favoring an increase in the SpoIIAA/SpoIIAB ratio in the prespore. As mentioned earlier, SpoIIIE is a DNA translocase, which transports the remaining two thirds of the chromosome into the prespore during stage II of sporulation. The *spoIIIE36* mutant contains a mutation in the DNA translocase; this mutation prevents the remaining two thirds of the chromosome from being pumped into the prespore, and increases expression of some σ^F-dependent genes (Wu and Errington, 1998, Sun et al., 1991, Arigoni et al., 1999, Schuch and Piggot, 1994). This mutant provides a phenotype that can be described as “permanent genetic asymmetry”. Since SpoIIAB protein is unstable and its gene is stuck in the mother cell in

the *spoIIIE36* mutant, it can be inferred that the increase in the activation of some σ^F-dependent genes can explained by the absence of SpoIIAB in the prespore. This hypothesis was tested by moving the gene for *spoIIAB* from its wild type position near the terminus to an ori-proximal position in a *spoIIIE36* background. The result was a strain where the increase in activation of σ^F-dependent genes was abolished (Dworkn and Losick, 2001). Although no single solution to the question of compartmentalization has been provided by the transient genetic symmetry line of research, enthusiasts of this model conclude that compartmentalization is the result of at least two partially redundant mechanisms which together result in the activation of σ^F in the prespore. These are: 1) localization of SpoIIE to the site of the sporulation septum results in an increased concentration of dephosphorylated SpoIIAA in the prespore (through a yet to be determined mechanism); and 2) the genetic asymmetry of *spoIIAB* combined with its product's instability results in a decrease of its concentration in the prespore. However it is still possible that genetic asymmetry of a yet to be described inhibitor of the activation of σ^F will be identified.

So far, in-silica analysis of Spo0A regulon (Fujita et al., 2005) has not provided evidence to support to the existence of an inhibitor of the activation of σ^F. Iber has recently argued against both transient genetic asymmetry and the existence of a SpoIIE inhibitor (Iber, 2006). The author employed a computational model to demonstrate that the duration of the transient genetic asymmetry was too short for the degradation of SpoIIAB to become responsible for the release of σ^F. Furthermore, the existence of an unidentified SpoIIE inhibitor was held to be inconsistent with current experimental data.

It is not clear to what extent the analysis provided by this computational model has removed genetic asymmetry from consideration. An in-depth discussion of a model based on a combination of computational analysis and experimental methods will follow shortly.

SpoIIAB Sink

As previously described, Spo0A induction of the *spoIIAC* operon results in the following two components; σ^F which is held inactive by a dimer of anti-sigma factor SpoIIAB, and SpoIIAA which is held inactive by SpoIIAB mediated phosphorylation. The conventional dogma holds that σ^F activity is the result of the following cascade: 1) SpoIIE dephosphorylates SpoIIAA, and 2) dephosphorylated SpoIIAA binds SpoIIAB removing σ^F from SpoIIAB (Fig. 2). However, this model gives rise to some remarkable inconsistencies. For example, a conditional mutant has been obtained where successful dephosporylation of SpoIIAA occurs yet σ^F remains inactive (King et al., 1999). This study found that in a strain carrying a mutant form of the late acting and essential division protein DivIC, SpoIIAA becomes dephosphorylated while σ^F remains inactive. Likewise, the *spoIIE48* mutant expresses a mutant form of SpoIIE, which allows for substantial dephosphorylation of SpoIIAA while σ^F remains inactive (Feucht et al., 2002). Biochemical analysis of the ratio of SpoIIAA to SpoIIAA-P in cells lacking its cognate kinase SpoIIAB found a small amount of SpoIIAA-P present nevertheless. Furthermore, in cells, which lacked both SpoIIAB and SpoIIE, almost all the SpoIIAA was phosphorylated (Carniol et al., 2002). These paradoxical results gave rise to a new model to explain how dephosphorylation of SpoIIAA frees σ^F from SpoIIAB. It was proposed

that SpoIIAB held SpoIIAA in a long-lived complex that holds dephosphoylated SpoIIAA inactive until a threshold concentration is reached which triggers its release. Thus, accumulation of the SpoIIAB-SpoIIAA complex serves as a sink that holds dephosphorylated SpoIIAA inactive until an excess of SpoIIAA overwhelms the sink's ability to restrain SpoIIAA activation of σ^F (Carniol et al., 2004). A previous line of biochemical research had also focused on the SpoIIAA-SpoIIAB-ADP interaction. The authors found that SpoIIAB-ADP sequesters SpoIIAA in an inactive form, and that this intermediary is long lived (Lee et al., 2000). Site-directed mutagenesis of SpoIIAA then provided two SpoIIAA mutants which could not activate σ^F. One of these mutants, SpoIIAAL90A was also unable to form a SpoIIAA-SpoIIAB-ADP complex (Lee et al., 2001). The authors concluded that it was the inability of SpoIIAAL90A to form a ternary complex with dephosphorylated SpoIIAA that was responsible for the block in σ^F activity. However, since this refers to the step where SpoIIAA binding of SpoIIAB releases σ^F, it does not necessarily conflict with the SpoIIAB sink model. In one case the SpoIIAA-SpoIIAB-ADP complex forms a pool of stored dephosphorylated SpoIIAA, in the other free SpoIIAB-ADP is unable to bind dephosphorylated SpoIIAA in order to free it from σ^F. In the context of the SpoIIAB sink model, if SpoIIAB could not bind SpoIIAA, the sink would not matter since SpoIIAB could not be freed from σ^F by SpoIIAA anyway.

Are these elegant models simply attempts to avoid the alternative interpretation that SpoIIAA does not directly release σ^F from SpoIIAB? Alternatively, these results could suggest a yet to be identified mechanism connecting SpoIIE, SpoIIAA and σ^F. For example there could be an unknown protein that facilitates a presently unidentified step

following dephosphorylation of SpoIIAA. Alternatively, SpoIIAA phosphorylation might be catalyzed by a yet to be identified kinase. An entirely new paradigm might arise out of further investigation of SpoIIIE's role in compartmentalization. It seems possible that new players could still be identified and revised roles might be assigned to presently known players. Such alternative mechanisms for the compartmentalized activation of σ^F are no longer necessary according to Yudkin and Clarkson, (2005), who think that they have solved the question. The next section will be an overview of the most recent, and perhaps the most convincing model for compartmentalization to date.

Mathematical Model

Using a combination of mathematical and experimental techniques Yudkin and Clarkson, (2005), devised a model that points to the volume difference in the two compartments as the determining factor of cell fate. Furthermore, they found that the localization of SpoIIE to the site of the asymmetric septum is sufficient for prespore specific activation of σ^F. The authors based their computational analysis on results from many key biochemical studies of SpoIIAA, SpoIIAB, SpoIIE and σ^F. What follows is a review of these studies, which will facilitate the subsequent discussion of this computational model.

Dephosphorylated SpoIIAA is necessary for the release σ^F from the inhibitory complex. The evidence for the mechanism by which SpoIIAA interacts with this complex will be considered first. Early biochemical studies of the direct interaction of SpoIIAA with σ^F-SpoIIAB-ATP demonstrated that the addition of SpoIIAA could release σ^F from an affinity matrix to which σ^F-SpoIIAB-ATP was bound (Duncan et al., 1996).

The same study also demonstrated that *in vitro* transcription by σ^F could be restored by adding SpoIIAA. These results suggest that direct interaction between SpoIIAA and the σ^F-SpoIIAB-ATP complex somehow releases σ^F. The question remained, how does this occur? Further dissection of this critical mechanism provided clues. X-ray crystallography of the σ^F-SpoIIAB complex strongly suggested that σ^F lies asymmetrically across mostly one molecule of the SpoIIAB dimer (Campbell et al., 2002). This would leave the other molecule of SpoIIAB open for "docking" by SpoIIAA, and the authors proposed that SpoIIAA could then release σ^F in a zipper like effect. Using these crystallography data, SpoIIAB was mutated at residue (Arg20), which was shown to interact with σ^F in one subunit of SpoIIAB and not the other (Ho et al., 2003). The ability of this mutant form of SpoIIAB to bind σ^F was negligible, however the dissociation constant (K_d) for a heterodimer of mutant SpoIIAB and wild type SpoIIAB was only slightly higher than seen with a homodimer of wild type SpoIIAB. In further experiments, Ho found that SpoIIAA could release σ^F from a homodimer of wild type SpoIIAB, but could not release σ^F from a heterodimer of mutant SpoIIAB/wild type SpoIIAB (Ho et al., 2003). These results support the docking model wherein one molecule of SpoIIAB interacts with σ^F and the other interacts with SpoIIAA. It is also worth mentioning that X-ray crystallography of SpoIIAA complexed with SpoIIAB revealed a negatively charged protrusion which could possibly repel a similar patch on σ^F thus providing a means for SpoIIAA mediated displacement of the sigma factor (Masuda et al., 2004). This same study found no differences in the structures of wild type SpoIIAA versus a non-phosphorylatable form of SpoIIAA.

The interactions of σ^F, SpoIIAA and SpoIIAB are complex and the sequence of these events has been the subject of much interest. For example, in once case, σ^F – SpoIIAB-ATP binds SpoIIAA and the result is free σ^F and a SpoIIAA-SpoIIAB-ATP complex. Alternatively, SpoIIAB kinase activity can result in phosphorylation of SpoIIAA via the SpoIIAA-SpoIIAB-ATP complex, whose products are SpoIIAB-ADP and SpoIIAA-ATP. The exchange of SpoIIAB-ADP to SpoIIAB-ATP is an additional event whose kinetics are of obvious relevance. The question then arises whether the release of σ^F is linked to phosphorylation of SpoIIAA, or whether these two events are separate. Furthermore, if these two events are linked, is release of σ^F simultaneous with phosphorylation of SpoIIAA or does it follow or precede it? An early observation provides some answers; prematurely active σ^F is observed in cells that carry a mutant SpoIIAA-Ser58 protein that is unable to be phosphorylated (Diederich et al 1994). This indicates that the ability of SpoIIAA to free σ^F does not rely on its phosphorylation by SpoIIAB. However, this does not shed light as to the sequence of events. To address this question, Clarkson et al. (2004a) employed the use of fluorescence spectroscopy of intrinsic tryptophan probes that enable one to detect formations or disassociations of protein-protein complexes in real time. They found that phosphorylation of SpoIIAA occurs after σ^F is released from its complex with SpoIIAB. Since SpoIIE would then be expected to dephosphorylate SpoIIAA-ATP, this indicates the possibility of a cycle wherein SpoIIAA is repeatedly phosphorylated and dephosphorylated. Sporulating *B. subtilis* avoids the waste of ATP this predicts via the biphasic nature of SpoIIAB's phosphorylation of SpoIIAA. Measurement of the kinetics of phosphorylation of SpoIIAA by SpoIIAB revealed a moderate presteady-state rate followed by a much

slower steady state reaction (Magnin et al., 1997). The result would be SpoIIE out pacing SpoIIAB in their cycling of SpoIIAA. The observed biphasic rate was first accounted for by the slow release of ADP following the first round of phosphorylation (Najafi et al., 1996). According to this model, SpoIIAB is left in an inactive state following the first round of phosphorylation and must be restored to an active form via this slow step. It was suggested by Garsin et al., (1998) that SpoIIAB-ADP might interact with SpoIIAA in a complex that would prevent SpoIIAB-ADP from being restored to a form capable of interacting with σ^F. Indeed it was shown that SpoIIAB binding to σ^F was not increased by adding σ^F to a reaction mixture of SpoIIAA/SpoIIAB in its steady state (Lee et al., 2001). SpoIIAB was unable to bind σ^F as long as dephosphorylated SpoIIAA was present. These results strongly suggest that SpoIIAA-SpoIIAB-ADP complexes do extend σ^F's time of activity. Although it was originally believed that SpoIIAB was left in an alternative form that tightly binds ADP as the result of the reaction where SpoIIAA is phosphorylated, subsequent evidence suggested otherwise. The biphasic time course of SpoIIAB phosphorylation of SpoIIAA was eliminated when SpoIIAB was preincubated with SpoIIAA (Clarkeson et al., 2004b). This result indicates that the interaction of SpoIIAA with SpoIIAB induces a conformational change in SpoIIAB, which results in the slower steady state. A model to explain the mechanism for this conformational change and slower nucleotide binding was formulated based on the structure of SpoIIAB (Clarkson et al., 2004b), (Fig. 5a). SpoIIAB was found to contain the GHKL superfamily fold that contains a nucleotide binding pocket partially covered by a flexible "ATP lid". According to this model, SpoIIAB binding of SpoIIAA results in a closure of the ATP lid that would obstruct the release of ADP. This model is supported by the crystal structure

Fig. 5a

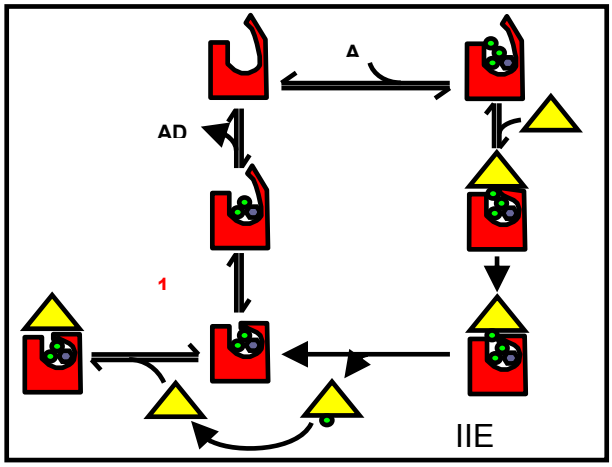


Fig. 5b

The σ^F regulatory interactions

$AB + ATP \rightarrow AB{\cdot}ATP$
$\sigma^F + AB{\cdot}ATP \rightarrow \sigma^F{\cdot}AB{\cdot}ATP$
$AA + \sigma^F{\cdot}AB{\cdot}ATP \rightarrow \sigma^F + AA{\cdot}AB^*{\cdot}ATP$
$AA{\cdot}AB^*{\cdot}ATP \rightarrow AA\text{-}P + AB^*{\cdot}ADP$
$AA\text{-}P + H_2O \rightarrow AA + P_i$
$AA + AB^*{\cdot}ADP \rightarrow AA{\cdot}AB^*{\cdot}ADP$
$AB{\cdot}ADP + ATP \rightarrow AB{\cdot}ATP + ADP$
$\sigma^F + \text{core RNAP} \rightarrow \sigma^F{\cdot}\text{holoenzyme}$

Fig. 5c

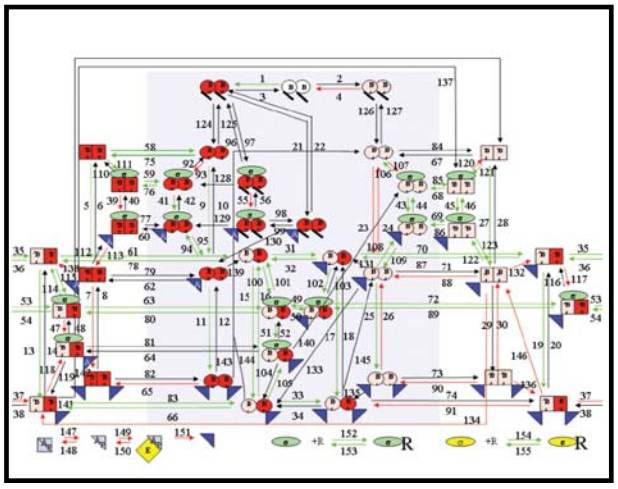


Fig. 5d

The model could successfully simulate results obtained *in vitro*:

Binding of AA to AB·ADP.
Binding of σ^F to AB·ATP.
Disruption of σ^F·AB·ATP complexes by AA.
Rebinding of σ^F to AB·ATP as AA is phosphorylated.
Response of this rebinding to IIE.
Time course of phosphorylation of AA.

Figure 5. Mathematical model for activation of σ^F. 5a: SpoIIAB binding of SpoIIAA results in a closure of the ATP lid that would obstruct the release of ADP. SpoIIAB (red boxes), when in its SpoIIAB-ATP form, interacts with SpoIIAA (yellow rectangles) and results in the closure of the ATP lid. 5b: The kinetic constants for all the initially considered σ^F regulatory interactions as well as the concentrations of the intermediates of these interactions were used in the mathematical model. SpoIIAB, AB; SpoIIAA, AA. 5c: A diagram depicting the revised version of the mathematical model which includes 155 interactions. This is too elaborate to describe in detail here, but rather is included to provide confidence in the Iber et al.'s (2006) attempt to be rigorous in their efforts to include all relevant interactions. 5d: A list of interactions the mathematical model could successfully simulate.

that demonstrates that SpoIIAA binds on top of the SpoIIAB ATP lid (Campbell et al., 2002). Further evidence was provided from fore mentioned fluorescence spectroscopy data that demonstrated large differences in the intensity of fluorescence of SpoIIAB-F97W upon binding with SpoIIAA. Since crystal data indicate that SpoIIAA does not bind near residue 97, it follows that the decrease in fluorescence represents a change in conformation (Clarkeson et al., 2004a). Before I discuss how this biochemically based data fit into the computational model, a review of relevant *spoIIAA* and *spoIIAB* mutants would also be helpful. The following section will be a review of these mutants followed by a description of the computational model. Four mutants have been identified in which the binding properties of SpoIIAB to SpoIIAA have been altered but which lead to varying and profoundly altered phenotypes. The first such mutant resulted from the replacement of Leu-90 with AA-90 in SpoIIAA (AA-L90), (Lee et al., 2001). The resulting phenotype is a Spo$^-$ strain that is unable to activate σ^F during sporulation. *In vitro* studies of purified AA-L90 showed a large decrease in its ability to bind SpoIIAB in the presence of ADP. Additionally, the time course of its phosphorylation reaction with SpoIIAA was linear instead of biphasic. The authors suggested a linkage between these two properties, which is crucial for the regulation of σ^F. This suggestion is supported by the following studies of three *spoIIAB* mutants.

Mutation of the *spoIIAB* gene led to isolation of two mutants that were defective in the activation of σ^F (Garsin et al., 1998a). One of them (AB-T49K) was defective in binding to SpoIIAA in the presence of ADP, the other (AB-104K) was defective in its ability to phosphorylate SpoIIAA. Premature and elevated activation of σ^F was observed in a SpoIIAB mutant (AB-R105A) which was constructed by replacement of an Arg

residue in the ATP lid (Arg-105) by Ala (Ho et al., 2003). Purified AB-R105A protein was found to be defective in kinase activity. Although the AB-104K and Arg-105 mutants stem from mutation of adjacent residues on the ATP lid, they have opposite phenotypes. Ho et al., (2003) suggested that the difference was the result of SpoIIAA's inability to release σ^F in the AB-104K mutant. Further characterization of all three mutants was undertaken to provide a more detailed analysis of their kinetics of phosphorylation and protein-protein binding properties.

In contrast to the original findings, Shu et al., (2004) found both AB-E104K and AB-T49K was also defective in binding to SpoIIAB in the presence of ADP. The linear rather than biphasic time course of phosphorylation of SpoIIAA was also similar for both mutants. However, these two mutants did vary in their binding properties to certain ligands. For AB-E104K, both the K_d for the SpoIIAA-SpoIIAB-E104K-ADP complex, and the K_m for SpoIIAA in the phosphorylation reaction were higher than in wild type. Interestingly, the K_d for the ADP or ATP dependent complex with σ^F was unchanged.

The defect in AB-T49K was in the interaction with ADP. In this mutant a complete absence of ADP-dependent binding of AB-T49K to SpoIIAA or to σ^F was observed, whereas the K_d for the SpoIIAA-SpoIIAB-E104K-ATP complex showed negligible difference from that for the wild type (Shu et al., 2004).

Opposite to what was seen in the first two mutants, AB-R105A formed stronger than normal complexes with SpoIIAA in the presence of ADP. The time course of phosphorylation of SpoIIAA also differed, and was strongly biphasic with a much faster pre-steady-state phase followed by a strikingly slow steady-state phase as compared to wild type. The authors concluded that AB-R105A has a highly elevated affinity for ADP

that would account for its unusual time course in phosphorylation of SpoIIAA. They also suggested that this affinity might result in stable complexes of SpoIIAA-SpoIIAB-ADP, which would prevent SpoIIAB from its anti-sigma factor role. Shu et al., (2004) concluded that taken together these results suggest that the K_d of the SpoIIAA-SpoIIAB-ADP complex was of critical importance to the correct regulation of σ^F. This interaction must be strong enough to keep SpoIIAB away from σ^F once released, but also weak enough to allow SpoIIAB to function in its role as anti-sigma factor.

A detailed reaction scheme for the regulation of σ^F was proposed which takes in consideration the results described above. It attempts to account for the release of σ^F from SpoIIAB-ATP and describes a cycling of SpoIIAA that allows for the maintenance of free σ^F (Yudkin and Clarkson, 2005). The model begins with the widely accepted mechanism of σ^F being held inactive by a dimer of SpoIIAB-ATP in vegetatively growing cells and in the mother cell. Early in sporulation, SpoIIAA is in its phosphorylated form, which can not interact with SpoIIAB, and SpoIIE is not present. SpoIIE is then made which hydrolyses SpoIIAA-P to SpoIIAA. SpoIIAA attacks the σ^F-SpoIIAB-ATP complex, releasing σ^F. This interaction of SpoIIAA and SpoIIAB-ATP results in the phosphorylation of SpoIIAA by SpoIIAB-ATP and the conversion of SpoIIAB-ATP to SpoIIAB-ADP. The authors of this model suggest that at this step, a conformational change occurs in SpoIIAB as a consequence of its interaction with SpoIIAA, and that this conformation change is a closing of the ATP lid when SpoIIAA binds. This conformation change has important consequences; firstly, this altered conformation of SpoIIAB binds ADP much more tightly than in its original form. Secondly, this new form of SpoIIAB-ADP is able to interact with SpoIIAA. Therefore

while phosphorylating SpoIIAA, SpoIIAB is converted to its new conformation whose accumulation greatly slows the phosphorylation of SpoIIAA. This prolonged phosphorylation of SpoIIAA serves to maintain activity of σ^F.

So far, this model has accounted for the activation of σ^F, but has not provided a basis for its compartmentalized activity. To address this question, the authors call into consideration the activity of SpoIIE. As previously mentioned, SpoIIE localizes to the asymmetric septum and dephosphorylates SpoIIAA. The question remained, how is SpoIIE activity restricted to the prespore? A mathematical model was employed to answer this and other questions, and will be described now. This mathematical model can be divided into its qualitative aspects and its quantitative aspects. Qualitatively, it must include all of the components in the system and their interactions, which are identified via genetics and biochemistry. The quantitative aspects include both the kinetic constants for all σ^F regulatory interactions as well as the concentrations of the intermediates of these interactions (Fig. 5b). The quantitative data are the parameters that are used to produce the set of linked differential equations, which constitute the model. The initial run indicated that some factor was missing from their mathematical model, (this is probable when the model fails to simulate the known behavior of the system). The authors determined that since SpoIIAB is a dimer that undergoes conformational change, perhaps it is an allosteric protein. Since SpoIIAB binds ADP more tightly following its interaction with SpoIIAA, it would make sense that this is an example of positive cooperativity. Based on these concepts, a revised model was created (Fig. 5c) which successfully simulated many of the results obtained *in vitro (*Fig. 5d). However, prior to the presentation of the mathematical model's accounting for

compartmentalization, a review of critical biochemical data concerning SpoIIE activity would be helpful. Although the difference in volume between the prespore and the mother cell is roughly four fold, each compartment contains an equal quantity of SpoIIE. This suggests that there is four times more SpoIIE available in the prespore than the mother cell. One possible explanation to account for the all or nothing effect seen in the activation of σ^F was a non-linear relationship between the concentration of SpoIIE and σ^F activity. Evidence to support such a non-linear relationship came from fluorescent spectroscopy of the release of σ^F from its complex with SpoIIAB-ATP in the presence of a fixed concentration of SpoIIAA and varying concentrations of SpoIIE (Clarkson et al., 2004c). A ten-fold increase in the quantity of SpoIIE was sufficient to increase the activity of σ^F by 90%. This clearly illustrates a strongly non-linear relationship between the concentration of SpoIIE and σ^F activity, albeit *in vitro*. The authors originally suggested that although this could not in itself explain compartmentalization, perhaps it could in concert with transient genetic symmetry.

The initial mathematical model could not accurately predict the formation of σ^F-holoenzyme, this led the authors to a revised model that took into account SpoIIAB allostery. The expanded model performed much better and was able to simulate results from many of the *in vitro* experiments described above (Fig. 5d). This gave the authors confidence to apply this model to the situation expected in the living cell. Surprisingly, when applied to the question of whether the increased concentration of SpoIIE in the prespore could account for the prespore-specific release of σ^F, the prediction was yes (Iber et al., 2006). When the authors modeled the system with known intracellular concentrations of SpoIIAA, SpoIIAB, SpoIIE and σ^F, the predicted increase in the

concentrations of SpoIIAA and SpoIIE in the smaller prespore was sufficient to initiate compartment specific activation of σ^F (Iber et al., 2006). This result indicates that the existence of an additional regulator such as the proposed mother cell inhibitor of SpoIIE is unnecessary. Furthermore it also suggests that the putative effects of transient genetic asymmetry are also unnecessary for the sporulating cell to initiate and maintain compartment specific activity of σ^F. Indeed, computational analysis of the effects of transient genetic asymmetry on compartmentalized σ^F activity concludes that the duration of this imbalance is too short to have an impact on the release of σ^F. Therefore, this computational model supports a model wherein the volume difference between the prespore and the mother cell is the main determinant of cell fate (Iber, 2006).

Although the computational model described above provides the most rigorous analysis of the pieces to the compartmentalization puzzle to date, it has at least two weaknesses. The first weakness lies in the author's decision to omit consideration of the role SpoIIIE must have in compartmentalization from their model. *spoIIIE* mutants display a complete lack of compartmentalized activity of σ^F from the earliest time points. Whether direct or not, SpoIIIE's activity is key to compartmentalization and this must be explained. Secondly, would another result been obtained if different sets of biochemical data where chosen? In other words, does the validity of this model extend beyond its limited choice of biochemical data? It still seems possible that a new role for SpoIIIE could be identified, as well as new mechanisms which would change the result if included in this computational model.

In summary, despite a vast amount of work, our efforts to definitively explain compartmentalization of σ^F have not succeeded. It still seems premature to consider the

case closed based on any of the prevailing models, and it will be interesting to see how future work either props them up or tears them down.

The σ^F Regulon

At least 48 genes are turned on by σ^F (Wang et al., 2006). As one might expect, σ^F turns on expression of many genes involved in morphogenesis of the spore and to its resistance and germination properties. Additionally, σ^F also directs the coupling of prespore and mother cell-specific gene expression. Classical genetic methods have identified at least four *spo* loci which are directly regulated by σ^F: *spoIIIG* which encodes σ^G (Sun et al., 1989, Sun et al., 1991); *spoIIR* which is required for activation of σ^E (Karow et al., 1995, Londõno-Vallejo and P. Stragier, 1995); *spoIVB* which is involved in the regulation of late mother cell-specific sigma factor σ^K (Cutting et al., 1991, Gomez and S. M. Cutting. 1996): and *spoIIQ* which encodes a regulator of *spoIIIG* expression and under certain conditions is required for engulfment (Londõno-Vallejo et al., 1997, Sun et al., 2000). Other genes whose expression is regulated by σ^F include *katX* which encodes a spore catalase required for resistance to hydrogen peroxide (Bagyan et al., 1998), *gpr* which encodes a protease specific for SASPs (Sussman and P. Setlow 1991), *bofC* which encodes a regulator of σ^K (Gomez and Cutting, 1997) and two additional genes encoding regulators of σ^F activity, *lonB* (Amaya et al., 2001, Serrano et al., 2001) and *rsfA* (Wu and J. Errington. 2000).

Activation of σ^E

Following asymmetric septation and activation of σ^F, σ^E becomes active and directs gene expression in the mother cell. σ^E was the first sporulation specific σ factor to be purified (Haldenwang et. al., 1981), which provided the first evidence for the existence of alternative sporulation-specific σ factors. Antisera raised to σ^E allowed for immunoblotting experiments that identified two forms of the protein, the larger of which being its precursor (P31), which is now called pro-σ^E (Trempy and W. Haldenwang, 1985). The processing of pro-σ^E to its active form involves the removal of 27 amino acids from its N-terminus (LaBell et al., 1987, Stragier et al., 1988). Both pro-σ^E and one of its regulators SpoIIGA are encoded by the two-cistron *spoIIG* operon, which is under the control of Spo0A. *spoIIGA* encodes an asparyl protease which along with pro-σ^E is bound to the asymmetric septum. SpoIIGA is though to cleave the 27 N-terminal amino acids of pro-σ^E thus activating σ^E and releasing it into the cytoplasm (Jonas et al., 1988; Peters and Haldenwang, 1994; Straiger et al., 1988). Since activation of σ^E follows that of σ^F, it would make sense that σ^F would control expression of a key regulator for the processing of pro-σ^E. That regulator turned out to be SpoIIR, which is required for the processing of pro-σ^E (Karow et al., 1995; Londono-Vallejo and Straiger, 1995). SpoIIR is secreted out of the prespore (Hofiester et al., 1995), where it is believed to interact with SpoIIGA and trigger the proteolytic activation of σ^E. Since SpoIIR is initially present in the prespore, how is σ^E activity is limited to the mother cell? The compartmentalization of σ^E activity will next be discussed.

Compartmentalization of σ^E Activity

As is the case for models created to explain compartmentalization, the most obvious ones turn out to be false. It would have been lovely if SpoIIGA and pro-σ^E localized to the mother cell side of the asymmetric septum (Hofmeister, 1998; Ju and Haldenwang, 1999), but early indications turned out to be false (Fujita and Losick, 2002). However, out of these early studies it was found that a pro-σ^E –GFP fusion was preferentially degraded in the prespore (Fujita and Losick, 2002). Furthermore, the *spoIIG* promoter was active in the mother cell after asymmetric septation. These results suggest two new mechanisms for the concentration of σ^E in the mother cell. They also raise the question of how *spoIIG* transcription begins prior to septation (Gholamhoseinian and Piggot, 1989), but then becomes confined to the mother cell following septation. A closer look at Spo0A activity provided clues. Fujita and Losick determined that Spo0A activity increases after asymmetric septation but only in the mother cell (Fujita and Losick, 2003). To explain this effect, the authors propose that transient genetic asymmetry results in the exclusion of important phosphorelay genes from the prespore. An additional mechanism has been proposed which is based on the observation that dephosphorylated SpoIIAA inhibits Spo0A activity (Arabolaza and Grau, 2003). Since dephosphorylated SpoIIAA is found predominantly in the prespore (Lewis and Errington, 1998) one would expect a decrease in Spo0A activity there. One must assume that efforts are being made to employ computational models to the question of σ^E compartmentalization. It will be interesting to see if enough biochemical data for the kinetics of σ^E activation exists to facilitate the creation of such a model.

The σ^E Regulon

The σ^E regulon includes *spoIID*, *spoIIM* and *spoIIP* which encode proteins required for engulfment, as well as preventing a second asymmetric division in the mother cell (Coote, 1972; Piggot and Coote, 1976; Rong et al., 1986; Eichenberger et al., 2001; Fransden and Stragier, 1995; Pogliano et al., 1999, Smith et al., 1993; Abanes-De Mello et al., 2002); *sigK* the composite gene for late acting mother cell specific sigma factor σ^k (Stragier et al., 1989; Kunkel et al., 1990); *spoIVCA*, the gene for a recombinase which facilitates the chromosome rearrangement that gives rise to *sigK* (Kunkel et al., 1990; Popham and Stragier, 1989; Sato et al., 1994); *spoIVA*, *cotE*, and *spoVID* which encode scaffold proteins for assembly of the spore coat (Beall et al., 1993; Roels at al., 1994; Stevens et al., 1994; Zheng et al., 1988, 1990); and the *spoIIIA* operon which is required for the activation of the late prespore specific sigma factor σ^G (Illing and Errington, 1991; Kellner et al., 1996).

Three separate microchip array studies have identified at least 171 genes (in 121 operons) that are under the control of σ^E (Eichenberger et al., 2003; Feucht et al., 2003; Steil et al, 2005). In addition to many of the previously identified genes, several novel genes were identified which are required for efficient sporulation (Eichenberger et al., 2003). GFP localization studies of several others showed localization to the outer membrane of the prespore (Feucht et al., 2003).

Engulfment of the Prespore by the Mother Cell

Following asymmetric septation, a phagocytic like process begins which culminates in the prespore being completely enclosed by the mother cell as a free-floating

protoplast. During engulfment, the membrane of the mother cell migrates around the prespore, until the leading edge meets and fuses to release the prespore into the mother cell cytoplasm. The first step in the process of engulfment is the removal of peptidoglycan from the septum by autolysins SpoIID and SpoIIM (Abanes-De Mello, 2003; Chastanet and Losick, 2007). *spoIID* (Coote, 1972; Piggot and Coote, 1976), *spoIIM* (Smith et al., 1993) and *spoIIP* (Fransden and Stagier, 1995) mutants display normal autolysis at the center of the septum, but not at the periphery. Recently, it has been reported that SpoIIM, SpoIID and SpoIIP form a complex with SpoIIM that localizes to the spore septum (Chastanet and Losick, 2007). The same study also reported that localization of SpoIIM was dependent on SpoIIB, a protein previously thought to play a role in septal thinning (Perez et al., 2000). In addition to that of SpoIIM, SpoIID and SpoIIP, a second mechanism has been proposed wherein SpoIIQ and SpoIIIAH function as a ratchet which facilitates the movement along the membrane forward (Broder and Pogliano, 2006). Expression of SpoIIQ is controlled by σ^F (Londono-Vallejo et al., 1997), and is only required for engulfment when sporulation is initiated by nutrient exhaustion in rich medium (Sun et al., 2000). It was also found that in a *spoIIQ* mutant, *spoIIIG*, the gene encoding σ^G (Karmazyn-Campelli et al., 1989; Sun et al., 1989) fails to be expressed (Sun et al., 2000), indicating an additional role for SpoIIQ in coupling engulfment to σ^G activation.

Following the phagocytic migration of the membrane around the prespore, the final stages of the engulfment process are chromosome separation and membrane fusion. Interestingly, both membrane fusion and chromosome separation are dependent on DNA translocase SpoIIIE (Bogush et al., 2007; Bath et al., 2000; Wu and Errington, 1994).

The Late Prespore-Specific Transcription factor σ^G

During engulfment, σ^F is active in the prespore and is directing expression of the next prespore specific sigma factor, σ^G. Likewise, σ^E is active in the mother cell directing synthesis of the late mother cell specific sigma factor, σ^K. Therefore compartmentalization of σ^G and σ^K is simply the result of their localization to separate enclosed compartments. Most work in this area has focused on exploring the activation of σ^G and not its compartmentalized activity. Although it is clear that some mechanism must exist to couple σ^G activation with the completion of engulfment, that mechanism remains unclear.

The promoter for the gene that encodes σ^G is recognized not only by σ^F but σ^G as well (Sun et al., 1991) resulting in an autocatalytic loop. Interestingly, its transcription is delayed relative to other genes under σ^F control (Karow and Piggot, 1995). σ^G transcription is also dependent upon activation of σ^E in the mother cell (Partridge and Errington, 1993), and transcription of spoIIQ in the prespore (Sun et al., 2000). σ^G activation requires the σ^E controlled expression of the *spoIIIA* operon, as well as the vegetatively expressed *spoIIIJ* gene (Kellner et al., 1996). The *Escherichia coli* homologue of SpoIIIJ is YidC (Murakami et al., 2000) a protein translocase which is involved in placement of proteins in the lipid bilayer (Samuelson et al., 2000; Scotti et al., 2000). Early studies of σ^G activation suggested that σ^G was held inactive by anti-sigma factor SpoIIAB (Kellner et al., 1996). However, a subsequent study has cast doubts on what role SpoIIAB might be playing in the regulation of σ^G activity if any (Serrano et al., 2004). The authors conclude that activation of σ^G is the result of a yet to be identified

mechanism. Finally, recent technological advancements have allowed for the study of temporal compartmentalization and have found that some σ^F controlled genes are turned off prior to activation of σ^G (Li and Piggot, 2001).

The σ^G Regulon

DNA microarray analysis has recently identified 113 genes under the control of σ^G (Steil et al., 2005; Wang et al, 2006). The function of these genes mostly fall into three categories; those involved in protection of the spore against DNA damage; in sporulation and in germination (Hilbert and Piggot, 2004). σ^G controlled sporulation genes include *spoIIIG* which as mentioned above results in an autocatalytic loop; *spoIVB* encoding a serine peptidase which links activation of σ^K to the prespore (Cutting et al., 1991); *spoVT* encoding a transcriptional regulator of σ^G dependent gene expression (Bagyan et al., 1996); and *bofC*, encodes a regulator of SpoIVB activity and its intercompartmental signaling role in the σ^K checkpoint (Gomez and Cutting, 1996; Wakely et al., 2000). The genes for protection against DNA damage include *splB*, encoding a spore photoproduct (thymine dimer) lyase involved in repair of UV radiation-induced DNA damage (thymine dimer) during spore germination (Fajardo-Cavazos et al., 2000; Pedraza-Reyes, 1997); *yqfS*, whose product participates in the repair of AP sites and 3' blocking groups of DNA generated during both spore dormancy and germination (Salas-Pacheco et al., 2003; Urtiz-Estrada et al., 2003); and *ssp* genes which encode SASP's, the most abundant protein of the spore coat (Helmann and Moran 2002). SASP's are DNA binding proteins that help protect the DNA from UV irradiation, desiccation, exposure to heat, and also provide a source of amino acids upon germination.

σ^G controls the expression of all *ssp* genes (which include *sspA* through *sspP*) except *sspF* and *sspG* (Piggot and Losick, 2002). The germination genes include the *gerA* operon which is involved in the germination response to L-alanine and related amino acids; *gerB,* which is involved in the germination response to the combination of glucose, fructose, L-asparagine, and KCl (Paidhungat and Setlow 2002); and *pdaA* whose product participates in muramic β-lactam formation whose structure is important for germination (Fukushima at al., 2002).

The Late Mother Cell-Specific Transcription Factor σ^K

Following activation of σ^G in the prespore, σ^K becomes active in the mother cell. σ^K is synthesized as an inactive precursor (pro-σ^K) in the mother cell. Expression of pro-σ^K is the result of developmental chromosome rearrangement, which involves the removal of approximately 48 kb of the sequence between *spoIVCB* and *spoIIIC*, the two genes that encode pro-σ^K (Stragier at al., 1989). Sequences for the two halves of pro-σ^K become joined in frame during sporulation by site-specific recombination within a 5-base pair repeated sequence, resulting in the formation of the single composite gene, *sigK*. Since transcription and chromosomal rearrangement of the pro-σ^K genes only occurs in the mother cell during sporulation, σ^K activity is thereby limited to the mother cell.

Activation of σ^K is the result of the proteolytic processing of membrane bound pro-σ^K, and is controlled by a signal transduction pathway emanating from the prespore. This processing is performed by SpoIVFB (Lu et al., 1995), a membrane bound zinc metalloprotease (Rudner et al., 1999; Yu and Kross, 2000). SpoIVFB is held inactive by two other membrane proteins; SpoIVA and BofA. Recently, it has been demonstrated that

SpoIVB, a signaling protease, triggers pro-σ^K processing by cleaving the extracellular domain of the SpoIVFA regulator at multiple sites (Dong and Cutting, 2003; Campo and Rudner, 2006). A previous study had identified CtpB (Pand et al., 2003), which also activates pro-σ^K processing by cleaving SpoIVFA. Therefore activation of pro-σ^K is the result of two separate and redundant proteolytic pathways.

σ^G controls expression of *spoIVB* in the prespore (Cutting et al., 1991; Lu et al., 1990), which is the only role for σ^G that is essential for pro-σ^K processing (Gomez et al., 1995). Although expression of *spoIVB* is reported to be under control of σ^G and σ^F, the importance of σ^F mediated expression during sporulation is minimal (Gomez and Cutting, 1996). Following its synthesis in the prespore, SpoIVB localizes to the inner prespore membrane where it is autoproteolytically activated (Wakeley et al., 2000). Activated SpoIVB then cleaves SpoIVFA, which allows SpoIVFB to proteolytically process pro-σ^K.

The σ^K Regulon

DNA microarray analysis has recently identified 132 genes under the control of σ^K (Steil et al., 2005). As expected, the known genes are involved in spore coat formation, spore maturation, and regulation of σ^K controlled genes. These genes include the following; the structural gene for σ^K (which sets up an autocatalytic loop) (Kunkel et al., 1990); *oxdD* and 14 *cot* genes, which encode spore coat proteins (Costa et al., 2004; Driks, 2002; Henriques and Moran, 2000); and *spoVD* and *spoVK* which are required for spore maturation (Daniel et al., 1994; Fan et al., 1992). σ^K also directs expression of the

transcriptional regulator, GerE (Zheng et al., 1994), which is a small DNA binding protein that can act as either an activator or repressor of σ^K directed genes.

Late Stages of Sporulation

A multilayered proteinaceous shell, called the coat, surrounds the spore core and provides resistance to a variety of environmental stresses. Expression of proteins responsible for assembly of the spore coat begins early with σ^E directed expression of: *cotE,* which encodes a morphogenetic protein required for the assembly of the outer coat layer and spore resistance to lysozyme (Driks et al. 1999; Nicolson et al., 2000); *spoIVA*, encoding a protein that localizes to the septum and is required for the assembly of CotE as a ring-like structure around the prespore (Driks et al., 1994); *safA* encoding a protein that is believed to contribute to lysozyme resistance and germination (Ozin et al., 2000; Ozin et al., 2001) and *spoIVD* encoding a protein that is required for the maintenance of the CotE ring (Driks et al., 1994) and localization of SafA to the spore surface.

Following engulfment σ^K becomes active and drives the expression of most of the genes responsible for spore coat structural proteins and their assemblers. In total, over 30 spore coat polypeptides are assembled in three distinct layers; an amorphous undercoat, a lamellar inner coat, and the electron-dense striated outer coat, which provide protection to the spore while allowing for monitoring of conditions favorable to germination (Aronson et al., 1989; Driks et al. 1999; Henriques and Moran, 2000; Lai et al., 2003). CotE is believed to direct outer coat assembly through a complex network of interactions which recruit spore coat proteins (Kim et al., 2006; Little and Driks, 1999). In the final of spore coat formation, coat proteins are cross-linked by superoxide dismutase SodA (Henriques

et al., 1998) and may also be modified by both glycosylation (Roels and Losick, 1995) and proteolytic processing (Bourne et al., 1991; Cutting et al., 1991b).

The final stage of sporulation is the mother cell death process, which releases the spore into the environment. Recently these final events have been characterized; the spore separates from the polar site of the mother cell, which is followed by a rupture in the membrane and mother cell lysis (Hosoya et al., 2007). The process of mother cell death requires the activity of autolysins (Foster and Popham, 2002). Two autolysins, CwlC and LytC, were found to have a mutually compensatory role in mother cell lysis. Double mutants lacking expression of both of these proteins were unable to lyse at the end of sporulation (Smith and Foster, 1995), however single mutants lysed normally. Since the temporal control of mother cell lysis must be tightly regulated, there is much room for future work in our understanding of the mother cell lysis process. It will be interesting to see how investigators overcome the formidable obstacles of studying the final moments of a dying cell.

Project Aims

The proteins encoded by the *spoIIA, spoIIE*, and *spoIIG* loci are expressed prior to sporulation, but neither σ^F nor σ^E are active until an asymmetric septum is formed. It is not fully understood why σ^F is active only in the prespore, yet is clear that this compartmentalized activity occurs after the formation of the septum and is followed by the activation of σ^E, whose activity is limited to the mother cell (Errington, 1993). Although σ^F has been implicated in the activation of σ^E, experiments have shown that the compartmentalization of this activity is the result of other yet to be determined factors

distinct of σ^F. Therefore, σ^F and σ^E each direct the compartmentalized programs of gene expression in the prespore and mother cell respectively, however they do not direct compartmentalization itself. Current thinking about compartmentalization of gene expression has centered on prominent features of the sporulating cell versus vegetatively growing cells. For example, asymmetric division results in the volume of the mother cell being at least four times the volume of the prespore (Zhang, Higgins and Piggot, unpublished observations). The spore septum is thinner than the vegetative septum and surrounding wall. There are also differences in the two faces of the spore septum however SpoIIE is located on both. Translocation of one of the chromosomes early in sporulation results in the origin-proximal 30% residing in the forespore, and the remaining 70% plus one complete chromosome in the mother cell. This means that during the 15-20 minutes that it takes for the remaining 70% of the chromosome to enter the prespore, some of the genes are unavailable for expression. Another complementary hypothesis is that the control of compartmentalization may be facilitated by proteolysis of transcription factors and their regulators. Differential proteolysis could explain the lack of activity when expressing σ^F or σ^E in the wrong compartment. The computer based mathematical analysis provided by the Yudkin group provides a compelling argument for compartmentalization being explainable biochemically without the need for further experiments, however doubt lingers among some that although rigorous, this model remains incomplete. For example, this mathematical model does not sufficiently assign a role for SpoIIIE.

Our laboratory has developed a two-part *sacB*/SacY probe to study the temporal and spatial compartmentalization of gene expression. It utilizes the anti-terminator protein

SacY to control the transcription of reporter *lacZ*, which is cloned downstream of the *sacB* gene and is regulated by anti-termination. Expression of *sacB* and sacY are regulated by a pair of promoters specific for σ^F (prespore specific) and σ^E (mother cell specific.) Both SacY and *sacB* must be in the same compartment of the sporulating cell in order to obtain β-galactosidase activity. Mutagenesis of *Bacillus subtilis* was employed to identify determinants of compartmentalization of gene expression during sporulation. Mutants were screened for loss of compartmentalization using the two-part probe. We have mutagenized *Bacillus subtilis* using both chemical (EMS) and transposon methods. The strains have been engineered such that expression of *sacB-lacZ* and sacY are under the control of σ^F and σ^E respectively. Mutations in the *spoIIIA* and *spoIIIJ* loci were found to disrupt the compartmentalization of gene expression during sporulation of *Bacillus subtilis*, identified by β-galactosidase activity. It was shown that the loss of compartmentalization was a secondary consequence of the instability of the mutant prespores.

In addition to the two part *sacB*/SacY probe, a second method was developed; transposon mutagenesis was performed on strains where expression of *gfp* was regulated by promoters recognized by either σ^F or σ^E. Cells deficient in sporulation were isolated and evaluated by fluorescence microscopy for uncompartmentalized *gfp* expression The goal of this project was to identify any additional proteins that may play a role in the regulation of compartmentalized activity of σ^F and σ^E. There remain sufficient gaps in the current models to assume that such a protein might exist. An alternative explanation is that all the proteins necessary for compartmentalization have been identified, but that their characterization needs to be expanded or revised. Since the possibility of a negative

result exists (no *new* proteins discovered), the screens need to be convincingly thorough, with all the known players of compartmentalization serving a positive controls.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains

The *Escherichia coli* strain used for cloning was DH5α [F^- *endA1 hsdR17* ($r_k^- m_k^+$) *supE44 thi-1 λ recA1 gyrA96 relA1* Δ(*lacZYA-argF)U169* Φ80d*lacZ*Δ*M15*] (Gibco BRL, Grand Island NY). The *B. subtilis* strains and their genotypes are listed in Table 1. All were constructed in the SL4 (BR151) genetic background.

Media

Luria Bertani (LB) medium (Appendix) was used for growth of *E. coli* strains. When appropriate, the medium was solidified with 1.5% Difco Bacto agar. Luria Bertani agar (LA) was used for routine growth of *B. subtilis*, and LB was used for growth for total DNA isolation. To induce sporulation, *B. subtilis* strains were grown in modified Schaeffer's sporulation medium (MSSM), in Ramaley and Burden medium, or on Schaeffer's sporulation agar (SSA) (Appendix). Strains were stored at -80°C in LB with 15% glycerol. When appropriate, antibiotics were used at the following concentrations: for *E. coli*, ampicillin at 100 μg/ml and neomycin at 100 μg/ml; for *B. subtilis,* chloramphenicol at 5 μg/ml, erythromycin at 1.5 μg/ml, neomycin at 3.5 μg/ml and spectinomycin at 100 μg/ml. When appropriate, the chromogenic substrate 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) was used in LB agar or SSA at 40 μg/ml. When necessary, isopropyl-beta-D-thiogalactopyranoside (IPTG) was used at concentrations from 10 μM to 1 mM as specified.

Table 1. *B. Subtilis* Strains

Strain	Relevant characteristics	Source
SA501		S. Aymerich (INRA, Thiverval-Grignon, France)
SL4	*trpC2 metB10 lys-3*	F.E. Young (BR151)
SL5740	SL4 transformed with pHV1249	Gryczan et al
SL7643	SA501 cured of prophage SPβ (and inserts within the prophage)	Li and Piggot, 2001
SL9106	SA501 cured of prophage SPβ; P_{cotEp1}-*sacB'*-'*lacZ*/P_{spoIIQ}-*sacY*(1-55)	Li and Piggot, 2001
SL9109	*trpC2 metB10 lys-3 spoIID-gfp*	David Hilbert and Vasant Chary
SL12046	*trpC2 metB10 lys-3 spoIID-gfp*	This Study
SL10253	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIJ:cat*	Li and Piggot, 2001
SL10280	*trpC2 metB10 lys-3 spoIIQ-gfp*	David Hilbert and Vasant Chary
SL10271	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIAB:cat*	Li and Piggot, 2001
SL10307	*trpC2 metB10 lys-3 spoIID-gfp spoIIIAB:cat*	Li and Piggot, 2001
SL10319	*trpC2 metB10 lys-3 spoIID-gfp spoIIIJ:cat*	Li and Piggot, 2001
SL12147	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIAA*::Tn*10*	This Study
SL12149	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIAB*::Tn*10*	This Study
SL12151	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIE*::Tn*10*	This Study
SL12155	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIJ*::Tn*10*	This Study
SL12161	*trpC2 metB10 lys-3 spoIIQ-gfp spIIM*::Tn*10*	This Study
SL12167	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIAB*::Tn*10*	This Study
SL12169	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIAA*::Tn*10*	This Study
SL12162	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIE*::Tn*10*	This Study

SL12170	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIAA*::Tn*10*	This Study
SL12172	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIE*::Tn*10*	This Study
SL12173	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIAB*::Tn*10*	This Study
SL12188	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIAB*::Tn*10*	This Study
SL12169	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIAE*::Tn*10*	This Study
SL12204	*trpC2 metB10 lys-3 spoIIQ-gfp ald*::Tn*10*	This Study
SL12232	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIJ*::Tn*10*	This Study
SL12769	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIAE*::Tn*10*	This Study
SL12784	*trpC2 metB10 lys-3 spoIIQ-gfp yqjF*::Tn*10*	This Study
SL12791	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIE*::Tn*10*	This Study
SL12799	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIAB*::Tn*10*	This Study
SL12807	*trpC2 metB10 lys-3 spoIIQ-gfp ald*::Tn*10*	This Study
SL12832	*trpC2 metB10 lys-3 spoIIQ-gfp spoIIIAB*::Tn*10*	This Study
SL12840	*trpC2 metB10 lys-3 spoIIQ-gfp kinA*::Tn*10*	This Study

DNA Manipulations

Enzyme Treatments

Restriction endonucleases, shrimp alkaline phosphatase (SAP), T4 kinase, and T4 ligase were obtained from Promega (Madison, WI). All enzymatic reactions were preformed according instructions provided by the manufacturer or as described by Sambrook et al (1989). Reactions were typically performed in a 25 µL volume in a 1.5 ml polypropylene microcentrifuge tube, and were scaled up when necessary.

Agarose Gel Electrophoresis for DNA Selection

1% LE agarose gels containing 0.5 mg ethidium bromide per ml were used for electrophoretic separation of DNA fragments (SeaKem, FMS Bioproducts, Rockland, ME). Agarose gels were run in 1X Tris-acetate buffer (TAE) running buffer, described in Sambrook et al., (1989). DNA was visualized by UVB radiation and photographed with an analog FisherBiotech Camera (Fisher Scientific, Pittsburgh, PA).

DNA Fragment Isolation and Ligation

DNA fragments for ligation were fractioned on agarose gels in TAE containing ethidium bromide at 125 volts for 40 mins. After photography, DNA fragments were excised with a razor blade and placed into 1.5 ml centrifuge tubes. DNA fragments were gel-purified using the Wizard® PlusMinipreps DNA Purification System (Promega, Madison, WI), or the Quiaquick PCR Purification Kit (Qiagen, Valencia, CA) as per the manufacture's instructions. Ligations were performed in a 10 µL volume comprised of 0.5 µL T4 DNA Ligase (1.5 units), 1 µL of 1 X Ligase Buffer. The amounts of vector

and insert DNA used was determined empirically. For all ligations, the vector was dephosphoryated with shrimp alkaline phosphatase (SAP), which was then inactivated by incubation at 95° Celsius for five minutes. All ligations were performed with a self-ligation of the vector as a control. Both cohesive-end and blunt-end ligation reactions were carried out overnight at room temperature, and the entire reaction volume was used for *E. coli* transformation.

Synthetic Oligonucleotides and DNA Sequencing

The oligonucleotides used for DNA sequencing, PCR, and in vitro site-directed mutagenesis were synthesized by either the Great American Gene Compnay of Ransom Hill Bioscience, Inc. (Ramona, CA) or Integrated DNA Technologies (Coralville, IA). They are listed in Table 2. DNA was sequenced by the Thomas Jefferson University Kimmel Cancer Center Nucleic Acid Facility (Philadelphia, PA).

Polymerase Chain Reaction (PCR)

Polymerase Chair Reactions were performed using the protocols provided by Promega (Madison, WI) or Stratagene (La Jolla, CA) for their respective DNA polymerases. The reactions typically contained an empirically determined amount of plasmid or chromosomal DNA template, 100 pmoles of each oligoneucloetide, 0.25 mM of each dNTP, either 25 units/ml of Taq (Promega) or 12.5 units/ml of Pfu (Stratagene) and the appropriate amount of 10X buffered supplied by the DNA polymerase supplier in a total volume of 25-100 μl. The reactions were performed in either a ThermoCycler 2400 with Heated Lid (Perkin-Elmer Corp., Foster City, CA) or Tgradient Thermocycler

Table 2. **Oligoneucleotides**

Name	**Sequence**
InversePCRMfesense	ATG CCTTTATTAAAATT
InversePCRMfeanti	TCCTAAATTTTTATCTAA
SequencingMfe	CATATCAAATGAACTTTA
SpoIIAAR	CATGATGCCACCCCCAGT
SpoIIAAL	ATGAGCCTTGGAATTGAC
SpoIIEL	ATGGAAAAAGCAGAAAGAAGAGTG
SpoIIER	TTATGAAATTTCTTGTTTGTTTTGA
SpoIIIABf	ATGCTGAAGCTGCTGCTGGGC
SpoIIIABR	GTGGCAAAGAAAAAACGAAAA
SpoIIIEL	CCTATGTAAACCACTTTCGATAAAAAT
SpoIIIER	GTCTACTCCCATTTTATTTTTGCTCC
Tn10frag2ANTI	ATTATTATTGCCGGCCTCTGGTATTTGGACTCC
Tn10frag2SNS	ATATTTGTGCATCACGGCAATCAACTTCTTCCA
Tn10frag1SNS	TATAGCTAGTTGGTTCAAATAATGAT
Tn10frag1ANTI	AGGATATCCTTATTATCAAGATAAGA
Tn10 seq 3601	TTCACACATGGTTACGCTT
Tn10 seq 3500	TCAACAGTTCGCTTAGGCA
#14SpoIID	TGCCCATAGACTAGACTAGA

2400 with Heated Lid (Perkin-Elmer Corp., Foster City, CA) or Tgradient Thermocycler (Whatman Biomentra, Gottingen, Germany). Cycling conditions consisted of an initial incubation of 5 minutes at 94°C to melt DNA, followed by either 25 or 30 cycles of 45 seconds at 94°C, annealing for 45 seconds at 50°C, and extension for 1 minute/Kb of anticipated product at 72°C. Samples were analyzed by electrophoresis and stored at 4°C.

Southern Blot

Total DNA was isolated from 18 *B. subtilis* mutant strains, and10 μg of each sample was digested with *Eco*R1 overnight at 37°C. The digested DNA was phenol/chloroform extracted and ethanol precipitated and centrifuged at 10,000 g for 15 mins. The dried pellets were resuspended in 10 μl TE, loaded onto a 0.8% TAE gel, and fractionated by electrophoresis overnight at 15V. Transfer of the DNA to filters and hybridization was done as described by Sambrook et al (1989). A plasmid template was PCR labeled with digoxigenin conjugated dUTP and visualized using the Genius^{TM} DNA detection kit for the alkaline phosphatase system (Boehringer Mannheim, Indianapolis, IN).

Transformation

E. coli Transformation

Competent *E. coli* cells were prepared using a protocol adapted from a standard $CaCl_2$ based method (Sanbrook, 1989). Single colonies of DH5α where used to inoculate 5 ml LB medium (Appendix) in a 15 ml disposable polypropylene culture tube (Fisher Scientific, Pittsburgh, PA). Overnight cultures were grown at 37°C with 220 rpm

shaking. The next morning, 0.5 ml of overnight culture was used to inoculate 150 ml LB medium (Appendix) in a 50 ml flask and incubated at 37°C with 220 rpm shaking until the OD_{600} reached 0.35. Cells were harvested into three 50 ml centrifuge tubes (Fisher Scientific, Pittsburgh, PA) and chilled on ice for 10 min., and centrifuged at 5000g for 15 min. at 4°C. The supernatants were removed; the cells resuspended in an ice-cold solution of 0.1 M $CaCl_2$, and cultures incubated for 30 min on ice. The cells were repelleted under the same conditions, and pellets were resuspended in 5 ml of a 60 mM $CaCl_2$, 15% glycerol solution. One hundred µl aliquots of the competent cell suspension were transferred into pre-chilled 1.5 µl centrifuge tubes, flash frozen, and stored at -70°C.

For transformation, 100 µl of competent cells (per reaction) was thawed on ice for 10 minutes. Plasmid DNA was added and the cultures gently mixed. Tubes were incubated on ice for 30 mins. The cells were heat shocked for one min. at 42°C, and incubated on ice for 2 mins. Following the addition of 800 µl of LB medium, the cells were incubated for one hour at 37°C with vigorous shaking. Cells were pelleted and resuspended in 200 µl LB. Between 20 µl and 200 µl of transformation mixture was plated on LA containing the appropriate antibiotic and X-Gal when necessary. Transformation plates were incubated overnight at 37°C.

B. subtilis **Transformation**

B. subtilis transformation was performed according to the method of Piggot et al. (1984). A single colony from an SSA plate with antibiotic, when appropriate, was used to inoculate 5 ml of GMI (Appendix) in a 50 ml centrifuge tube and incubated overnight at 30°C. The overnight culture was diluted 10-fold in GM1 (Appendix) in a 125 ml flask and incubated at 37°C with 175 rpm shaking. 90 minutes after the OD_{600} of the undiluted

culture reached 0.9, it was diluted 10-fold in GM2 (Appendix), followed by incubation for 90 minutes at 37°C with 175 rpm shaking. At this point, the cells are expected to be ready to accept plasmid or chromosomal DNA, which is added to the competent cells in volumes ranging from 10-50 µl. The total amount of DNA used was determined empirically. The transformation mixture was incubated for one hour at 37°C with 150 rpm shaking. 200 µl was plated on either LBA or SSA plates containing the appropriate antibiotic and IPTG and X-Gal when appropriate. Plates were incubated for 18 hours or two days at either 30°C or 37°C.

Plasmids

All plasmids were maintained in *E. coli* DH5α. All constructions were verified by restriction digestion, PCR, and when possible, phenotypic analysis in *B. subtilis*. Details of their construction are listed in Table 3.

DNA Isolation

Isolation of Plasmid from *E. coli*

Plasmids were isolated from *E. coli* using the Wizard Plus Midiprep DNA Purification system (Promega, Madison WI). A single colony was chosen from a LA plate to inoculate 10 ml of LB with the appropriate antibiotic in a 50 ml centrifuge tube and incubated overnight at 37°C with 220 rpm shaking. DNA was prepared from the overnight culture as per the manufacturer's instructions. Plasmid was eluted in 200 µl of TE (Appendix).

Table 3. **Plasmids**

Plasmid	**Description**
pBS-SK-	pBluescript SK- (Stratagene, La Jolla, CA). pBluescript II phagemids are designed for DNA cloning, dideoxy DNA sequencing, *in vitro* mutagenesis and *in vitro* transcription.
pBluespec	A *spcE* resistance cassette was cut out of pDG1727 using blunt-end cutters *Eco*RV and *Stu*I and ligated in reverse orientation into pBLuescript SK^- which had been linearized with *Bsa*I and *Xmn*I to delete approximately 2/3 of the ampicillin resistance (*bla*) gene
pDG1727	Contains a Sp^R cassette corresponding to bases 1-1158 in GeneBank sequence M69221 (*Enterococcus faecalis* spectinomycin adenyltransferase gene), which was cloned in the *Sma*I site of pBluescript II SK- (Stratagene, La Jolla, CA).
pHV1249	A vector used for transposon mutagenesis. pE194 (*Staphylococcus aureus* temperature sensitive Ori) which contains a mini-Tn*10* transposon. Tn*10* is linked to *cat*. This plasmid also has erm^R (Petit et al, 1990).
pIC216	We expected this to be a delivery vector for Tn*10* cloning (Steinmetz, 1994). This plasmid contained *amyE* sequences, rendering it unusable for this purpose.
pZS139	A 5.6 Kb pBluescript based vector containing CAT and amyE-bk. Used to integrate a pBLuescript vector into Tn*10;* which can then be excised and used to recover flanking DNA for sequencing..

Isolation of Total DNA from *B. subtilis*

Isolation of DNA from *B. subtilis* was performed using a protocol based on that of Piggot et al., 1984. A single colony was picked from an LA or SSA plate and used to inoculate 6 ml of LB with the appropriate antibiotic in a 50 ml centrifuge tube (Fisher Scientific, Pittsburgh PA) and incubated overnight at 37°C with 150 rpm shaking. The overnight culture was centrifuged for 5 min at 5000 g and the supernatant was discarded. The pellet was resuspended in 1 ml SET (20% sucrose, 10 mM EDTA pH 8.0, 20 mM Tris-HCI pH 8.0) with 10 mg/ml of lysozyme, and incubated at 37°C for 30 minutes. Cells were lysed in 200 µl of 10% SDS, 200 µl of 10X Proteinase K Buffer (250 mM Tris-HCI pH 8.0, 100 mM EDTA), 10 µl of 10 mg RNAse A/ml, and 10 µl of 20 mg Proteinase K/ml were added, and incubated at 50°C for 120 min to allow for protein and RNA digestion. The cell lysates were extracted with Tris-buffered phenol and chloroform (1:1). The DNA was precipitated by addition of 2.5 volumes of cold ethanol. DNA was harvested by centrifugation at 5000g for 5 min at 4°C, washed 1X in 70% ethanol, and resuspended in 500 µl of TE (Appendix).

Construction of a Tn*10* Library

B. subtilis strain SL5740 containing pHV1249 was revived on an LA plate containing chloramphenicol (Cat) and erythromycin (Erm) and grown overnight at 30°C. A single colony from this plate was used to inoculate a 10ml culture of LB-Cat/Erm and grown overnight at 30°C, with gently aeration in a water bath (175 rpm). The next morning, this culture was centrifuged at 5K for 10 min, and cells washed 3 times in LB-Cat. The resulting pellet was used to inoculate 10ml LB-Cat at a starting density of

OD_{600} of 0.03, and incubated with 175rpm shaking until the OD_{600}=0.15. The temperature was then increased to 50°C and culture incubated for an additional 3 hours with 175rpm shaking, (this temperature is restrictive for plasmid replication). This culture were then plated onto 72 LB-Cat plates and grown overnight at 50°C. After 12 hours of growth, plates were evaluated hourly to prevent overgrowth. At the time the cells were harvested, colonies had not yet grown large enough to touch each other, thereby preventing the possibility of disproportionate amplification of a few clones. Prior to harvesting of cells from the plates, 60 colonies were streaked out onto both LB-Cat and LB-Erm plates to test for efficiency of insertion. Colonies were harvested in 10 ml LB per plate with a glass cell spreader. Total DNA was isolated from cells harvested from all 72 plates using our standard protocal for genomic DNA extraction.

Recovery of Tn*10* flanking DNA Using pZS139

Mutant *B. subtilis* strains containing Tn*10* sequences were transformed with pZS139 DNA which integrates a pBluescript vector into the *Cat* gene of Tn*10* by single crossover. The plasmid was then excised and the Tn*10* flanking sequences identified by DNA sequencing. Mutant strains containing Tn*10* were made competent using our standard protocol. For transformation, 1.5 μg of pZS139 was added to 500 μl of competent cells and incubated for 1 hour at 37°C with 220 rpm shaking. The amount of culture per SSA-Spc plate varied for each mutant; however, 200ul of culture would typically yield 100-200 transformants/plate.

Transformants were streaked to single colonies and passed two times on SSA-Spc plates. Single colonies from the twice passed plates were used to inoculate 10 ml LB-Spc

and grown overnight 37°C with 220 rpm shaking. The next day, cells were harvested, and total DNA purified using our standard method for isolation of total DNA in *B. subtilis.* The integrated plasmid was then excised from 15 μg of purified total DNA by *Eco*R1 or *Cla*1 restriction digestion. Samples were then phenol/chloroform extracted, and precipitated in ethanol. The purified DNA pellets were resuspended in 600μl 1X ligation buffer Promega, Madison WI) and 200μl was self-ligated overnight at room temperature with 1.0 μl T4 DNA Ligase (3.0 units). The following day, the entire transformation reactions were purified using the Wizard® DNA Clean-up System (Promega, Madison, WI), and eluted in a total volume of 30 μl.

Tranformation of DH5α using the entire 30 μl of purified DNA was carried out using our standard methods. Tranformants were plated on LB-Amp and incubated overnight at 37°C. Tranformants from the self-ligation reaction were used to inoculate 1.5 ml LB-amp and incubated overnight at 37°C with 220 rpm shaking. The next morning, the Wizard® PlusMinipreps DNA Purification System (Promega, Madison, WI) was used to isolate plasmid DNA. The isolated plasmids were evaluated for presence of Tn*10* flanking DNA by restriction analysis prior to being sent to the Thomas Jefferson University Kimmel Cancer Center Nucleic Acid Facility (Philadelphia, PA) for sequencing.

Recovery of Tn*10* flanking DNA Using pJP17

This protocol is a slightly modified version of the previous protocol for recovery using pZS139. Mutant *B. subtilis* strains containing Tn*10* sequences were transformed with pJP17 DNA which integrates a pUC ColE1 ori linked to a spectinomycin resistance

gene into the *cat* gene of Tn*10* by double crossover. The plasmid is then excised and the Tn*10* flanking sequences identified by DNA sequencing. Mutant strains containing Tn*10* were made competent using our standard protocol. For transformation, 1.5 μg of pJP17 was added to 500 μl of competent cultures and incubated for 1 hour at 37°C with 220 rpm shaking. As found when transforming with pZS139, the amount of cells per SSA-Spec plate varied for each mutant.

DNA isolation, plasmid excision, ligation, and transformations were performed as previously described. To expedite the process of determining which plasmids had inserts, that could be used for sequencing, we used colony-PCR to amplify the DNA flanking our Tn*10* inserts. Colonies from transformation plates were picked and added directly to our standard PCR reaction mixtures, and amplified using primers designed to amplify the *B. subtilis* sequences flanking Tn*10*. PCR products were purified and sent the Thomas Jefferson University Kimmel Cancer Center Nucleic Acid Facility (Philadelphia, PA) for sequencing.

Inverse PCR

There are 3 basic steps to inverse PCR: restriction digestion, self-ligation, and amplification. Each step requires optimization which is specific to the template being amplified. The following reactions could be scaled up or down with good results. Total DNA purified from the mutants was digested as follows: 6.0 μg total DNA was digested in a total volume of 100 ul, in the appropriate 1 X restriction buffer containing 60 U *Eco*R1 (Promega, Madison WI). Samples were incubated for 3 hours and heat inactivated for 20 min at 65°C. To this sample, we added 100 μl containing 60 U *Mfe*I in

the appropriate 1 X Restriction Buffer (New England Biolabs, Ipswich, MA), incubated the reactions for an additional 3 hours, and heat inactivated for 20 min at 65°C.

The entire unpurified restriction digestion was self-ligated with the addition of 5.0 µL T4 DNA Ligase (15 units) and 22.5 µL of 10 X Ligase Buffer (Promega, Madison WI). Samples were incubated for either 2 hours at room temperature or overnight at 4°C and heat inactivated for 20 min at 65°C.

Primers were designed to facilitate amplification of a fragment of the recircularized Tn*10* and a stretch of flanking DNA (Table 2). Standard amplification conditions were used with the following modifications: 50 µl samples were amplified for 35 cycles; annealing temperature was 55°C, and extension time ranged from 1 – 10 mins at 72°C (1 min/kbp). Samples were analyzed by electrophoresis and stored at 4°C. Amplified products were gel-purified for sequencing.

Sporulation

B. subtilis strains were induced to sporulate in MSSM (Piggot and Curtis, 1987) and sporulation was determined by heat survival (Nicholson and Setlow, 1990). A single colony from an SSA plate was picked and used to inoculate either a 125 or 250 ml flask containing either 10 or 20 ml of MSSM, respectively. The culture was incubated overnight without shaking at either room temperature or 30°C, followed by incubation at 37°C (30°C for GFP-expressing strains) with 150 rpm shaking the next day. Samples were taken at regular intervals, and the OD_{600} was determined with a spectrophotometer. The end of exponential growth was considered the initiation of spore formation in MSSM. 20 hours after the initiation of sporulation the cultures were serially diluted in

PBS (Gibco BRL, Grand Island NY) and plated onto SSA plates. The dilutions were treated for 20 minutes at 80°C (a temperature at which only spores will survive) and plated in a likewise manner. The plates were incubated overnight at 37°C and the number of colonies determined.

Fluorescence Microscopy

Cultures were grown in MSSM at either 30°C or 37°C. 200 µl of cultures were mixed with 0.2 µl of 1 mg FM4-64 (Molecular Probes)/ml phosphate-buffered saline (Gibco-BRL). Samples were incubated at room temperature for 5 min. 0.75 µl of sample was transferred to a microscope slide, and a glass cover slip was placed on top of it. Gentle pressure was applied to avoid disruption of prespores. All microscopy was performed using either a Photomicroscope II (Carl Zeiss, Oberkochen, Germany) with a 100-W mercury lamp source and a 100X Neofluor oil immersion objective lens; or a DM IRE2 microscope with the TCS SL confocal system (Leica), using a 100X oil immersion objective and Leica imaging software. GFP emission was captured between 500 and 550 nm and FM4-64 emission between 600 and 730 nm; excitation for both fluorophores was at 488 nm. All scoring of cells was performed manually.

CHAPTER 3

RESULTS

Loss of Compartmentalization in *SpoIIIA* and *SpoIIIJ* Mutants

Zusheng Li had previously used mutagenesis with a Tn*10* derivative to generate mutants defective in the compartmentalized activity of σ^F and σ^E (Li et al, 2004). A genetic test based on the *sacB*/SacY transcriptional antitermination system was employed to evaluate the compartmentalization of gene expression (Li and Piggot, 2001). The SacY protein regulates transcription of *sacB* (encoding levansucrase) by antitermination. Transcription of *sacB* will terminate in the region 5' to the *sacB* structural gene in the absence of SacY. When SacY is present, it binds to RAT site in the 5' region, which prevents termination, and thereby allows transcription of the *sacB* structural gene. In the two-part probe system used in this screening, SacY needs to be present during transcription of *sacB* in order to obtain β-galactosidease from a *sacB*'-'*lacZ* translational fusion. By placing *sacB*'-'*lacZ* and *sacY* under the control of promoters directed by σ^F and σ^E, we were able to identify Tn*10* mutants which were defective in the compartmentalized activity of these sigma factors in collaboration with Zusheng Li.

The initial mutagenesis was performed by Zusheng Li in strain SL7643; a derivative of SA501 cured of SPβ, which has deletions of *sacY, sacB, licT* and *sacT* to avoid artifacts relating to potential interference with the antitermination system. Total DNA of the pooled mutants was purified and used to transform the tester strain SL9106 which contains P_{cotEp1}-*sacB'*-'*lacZ*/P_{spoIIQ}-*sacY*(1-55) (σ^E/σ^F –dependent promoters); selecting for Tn*10* associated chloramphenicol resistance. Out of approximately 30,000

Cm^r transformants, 32 displayed β-galactosidase activity, indicating uncompartmentalized activity of the σ^E/σ^F directed reporter. After elimination of artifacts, 8 strains remained with mutations that caused a significant loss of compartmentalization. Flanking DNA from the sites of Tn*10* insertions were recovered using the pBluescipt-based integration vector pZS139 and sequenced. Seven mutants were identified by Zusheng as *spoIIIJ* and one mutant was identified as *spoIIIAB*.

In order to determine whether the mutations in *spoIIIJ* and *spoIIIAB* affect the compartmentalization of σ^E and σ^F activity directly or indirectly, Zusheng Li transferred the mutations into strains with σ^E and σ^F directed *gfp* fusions (SL9109 *spoIID-gfp*, and SL10280 *spoIIQ-gfp*). Expression of the *spoIID-gfp* transcriptional fusion is regulated by σ^E and expression of *spoIIQ-gfp* is regulated by σ^F. Therefore, following septation, GFP localizes to the mother cell in the *spoIID-gfp* strain, and to the prespore in the *spoIIQ-gfp* strain. Whole cell fluorescence would indicate loss of compartmentalization. Mutations which block sporulation at stage III potentially generate a mutant prespore that is unstable and will break down; the consequence is a loss of compartmentalization not directly related to the regulation of compartmentalization. If the primary effect of the mutations in *spoIIIJ* and *spoIIIAB* are on compartmentalization, then we should be able to visualize whole cell fluorescence in single cells as soon as green fluorescent protein (GFP) is expressed. If the loss of compartmentalization is a consequence of defective engulfment or other genetically indirect defects, whole cell fluorescence of GFP will become apparent much later in the sporulation process, when they reach the impaired process. I took over this project at this point and performed the remaining experiments.

The following strains were induced to sporulate in modified Schaeffer's spoulation medium: SL10271 (*spoIIIAB, spoIIQ-gfp*); SL10253 (*spoIIIJ, spoIIQ-gfp*); SL10307 (*spoIIIAB, spoIID-gfp*); SL10319 (*spoIIIJ, spoIID-gfp*); SL9109 (*spoIID-gfp*); and SL10280 (*spoIIQ-gfp*). Beginning at the start of spore formation, defined as the end of exponential growth, samples were examined hourly and examined by fluorescence microscopy. The percentage of cells displaying compartmentalized verus uncompartmentalized GFP expression was determined for each strain. At least 200 GFP-expressing bacteria were counted for each sample. Consistent with the time that *B. subtilis* is expected to reach stage II of sporulation, GFP fluorescence was first detected about 3 hours after the end of exponential growth. GFP expression for both the *spoIIIAB* and the *spoIIIJ* mutants was almost completely compartmentalized at the earliest time points, as assayed with either the σ^E or σ^F directed *gfp* fusions. However, by 8 to 10 hours after the initiation of sporulation, uncompartmentalized fluorescence was observed in an increasing proportion of both mutants (Table 4). At least 40% of cells expressed GFP, except for SL10253 (14%) and SL10280 (38%) at the earliest time point. The observed breakdown in compartmentalized expression was consistent for both mutants between experiments, although the extent of the breakdown varied.

Stage III Loss of Compartmentalization in Mutants

Determination of which stage the loss of compartmentalization of gene expression in the *spoIIIA* and *spoIIIJ* mutants occurs is of critical importance. We observed significant

Table 4. Compartmentaliztion of σ^F and σ^E in mutant *B. subtilis* Strains

Strain	Relevant genotype	σ factor[a]	Proportion (%)[b] of GFP-expressing bacteria displaying uncompartmentalized expression at time (h) after the end of exponential growth					
			5	6	7	8	9	10
SL10280	+	σ^F	0	0	0	1	0	1
SL10271	*spoIIIAB*	σ^F	0	0	0	3	21	31
SL10253	*spoIIIJ*	σ^F	0	0	0	0	5	23
SL11371	+	σ^E	0	0	0	0	0	
SL10307	*spoIIIAB*	σ^E	0	0	0	0	24	62
SL10319	*spoIIIJ*	σ^E	1	0	2	5	14	50

[a] σ factor directing *gfp* expression; *spoIID-gfp* is regulated by σ^E and *spoIIQ-gfp* is regulated by σ^F.

[b]At least 100 GFP-expressing organisms were counted for each sample.

breakdown in compartmentalization in the *spoIIIA* and *spoIIIJ* mutants beginning 8 hours after the end of exponential growth. To evaluate what stage of sporulation this correlates with, we examined the cultures in detail 7 hours after the end of exponential growth before the loss of compartmentalization occurs.

Strains SL10280, SL10271, SL10253, SL11371, SL10307 and SL10319 were induced to sporulate in MSSM and samples examined by fluorescence microscopy and scored for stage of sporulation as follows: spore septum formed, (II_i); partial engulfment of the prespore by the mother cell, (II_{ii}-II_{iii}); and complete engulfment (III). In bacteria

reaching stage III, the prespore had clearly detached from the mother cell, and prespore was only detectable by GFP, and not typically detected by FM4-64 staining. The prespore was visualized by prespore- or mother cell-specific GFP expression. At 7 hours after the end of exponential growth, engulfment was complete in about 50% of the GFP-expressing bacteria in the s*poIIIJ* mutant and control strains (SL10280 and SL10253). Similarly, engulfment was complete in 25% and 48% of the *spoIIQ-gfp* (σ^F directed) and *spoIID-gfp* (σ^E directed) *spoIIIAB* strains, respectively (Table 5). The percentage of these cells that reached engulfment at this time point reached 50% in other experiments. Similar results were obtained in the *spoIIQ-gfp* and the *spoIID-gfp* strains These results indicate that the completion of engulfment occurred well before significant numbers of mutant bacteria displayed the uncompartmentalized phenotype (Table 4). Therefore, it is highly unlikely that SpoIIIAB or SpoIIIJ are regulators of the compartmentalizated activities of σ^F and σ^E.

Table 5. Compartmentaliztion of mutants 7 hours after initiation of sporulation

Strain	Relevant Genotype	σ factor[a]	Proportion (%)[b] of bacteria displaying compartmentalized GFP expression at the indicated stage[c]		
			II_i	II_{ii}-II_{iii}	III
SL10280	+	σ^F	18	17	65
SL10271	*spoIIIAB*	σ^F	35	40	25
SL10253	*spoIIIJ*	σ^F	15	18	67
SL11371	+	σ^E	11	38	51
SL10307	*spoIIIAB*	σ^E	19	33	48
SL10319	*spoIIIJ*	σ^E	21	23	56

[a]σ factor directing *gfp* expression; *spoIID-gfp* is regulated by σ^E and *spoIIQ-gfp* is regulated by σ^F.

[b]At least 100 GFP-expressing organisms were counted for each sample.

[c]The stages of sporulation are as follows: II_i, the spore septum formed; II_{ii}-II_{iii}, partial engulfment of prespore by the mother cell; III, complete engulfment.

Construction of a Tn*10* Library

Since we were unable to identify novel genes essential for the regulation of compartmentalization using transposon mutagenesis, we decided to try an alternative method of mutagenesis. Our goal was to create point mutations which, for example, could result in conditional mutants. Such mutants might provide phenotypes not possible with transposon-based mutant strains. Therefore, we attempted a chemical mutagenesis screening of our 2-part probe strain, SL9106 [P_{cotEp1}-*sacB'*-'*lacZ*/P_{spoIIQ}-*sacY* (1-55)].

This screen proved problematic and was abandoned for several reasons: first, the initial chemical mutagenesis was at too low of an efficiency; second, many of the mutants generated were artifacts, and difficult and time consuming to eliminate; and third, we

were not able to identify the mutations responsible for the loss of compartmentalization in the few remaining strains. The method we used to locate the point mutations created by chemical mutagenesis was as follows: transform our mutant strains with total DNA purified from a Tn*10* library, and isolate transformants where the loss of compartmentalization has been corrected; identify the site of Tn*10* insertion, attempt to narrow down the possible regions for sequencing; and finally sequencing to identify the point mutation. During preparation of the Tn*10* library, an idea for an alternative screening method came about: we would use total DNA from the Tn*10* library to mutagenize a GFP based reporter strain.

In order to search for novel genes that might regulate compartmentalization, and were missed in our first two screenings, a third genetic screen was undertaken. Total DNA from a newly prepared Tn*10* library (prepared from a Tn*10* infected wild type strain) was used to transform *spoIIQ-gfp* (σ^F directed) or *spoIID-gfp* (σ^E directed) strains, selecting for translucent colonies (Spo^-) on SSA. Loss of compartmentalization of σ^E or σ^F activity typically result in Spo^- bacteria, which can be easily differentiated from the usual opaque colonies of sporulating wild type *B. subtilis* or the tester stains (SL101128 and SL10146). It seemed reasonable to believe that we our first Tn*10*-based screening was not exhaustive in its goal of identifying all the critical genes responsible for compartmentalization. Therefore we undertook a second transposon based screening.

In order to construct a highly representative library with as little bias as possible, we used a plate purification method for isolation of total DNA from the initial transposon culture. The transposon library culture was plated at high density (approximately 400 colonies/plate) and colonies grown to a maximum of approximately 2 mm to prevent

overgrowth. In total, 4.2 mg of total DNA (Fig. 6) was purified from a pool of approximately 21,000 colonies, of which, 78% were erythromycin sensitive and chloramphenicol resistant (Fig. 1: a measure of the efficiency of transposition).

To test for the randomness of insertion, Southern blot analyses was performed on 18 independent Erm^S and Cat^R clones using a digoxigenin-labeled probe specific for the Cat^R gene in Tn*10*. The chromosomal DNA was cut with *Eco*R1, which does not cut Tn*10*. Therefore, we would expect to see single bands of differing size if the Tn*10* transposition was random. The resulting autoradiograph revealed that each of the 18 samples appeared to have single bands of unique size (data not shown). This result indicates that single copies of the transposon had inserted randomly into the chromosome.

Pilot Test of Screening Strategy

Prior to our large scale screening, a pilot test of our novel screening method was undertaken. Total DNA from our newly constructed transposon library was used to transform the σ^F directed *spoIIQ-gfp* fusion reporter strain SL10280. The transformation mixture was incubated for 2 days on an SSA-CAT plate, and translucent colonies evaluated for compartmentalization of GFP expression by fluorescence microscopy. Four translucent colonies were obtained indicating Spo^- mutations (Fig. 7). Samples from all four colonies were viewed under fluorescence microscopy, revealing one with whole cell fluorescence in 30-40% of cells expressing GFP (Fig. 8). This result demonstrates that our screening strategy could be used to isolate mutants defective in compartmentalization and provided a basis for proceeding to a large scale screen.

Large Scale Screening

One 25 ml culture of each of the competent reporter strains [SL10280 (*spoIIQ-gfp*); SL10246 (*spoIID-gfp)*] was transformed with 12.5 μg of total DNA purified from this new Tn*10* library. Two-hundred microliter samples of the two transformed cultures were spread on each of 100 SSA-CAT plates, which was empirically determined to yield approximately 200 colonies/plate. Both sets of plates were incubated for two days at 30°C. Translucent colonies were identified and streaked out to single colonies three consecutive times each to ensure homogeneity. For the SL10280 strain, 13,460 colonies with Tn*10* inserts were analyzed, of which 316 were Spo^- (2.4%); for the SL10246 strain, 11,812 colonies with Tn*10* inserts were analyzed, and 294 were Spo^- (2.5%). Samples from all 610 Spo^- colonies were scored for the percent of cells which displayed a loss of compartmentalization by fluorescence microscopy. Loss of compartmentalized GFP expression was identified in 75 out of the 316 Spo^- mutant strains obtained in our tranformation of SL10280, and 24 out of the 294 Spo^- mutants obtained in our tranformation of SL10246 (Table 6). In the SL10280 group, 12 mutants were obtained that had 75-100% percent loss of compartmentalization; and in the SL10280 group, 4 mutants had had 75-100% percent loss of compartmentalization. These numbers were consistent through 3 subcultures of the mutant strains.

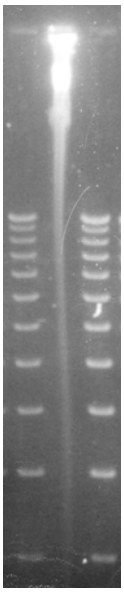


Figure 6. Total DNA isolated from our transposon library. Electrophoresis of total DNA isolated from our transposon library. This minigel was intentionally overloaded with 5mg of DNA sample to demonstrate the high percentage of high molecular weight sample. The molecular weight markers are the 1 Kb ladder (Promega, Madison, WI), the highest band is 10kb.

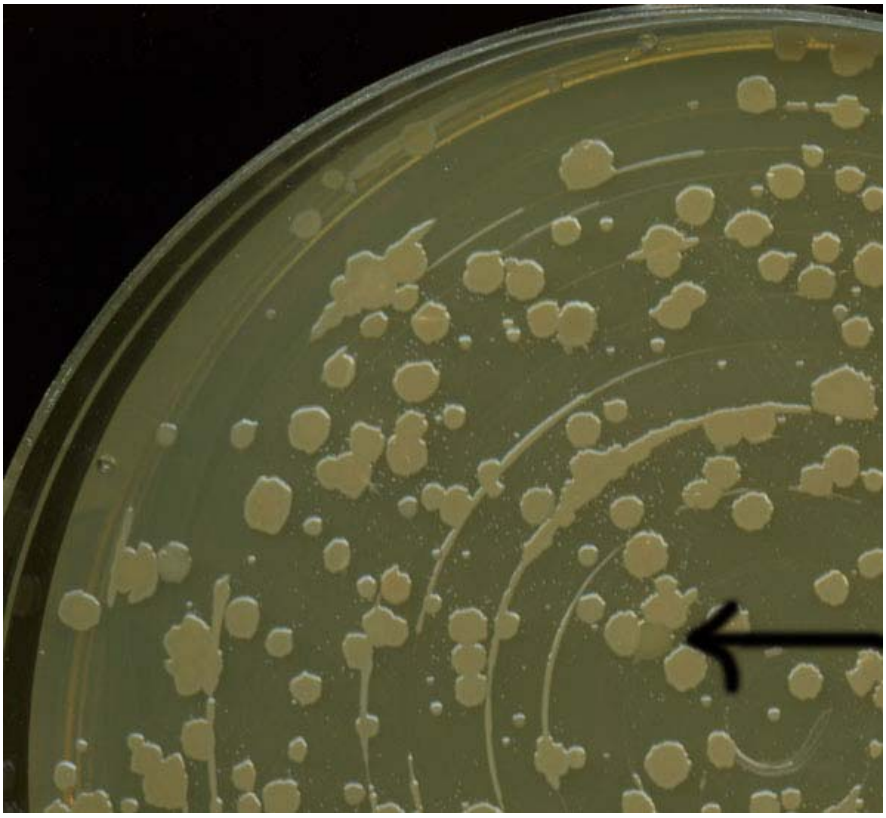


Figure 7. Transformants of the Tn*10* library. Transformant colonies of the *spoIIQ-gfp* reporter strain transformed with the Tn*10* library, on sporulation medium. The arrow is pointing to a translucent colony which is typical of a spo minus phenotype.

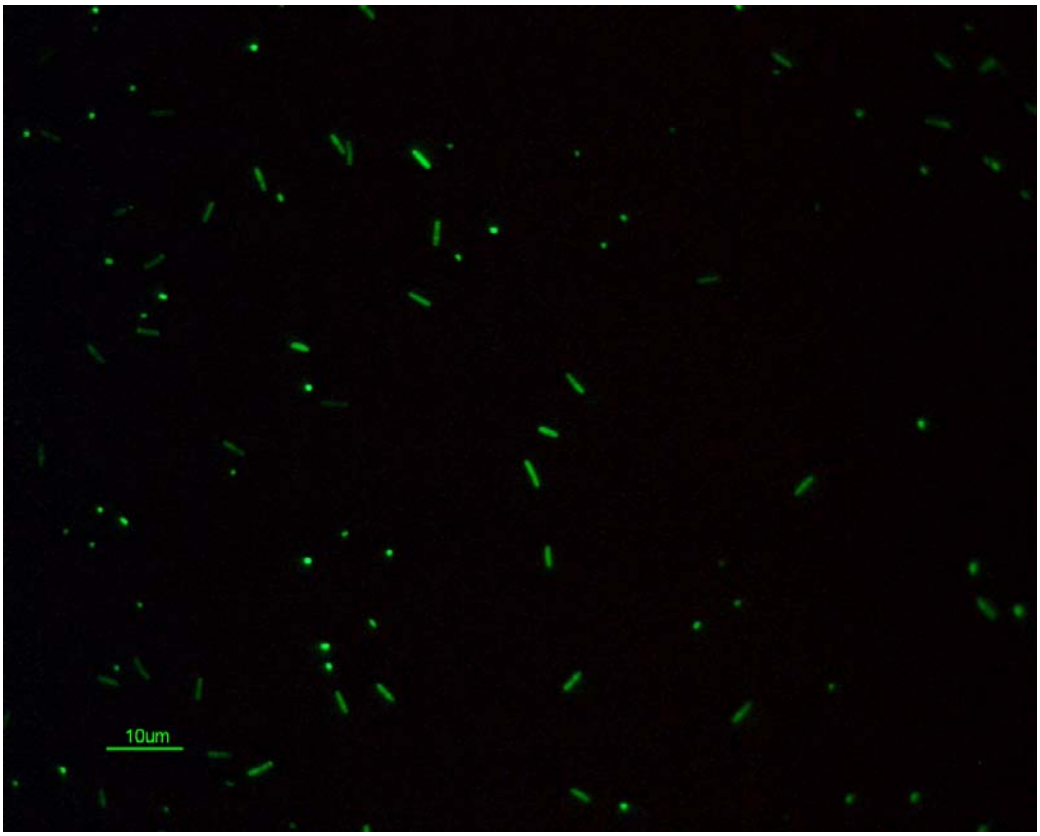


Figure 8. Fluorescent image of SLl0280 transformed with the Tn10 library. Fluorescent image of a mutant derivative of the *spoIIQ-gfp* reporter strain SL10280, displaying loss of compartmentalization. The fluorescent spheres indicate compartmentalized GFP in the prespore; rods indicate whole cell GFP fluorescence.

In order to identify the genes responsible for the loss of compartmentalization, the next step was to recover flanking DNA from the sites of Tn*10* insertions for sequencing. Several different methods were employed to overcome unexpected obstacles that arose during efforts to identify the sites of Tn*10* insertions; these methods included inverse PCR, and use of two different plasmid systems.

Use of pZS139 to Clone Tn*10* Inserts

As was done in our previous screening, we attempted to recover flanking DNA from the sites of Tn*10* insertions using the pBluescipt-based integration vector pZS139. Mutants which displayed the most dramatic loss of compartmentalization were given the highest priority for sequencing. First attempts to identify the mutations failed and it became apparent that we would need to develop an alternative to the pZS139 based method for to recovery of flanking DNA. The problem with pZS139 lies in its single-crossover design which can result in the formation of tandem repeats of the plasmid during recombination. Having multiple copies of the plasmid in tandem, resulted in the cloning of empty plasmids. Therefore, an inverse PCR protocol for the amplification of Tn*10* and its flanking DNA was developed to overcome the problems encountered using pZS139.

Genomic DNA was isolated from 60 mutants with the highest percentage loss of compartmentalization and fractionated by electrophoresis in a 0.6% TAE gel to test for quality and yield. Purified total DNA was digested with *Mfe*I and fractionated on a 1.0% TAE gel to test for complete digestion. Samples were self-ligated and amplified as described in the Methods and Materials section.

Table 6. Large scale screening for loss of compartmentalized σE and σF activity.

Reporter Strain	The number of Spo minus mutants that displayed different extents of whole cell fluorescence of GFP compared to compartmentalized GFP fluorescence.[a]				
	75-100%	40-60%	15-30%	5-10%	Total
SL10280	12	10	26	27	75
SL10246	4	4	6	10	24

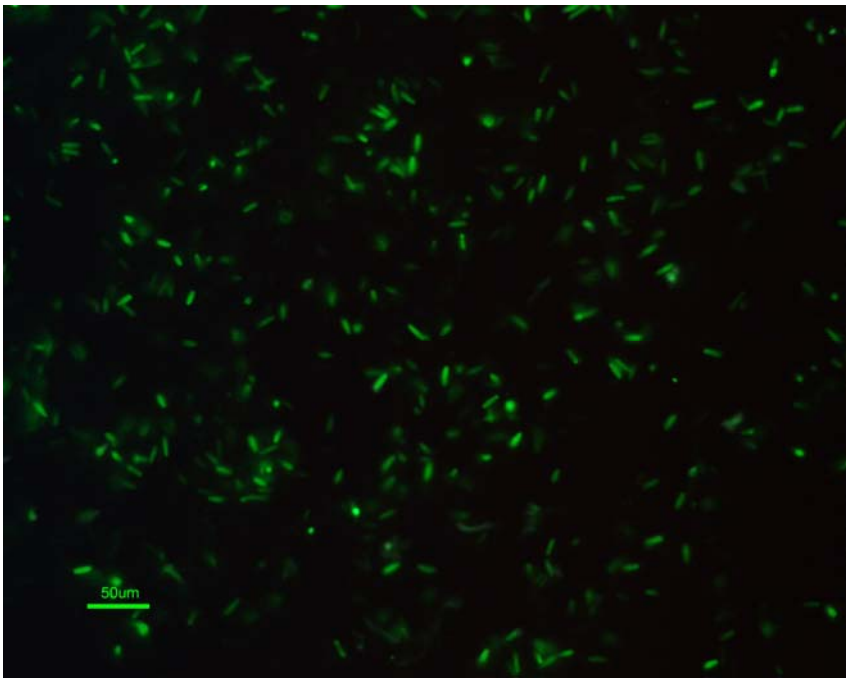
[a]Out of the 25,000 transformants screened; approximately 12,500 from both SL10280 and SL10246.

The Tn*10* site of insertion was determined for 8 mutants by this method (four examples of the resulting gfp expression are shown in fig. 9): two distinct mutations in *spoIIIE*; two distinct mutations in *spoIIIAB; a*nd one each in *spoIIAA*, *spoIIE*. *yitG* (encoding a multidrug resistence protein homolog) and *ywqA* (encoding a SNF2 helicase homolog) (Fig. 4.) YitG and YwqA were later shown to be artifacts of promiscuous GFP expression due to defects in the *spoIIQ-gfp* reporter, and were abandoned.

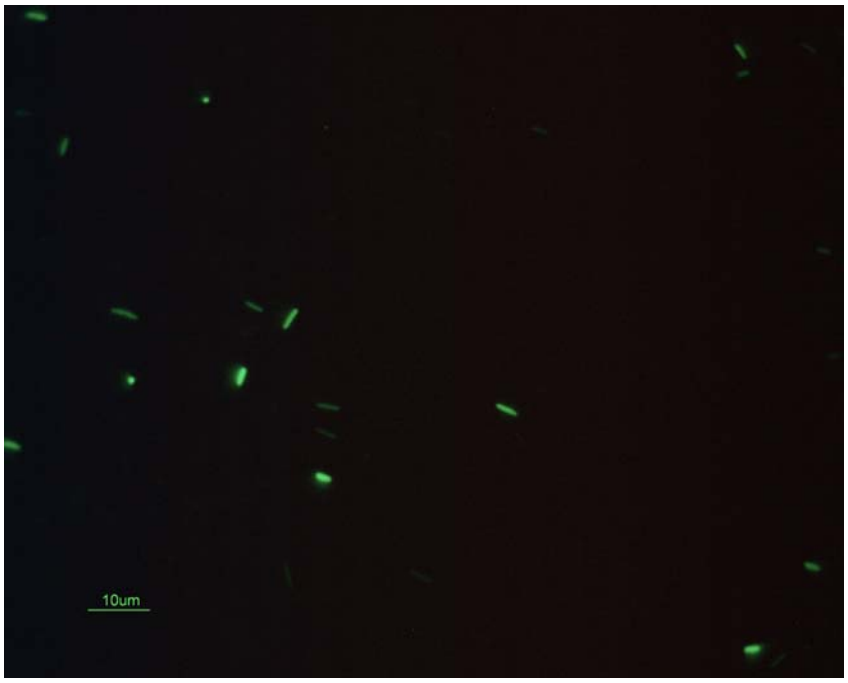
Since we had multiple *spoIIIE and spoIIIAB* mutants, it seemed prudent to screen total DNA preps from our remaining unidentified mutants by PCR for genes which have already been identified. Amplification of the remaining unidentified mutants in the 50-100% loss of compartmentalization group (as seen in cells taken from SSA plates which were incubated overnight) was performed using primers specific for *spoIIAA, SpoIIE*, *spoIIIE, yitG, ywqA* and *spoIIIAB*. Using this approach, two additional mutants were

identified, one in *spoIIAA,* and one in *spoIIIAB*. Further attempts to identify additional mutants using inverse PCR failed, and a new approach was needed.

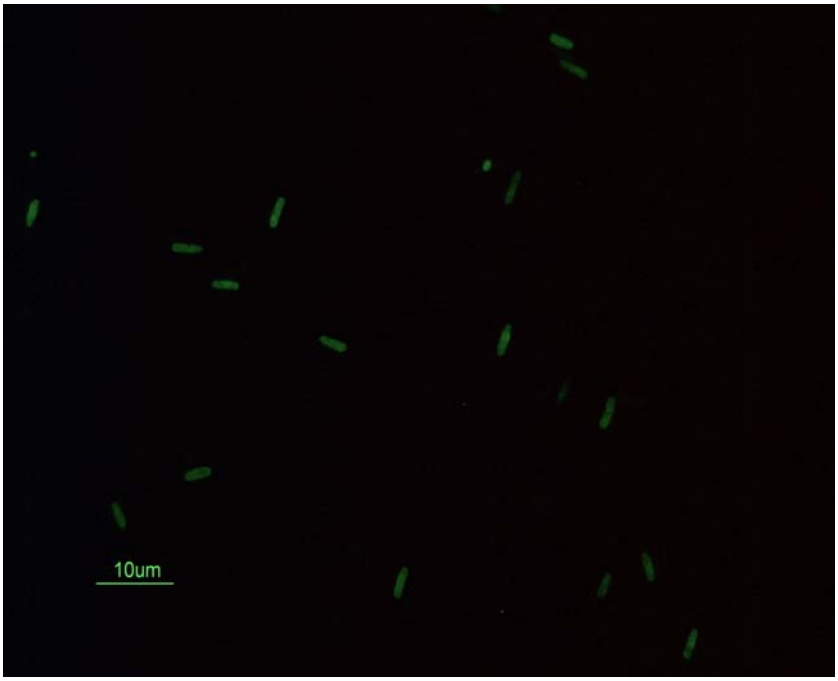
9A.



9B.



9C.



9D.

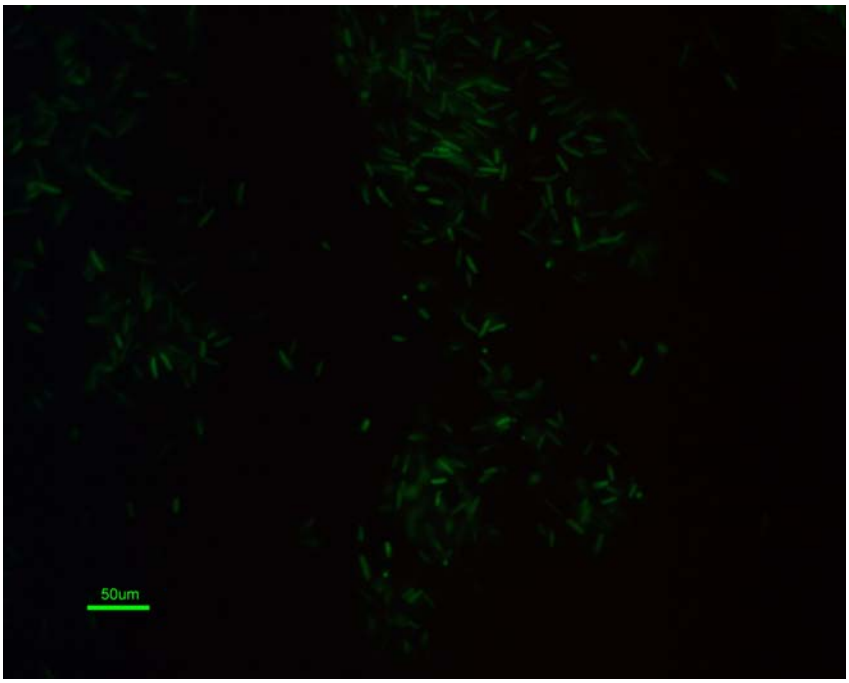


Figure 9. Fluorescent images of the *spoIIE*, *spoIIAA* and *spoIIAB* mutants. Identified using inverse PCR: 4A) Strain SL12162 = *spoIIIE*::*Tn10;* 4B) SL12172 = *spoIIIE*::*Tn10*; 4C) SL12149 = *spoIIIAB*::*Tn10*, and 4D) SL12170= *spoIIAA*::*Tn10*

Attempted Use of pIC216 to Clone Tn*10* Flanking DNA

Steinmetz et al (1994) reported construction of a plasmid that would allow for easy cloning of Tn*10* insertions (pIC215). Since this plasmid was designed to integrate by double-crossover, it should avoid the problem of tandem repeats. We requested a sample of this plasmid and were kindly provided pIC216 DNA from Stephane Aymerich, a colleague of the original authors. Although we obtained pIC216 and not pIC215, he thought that it was an alternate preparation of the same plasmid. Approximately 0.1 μg of pIC26 was used to transform competent DH5α, which were incubated on LA-Spc overnight. Tranformants from this procedure were used to inoculate 25 ml LB for plasmid isolation. The size of the purified plasmid was confirmed by restriction analysis. Although this plasmid transformed our mutant strains effectively and was efficiently rescued, all of the resulting clones contained sequences for *amyE*. Restriction analysis of pIC216 rescued from transformants from 10 different mutant strains indicated that all rescued plasmids were identical, despite the difference in their sites of transposition. We concluded that pIC26 containted *B. subtilis* DNA, and would therefore integrate into *amyE* sequences instead of Tn*10*. Therefore, we decided to construct a plasmid of similar design that would allow for cloning of Tn*10* insertions.

Construction of pJP17

A plasmid was designed that would integrate into Tn*10* sequence in the *B. subtilis* chromosome by double-crossover. We hoped that this would eliminate the generation of tandem repeats, and allow for easy cloning of Tn*10* insertions. This plasmid (pJP17) was designed to contain a *spcE* resistance cassette, and a pUC replication origin, flanked by two fragments homologous to the CAT gene present in Tn*10* (Fig. 10). This plasmid was

constructed as follows: a *spcE* resistance cassette was cut out of pDG1727 using blunt-end cutters *Eco*RV and *Stu*I and ligated into pBluescript SK$^-$ which had been linearized with *Bsa*I and *Xmn*I to delete approximately 2/3 of the ampicillin resistance (*bla*) gene; this construct was named pBluespec. Next, primers were designed to amplify two adjacent stretches of the *cat* resistance gene found in Tn*10*, bp 2315-2659 (CATI) and 2881-3519 (CATII); for directional cloning into pBluespec. The amplification products CATI and CATII were digested using the appropriate restriction enzymes, and were then sequentially ligated into pBluespec. The orientation of each insert was verified by DNA sequencing. The entire sequence of pJP17 is provided in the appendix.

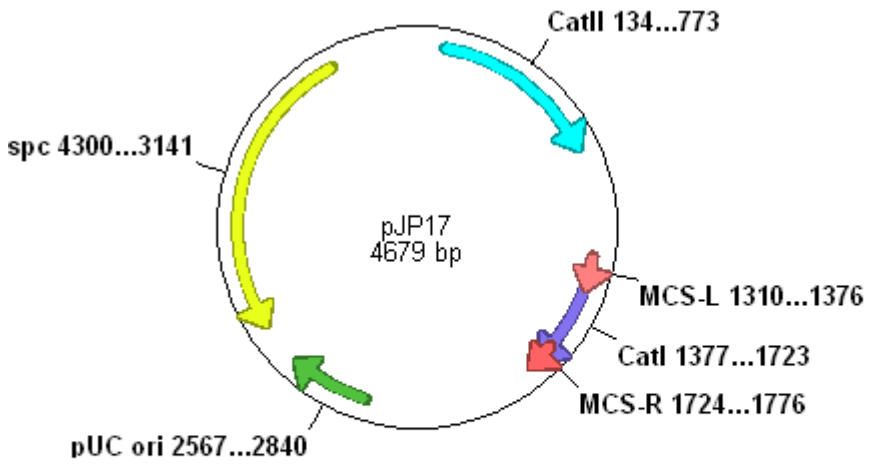


Figure 10. A map of pJP17. Plasmid pJP17 can be used as a shuttle vector to insert a pUC ori and spc resistance determinant into a Tn*10* transposon by double crossover. The numbering of bases was done such that base number one corresponds with the published sequence of it's parent vector, pBluescript II KS-.

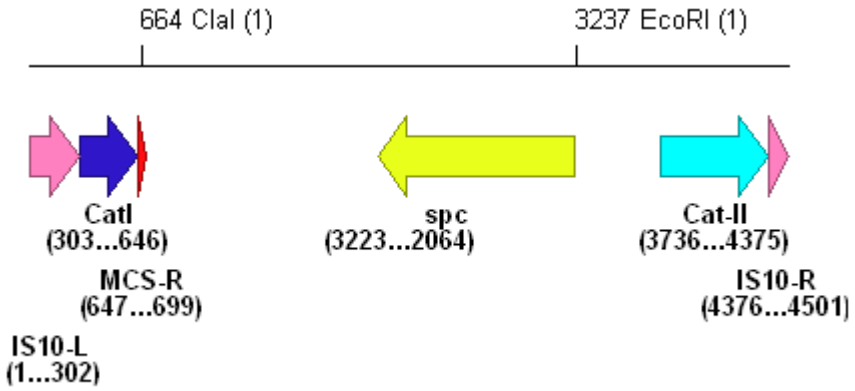


Figure 11. A map of pJP17 inserted into Tn*10*. The expected map of the pJP17 fragment after double crossover recombination in Tn*10* showing flanking sequences. IS10=inverted repeats of the insertion sequence (IS) at the ends of Tn*10* inserted sequences, used for normal Tn*10* transposition; IS10-L=bp 2016-2316 of the published Tn*10* delivery vector pHV1249 sequence containing IS10 sequences, IS10-R=bp 3520-3646 of the published Tn*10* delivery vector pHV1249 sequence containing IS10 sequences.

Pilot Test of pJP17: Cloning Of the *SpoIIAA*::Tn*10* Insert

To ensure that pJP17 could be used to identify the sites of *Tn*10 insertions, we conducted a pilot test using one of our previously identified mutants, *spoIIAA*::Tn*10*. Strain SL12147 was transformed with linearized pJP17 to insert a 4076bp fragment containing a ColE1 origin present in pUC vectors linked to a spectinomycin resistance gene (Fig. 11) The resulting transformants were streaked out on SSA plates containing either spectinomycin, erythromycin or chloramphenicol and incubated overnight to test for resistance; all strains were Spc^R, Erm^S and Cat^S. Total DNA was isolated from the resulting transformants and digested with *Cla*1, which cuts once in the insert proximal to the ColE1 and *spc* sequence (Fig. 11, appendix). Ligation of the digested DNA was performed as previously described, and resulted in Spc-resistant transformants in DH5α.

Six transformants were grown up in 1.5 ml LB, and plasmids purified. Plasmids were digested with either *Eco*R1, *Pst*1, *Xho*1, or *Nae*1 to test for the presence of inserts. All six plasmids were approximately 5.5 Kb and gave identical banding patterns which indicated a set of identical plasmids containing inserts of approximately 1.5kb. Sequencing data from one of these clones confirmed a Tn*10* insertion in *spoIIAA* (figure 12)

TNNNTAGGGANCCNNGNACAAGCACAGNATTCTTTGACATTC
ATGNTCAATTCCAAGGGCTCATGCTCACCCCCTCCNTGGATA
TGATCGGATAATGAGTGTTTCGATTTCGACGGAATGAATTCC
TTCACCGTACAAAACTAGTGGTCATTCGGCATAATTACTTAA
ATTTTGTCCAGTCTCCCATCGTCCGCTTTAAGAATGTGATAA
ACCCGGCTTTCTTCATATCTTCTTTTGCAGCAACAGGACTTT
CAGCGAGTACTTCTCCATCCTTTTTCAGAACAAGAGTGCCAA
GCTCTTGGCCTTTTTGAATCGGAGCACTAATATTGTCCTTCA
TCTTGATTTCTTTTTTCACATCGTTCATATCCTCGCCTTTTTT
CGTCAATATTGAAATCGGCTCAGATGTAGTGAGTTCGATAAA
TTTTTGTTTCCCTTTTTTGACCTTTACTTTTGCTACGTTTGAT
TTCGTTTATATAAAGGATGCGTTTCATATTGGCTAAAGGCGA
AGTCAAGCATTTTTGTCACTTGCGCGTTTCTTTCTTTAGGCG
TCTCGCTCCGAATACAACCGCTATGGCCCGCATGTTTCCTTT
TTTAGCGAAGCAGTCAGACAATATTTCGCTTCGCCTGTATAG
CCTGTTTTTACGCCGTCTACACCAGGATAAAATTTGATAAGG
CGATTTGTATTTCAAAANTTTNCCTTC

Figure 12: Sequencing data for the rescued pJP17 plasmid. pJP17 integration into SL12170 (previously identified as Tn*10*:*spoIIAA*; Tn*10* is in reverse orientation*)*. The beginning sequences agree with end of the expected Tn*10* insertion. The readable sequence highlighted in yellow correspond to *spoIIAA*, bp 42-6; the sequences highlighted in red correspond to *dacF* (which is just upstream from *spoIIAA*). This sequence agrees with our original sequence data for the site of Tn*10* insertion in this strain.

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Most Mutants Loss of Compartmentalization is Post-Stage II

Although more than 60 mutants remained to be identified, only a few displayed >50% loss of compartmentalization based on fluorescence microcopy of strains taken from plates that had been incubated overnight on SSA. To narrow down the number of mutants left to study, we decided to ensure that the defect in compartmentalization was significant by performing two tests: first, a backcross of our mutant strains was done to ensure that the loss of compartmentalization was actually linked to the Tn*10* insertion; and second, we evaluated loss of compartmentalization at an early time point.

Total DNA purified from all remaining strains was used to transform SL4 selecting for Tn*10*-linked chloramphenicol resistance. The tranformants were then subcultured two times; once on LBA and once on LBA-chloramphenicol. Single colonies were picked from the second subculture and used to inoculate a liquid culture. Total DNA was isolated and used to transform the reporter strain SL10280. Transformants were streaked to single colonies, and subcultured two times as done previously. Single colonies from the second subculture were inoculated into modified Schaeffer's spoulation medium. Mutants were scored for loss of compartmentalization, at 4 and 10 hours past the end of exponential growth by fluorescence microscopy as previously described. Only three of the backcrossed strains showed a loss of compartmentalization in >10% of the cells at the 4 hour time point. Ten hours after the end of exponential growth, a breakdown in compartmentalization was seen in most

Table 7. Phenotype of backcrossed Tn*10* mutants.

Strain	Proportion (%)[a] of GFP-expressing bacteria that displayed uncompartment-alized expression in samples taken from 2 day old SSA plates	Proportion (%)[a] of GFP-expressing bacteria that displayed uncompartment-alized expression 4 hours after the end of exponential growth	Proportion (%)[a] of GFP-expressing bacteria that displayed uncompartment-alized expression 10 hours after the end of exponential growth
SL12149	100	No GFP Expression	100 / few transformants
SL12769	100	90	100
SL12160	95	40-50	40-50
SL12207	90	0	<10
SL12161	80	<5	<5
SL12155	80	0	<5
SL12148	75	<10	10
SL12807	*50-75*	<5	20
SL12150	50	0	0
SL12153	50	0	15
SL12166	50	<5	10
SL12167	50	<5	5
SL12177	50	No GFP Expression	No GFP Expression
SL12180	50	0	15
SL12232	50	0	0
SL12233	50	0	0
SL12791	50	0	15
SL12832	40-50	10	10
SL12169	40	40-50	40-50
SL12175	40	0	<5
SL12840	Hyper GFP fluoresence, 5	0	0, <5 expressing GFP

[a]At least 100 GFP-expressing organisms were counted for each sample

strains, although at a lower percentage than previously seen in samples taken from two day old plates (Table 7).

pJP17 Efficiently Clones Tn*10* Inserts

Based on the results of our backcrosses, we focused on only those strains that demonstrated more than 30% a loss of compartmentalization at 10 hours past the end of exponential growth. Using pJP17 we were able to clone *B. subitlis* DNA flanking Tn*10* insertions from most of the remaining mutant strains which were defective in compartmentalization. Sequencing data revealed Tn*10* insertions in *spoIIAA, spoIIE, spoIIM, spoIID, spoIIIAA, spoIIIJ, spoIIIAB, spoIIIAE, ald,* and *kinA*. The final results of our screen are presented in Table 8. The mutants which displayed the most profound loss of compartmentalization were the *spoIIIE* and *spoIIAA* mutants; both of which had whole cell GFP from the earliest points of detection. However, the intensity of GFP fluorescence was far more intense in the *spoIIIE* versus the *spoIIAA* mutant. All other mutations resulted in a loss of compartmentalization too late in the sporulation process to indicate a regulatory role; mutations in the *spoIIIA* operon and *spoIIIJ* behaved according to what we had originally observed in our initial screening using the 2-part probe. Our *spoIIM* and *spoIID* mutants were blocked at stage two and developed a bi-spheric pinched prespore, as previously reported by Smith et al (1993). Somewhat unexpectedly, we observed a 20% loss of compartmentalization in our *ald* mutant, in cells viewed 10 hours past the intiation of sporulation. Although there were many additional mutants left to be identified, they did not display a sufficient loss of compartmentalization to suggest a mutation that could potentially control the regulation of compartmentalized σ^E or σ^F

activity directly. This indicated to us that this approach had been exhausted in its ability to identify new proteins which *directly* control the regulation of compartmentalized σ^E or σ^F activity. we did not seek to characterize our mutants any further. The mutation which appeared to have the most profound effect on compartmentalization was in *spoIIIE*.

Table 8. The sites of Tn*10* Insertions, and their respective phenotypes.

Strain	Identity of flanking sequences for Tn*10* insertions	Exact site of insertion:(bp)[b] and orientation of Tn*10*	Proportion (%)[a] of GFP-expressing bacteria displaying uncompartmentalized expression in samples taken from 1 or 2 day old SSA plates	Proportion (%)[a] of GFP-expressing bacteria displaying uncompartmentalized expression at 10 hours after the end of exponential growth
SL12147	*spoIIAA*	42 reverse	100	100
SL12149	*spoIIIAB*	437 reverse	100-few fluor. / 100	Not fluorescing
SL12151	*spoIIE*	1771 forward	100	Not fluorescing
SL12155	*spoIIIJ*	21 forwad	30 / 80	<5
SL12161	*spIIM*	600 forward	10 / 80	<5
SL12167	*spoIIIAB*	188 reverse	>25	< 5
SL12169	*spoIIIAA*	46 reverse	> 20 / 100	100
SL12162	*spoIIIE*	323 forward	80	100
SL12170	*spoIIAA*	42 reverse	100	100
SL12172	*spoIIIE*	397 forward	100	100
SL12173	*spoIIIAB*	429 forward	100	<10
SL12188	*spoIIIAB*	429 forward	100	<10
SL12232	*spoIIIJ*	36 forward	40 / 50	0
SL12769	*spoIIIE*	446 reverse	100	100
SL12779	*spoIID*	NA	30 / 50	0
SL12791	*spoIIE*	1125 forward	50 / >50	15
SL12799	*spoIIIAB*	437 reverse	0	0
SL12807	*ald*	188 reverse	75	20
SL12832	*spoIIIAB*	429 forward	45	10
SL12840	*kinA*	26 Forward	Hyper GFP fluoresence[c], 5	0, <5 expressing GFP

[a]At least 100 GFP-expressing organisms were counted for each sample
[b]bp numbers refer to the first base of the open reading frame following the ISO left end of the inserted Tn*10*
[c]Hyperfluoresence was evaluated visually,and is defined as more than twice the normally observed maximum fluorescence typical of our reporter control strains.

CHAPTER 4

DISCUSSION

Despite the robust screenings of two Tn*10* libraries, we failed to identify any new regulators of compartmentalization. We did however identify a number of mutations that, to varying degrees, affect the integrity of compartmentalized $\sigma^F$and σ^E activity. As expected, most of our mutations identified genes associated with stages II and III of sporulation. I will begin this discussion with an evaluation of each of our mutants to determine how they might have impacted compartmentalization. A look at the results of our screening could provide a basis for thinking that no important regulators of compartmentalization were missed that could be identified by transposon mutagenesis.

spoIIIE*::Tn*10

The most dramatic effect on compartmentalization was observed in the *spoIIIE* mutant; GFP was visible throughout the entire cell at the earliest point of detection in both our *spoIIQ-gfp* (σ^F directed) and *spoIID-gfp* (σ^E directed) reporter strains. Mutations in *spoIIIE* were the most robust examples of significant loss of compartmentalization at the onset of GFP detection. Furthermore, we saw the highest intensity of whole cell GFP fluorescence at earliest time points versus other mutants which displayed a loss of compartmentalization. According to Hilbert et al. (2004), the loss of compartmentalization in *spoIIIE* mutants results from diffusion of GFP through a hole in the septum. Although the septal peptidoglycan is initially able to provide a barrier between the prespore and mother cell, because of peptidoglycan hydrolysis, the septal

membranes retract, allowing diffusion of GFP across the septum. If this were true, we might not expect to see uniform distribution of GFP fluorescence in all cells but rather a concentration of GFP in the prespore in at least a few of the *spoIIQ-gfp* (σ^F directed) cells before the diffusion is complete. However, the whole cell fluorescence we observed in our *spoIIIE* mutants appeared to increase uniformly across the entire cell beginning at the point of its faintest visibility. Although we can not discount the possibility that diffusion is a factor, it might be more complicated. Perhaps activation of σ^F occurs in a cascade of activation which is regulated by SpoIIIE on one side of the septum; (as opposed to the occurrence of a hole and its resulting "hole cell fluorescence"). Furthermore, Burton et al (2007) reported that fusion of the septal membranes during cytokinesis does not require SpoIIIE.

spoIIAA*::Tn*10

Apart from these *spoIIIE* mutants, the only other mutant that displayed an early loss of compartmentalization was the *spoIIAA* mutant. The intensity of GFP fluorescence was significantly lower in the *spoIIAA* mutant than is normal for the *spoIIQ-gfp* reporter (Fig. 4D). The Tn*10* insertion was in reverse orientation at bp 42 of the *spoIIAA* gene. Since such a mutation should effectively turn off expression of the entire operon including *spoIIAC*, it is difficult to explain what is causing the expression of the *spoIIQ-gfp* reporter in this strain. This result suggests the possibility that either SpoIIAA or SpoIIAB repress the activity of a yet unknown alternative sigma factor for σ^F. However, there is no evidence to suggest such an alternative sigma factor does exist. Given that the

purpose of this work was to explain the compartmentalization of σ^F, it seemed inappropriate to pursue the analysis of a mutant that does not express σ^F

Another possible explanation for the observed uncompartmentalized σ^F activity in this mutant is that promoters for either the Tn*10* *cat*^{R} gene or the pJP17 *spc*^{R} gene drive promiscuous expression of the *spoIIA* operon downstream from bp 42 of the *spoIIAA* gene. In this scenario, there could be expression of *spoIIAC* and a truncated SpoIIAA protein of unknown activity.

spoIIAA*::Tn*10* and *spoIIAA*::Tn*10

Our screening identified three unique mutations in *spoIIIAB*, and one in *spoIIIAA*. The *spoIIIA* locus encodes a total of eight proteins, SpoIIIAA to SpoIIIAH, which are expressed in the mother cell during sporulation. Guillot et al (2007) have identified a second essential promoter in the *spoIIIA* locus which was originally proposed to exist approximately 10 years earlier by Straiger et al. (1996). This second promoter is located in *spoIIIAF* and is necessary and sufficient for the expression of both *spoIIIAG* and *spoIIIAH*. Expression of both promoters is directed by σ^E in the mother cell.

Transcription of each of the eight proteins encoded in the *spoIIIA* locus is required for σ^G activation, and all but one of these proteins are predicted to be membrane associated. Little is known about the function of these proteins, however SpoIIIAA shows homology to AAA-type ATPases. We had originally identified a mutation in *spoIIIAB* in our initial screen, and found that the defect in compartmentalization occurred during stage III. We proposed that since the defect occurred past stage III, it is highly unlikely that SpoIIIAB is a regulator of the compartmentalized activities of σ^F or σ^E.

Each the *spoIIIA* mutants from the second screen also lost compartmentalization after the completion of stage III. Therefore, our data are consistent with regards to SpoIIIAB being only indirectly responsible for compartmentalization.

In both screenings, we did not identify mutations in any genes downstream from *spoIIIAB*. Since it is now known that two of the genes downstream from *spoIIIAB* are regulated by a separate promoter, we can therefore eliminate *spoIIIAG* and *spoIIIAH* from being responsible for the indirect loss of compartmentalization seen in these mutants.

spoIIE*::Tn*10

Two unique *spoIIE* mutants were identified: SL12151 with a mutation at bp 1771 (aa 590); and SL12791 with a mutation at bp1225 (aa 408); in both mutants, the Tn*10* transposition was in the same orientation as spoIIE. The multi-domain structure of SpoIIE contains 10 membrane spanning segments (domain I), followed by two cytoplasmic domains. Domain II is involved in the oligomerization of SpoIIE and its interaction with FtsZ. Domain III (residues 568–827) contains a serine phosphatase. SL12151 has a Tn*10* insertion at the beginning of the serine phosphatase domain, and SL12791 has a Tn*10* insertion in domain II, upstream from the phosphatase.

Both *spoIIE* mutants had 80-100% loss of compartmentalization when taken from SA after overnight incubation at 37°C. Fluorescence microscopy of samples taken from liquid cultures at 4 and 10 hours past the initiation of sporulation showed a difference between the two strains. None of the SL12151 cells expressed GFP at either time point, indicating activation of σ^F had been blocked in SL12151. Compartmentalized GFP

expression was detected in SL12791 at 4 hours past the initiation of sporulation, and a 15% loss of compartmentalization was observed at the 10 hour time point in this strain.

As with our *spoIIAA* mutant, the most straightforward explanation for *spoIIE*'s uncompartmentalized σ^F activity is transposon-induced expression of sequences downstream from the transposon, in this case, the serine phosphatase. If the transposon completely disrupted transcription downstream from its site of insertion, than there would be no serine phosphatase available to generate active SpoIIAA, and therefore no σ^F activity. Although a lack of SpoIIAA activation could explain the disruption of σ^F activity seen in SL12151 at the 4 and 10 hour time points, it does not explain why we are seeing σ^F mediated GFP expression in SL12791 at those time points or in both strains in samples taken from SSA plates. For GFP expression to occur, promiscuous expression of the serine phosphatase seems likely. The difference in the two strains might be accounted for by the sites of insertion either allowing for translation of an intact serine phosphatase (SL12791) or a slightly impaired product (SL12151.)

spoIIM*::Tn*10* and *spoIID*::Tn*10

SpoIID and SpoIIM are required for complete dissolution of the asymmetric septum, and engulfment. SpoIID is an autolysin and the function of SpoIIM is unknown. The expected phenotype for both a s*poIID* and *spoIIM* null mutant is identical: dissolution of the septum is prevented, leading to bulging of the prespore into the mother cell, and a blockage of engulfment. At low magnification, these mutants can almost look disporic, with two equal sized lobes pinched by the septum. The uncompartmentalization

typical of these mutants at or beyond stage III is the expected consequence of a rupturing of the prespore, and spillage of its contents into the mother cell.

spoIIIJ*::Tn*10

The *spoIIIJ* mutants obtained in both screenings showed a loss of compartmentalization too late (stageIII) to seem worth pursuing. Although *spoIIIJ* is active in vegetative growing *B. subtilis*, during sporulation *SpoIIIJ* locates to the asymmetric septa. This agrees with most of the genes identified in this screen.

kinA*::Tn*10

If the compartmentalization of σ^F activity was a simple consequence of its activating protein being located only in the prespore, than the phenotype of an activating-protein-null mutant strain, would be a complete disruption of σ^F activity, and not uncompartmentalization. Therefore, a Spo$^-$ strain which did not express GFP was identified. The Tn*10* transposition of this strain occurred in the gene for *kinA*; a sensor histidine kinase involved in the initiation of sporulation. Fluorescence microscopy revealed few fluorescing cells at all time points, and a small percentage of hyper fluorescent cells. Thus this mutant did not appear to be involved in compartmentalization during sporulation. Based on the possibility that we would identify other well characterized genes, we did not further pursue the identification of weakly-fluorescing mutants.

ald*::Tn*10

The *ald* gene encodes an alanine dehydrogenase, which catalyzes the deamination of alanine to pyruvate and ammonia and is required for growth when alanine is the sole carbon or nitrogen source. Siranosian et al. (1993) reported that Ald is required for normal sporulation in *B. subtilis*; and speculated that its role in sporulation was to generate pyruvate which is metabolized in the tricarbocylic acid cycle to generate energy. The effect Ald has on compartmentalization is likely to be a consequence of the *ald* mutant's failure to generate the energy it needs to facilitate sporulation. Although we observed a <5% loss of compartmentalization 4 hours after the initiation of sporulation, that number jumped to 20% by the 10 hour time point. This indicates an important role in stages I and II of sporulation, but does not indicate an important role in compartmentalization.

Conclusion

Two different reporter systems were used to screen two unique transposon libraries in an effort to identify determininants of the compartmentalized activity of σ^F. Of the 10 different mutants identified in this study which caused a loss of compartmentalization, the *spoIIIE* mutant was unique in its early and profound disruption of compartmentalization. It appears that we have exhausted the potential for transposon mutagenesis to shed new light on the regulation of compartmentalization. This contention is supported by several factors: we had taken great care in construction of a fully representative library; this library was fully screened; and all of the mutants identified were known to be involved in sporulation.

The original question of what the role of SpoIIIE is in compartmentalization remains unanswered. All current models assign chromosome translocation as SpoIIIIE's main role in sporulation. The disruption of compartmentalization seen in *spoIIIE* null mutants is typically regarded as an indirect effect caused by diffusion of prespore contents into the mothercell. However, my results suggest otherwise. I was able to compare examples loss of compartmentalization caused by diffusion as seen in the *spoIID* and *spoIIM* mutants, with that of our *spoIIIE* mutants, and the *spoIIIE* mutant seemed to be in a different class. This class difference was true when comparing *spoIIIE* mutants with all other mutants obtained in these screens. Only the *spoIIIE* mutant qualifies to be a candidate for regulation of compartmentalization based on the following observation; the *spoIIIE* mutant displayed approximately 100% loss of compartmentalization by the end of stage II, as determined by GFP fluorescence in our σ^F directed reporter.

The robustness of our screening procedure was aided a great deal by the construction of the Tn*10* cloning vector, pJP17. The efficient cloning of Tn*10* insertions and *B. subtilis* flanking sequences by pJP17 made it possible to identify mutants we could not identify otherwise. This vector will be a useful tool in the future for others needing to clone Tn*10* transpositions. Without pJP17, we would not have identified enough of the mutants to support our contention that a transposon approach had been exhausted, and suggest that SpoIIIE is a direct regulator of compartmentalization.

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APPENDIX A

MEDIA AND SOLUTIONS

Bott and Wilson Amino Acids

Val, Lys, Thr, Gly, Asp, Met, His, Trp, and Arg all at 0.5 mg/ml in water.

Bott and Wilson Salts:1.4% K_2HPO_4; 0.6% KH_2PO_4; 0.072% anhydrous $MgSO_4$; 0.2% $(NH_4)_2SO_4$; 0.19%Na Citrate- $2H_20$

Growth Medium 1 (GM1)

Bott and Wilson salts	100 ml
Bott and Wilson Amino Acids	5.0 ml
50% Glucose	1.0 ml
1 M $MgSO_4$	0.1 ml
Yeast extract (Difco)	0.5 g
Water	to 500 ml
Filter sterilized, stored at room temperature	

Growth Medium 2 (GM2)

GM1 adjusted to 0.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, immediately before use.

Luria Bertani broth (LB)

Yeast extract (Difco)	2.5 g
Sodium Chloride	2.5 g
Tryptone (Difco)	5.0 g
Water	to 500 ml
Autoclaved, stored at room temperature	

Luria-Bertani Agar (LB agar)

LB containing 1.6% Agar (USB)

Lysis Buffer

1 M Tris•HCI pH 7.5	40 µl
0.5 M EDTA	40 µl
20 mg PMSF (phenylmethylsulfonylfluoride)/ml	20 µl
10 mg lysozyme (Sigma)/ml	200 µl
10 mg DNAse I (Sigma)/ml	10 µl

Modified Schaeffer's Sporulation Medium (MSSM)

Nutrient broth (Difco)	8.0 g
$MgSO_4 \bullet 7H_20$	0.25 g
KCl	1.0 g
Water	to 500 ml

Autoclaved, cooled to 55°C, then the following were added:

1 M $Ca(NO_3)_2$	0.5 ml
0.1 M $MnCl_2$	0.5 ml
0.1 M $FeSO_4$	0.005 ml

Schaeffer's Sporulation Agar (SSA)

Nutrient broth (Difco)	4.0 g
$MgSO_4 \bullet 7H_20$	0.125 g
KCl	0.5 g
Water	to 500 ml

Autoclaved, cooled to room temperature, then the following were added:

1 M $Ca(NO_3)_2$	0.5 ml
0.1 M $MnCl_2$	0.5 ml
0.1 M $FeSO_4$	0.005 ml

SP10 (10X)

K_2HPO_4	70.0 g
KH_2PO_4	30.0 g
Sodium citrate	5.0 g
$(NH_4)_2SO_4$	10.0 g
$MgSO_4 \cdot 7H_20$	1.0 g
$MnCl_2$(0.1 M)	0.5 ml
Water	to 500.0 ml

Tris-Acetate EDTA (TAE) Buffer, 50X

Tris base (Sigma)	242.0 g
Glacial acetic acid	57.1 ml
EDTA (sodium salt)	37.2 g
Water	to 1000 ml

Autoclaved, stored at room temperature

TE

1 M TRIS-HCl, pH 8.0	10 ml
1 M EDTA pH 8.0	1 ml
Water	1000 ml

Autoclaved, stored at room temperature

APPENDIX B

THE COMPLETE SEQUENCE OF PJP17

Ligated fragments are indicated by a change in case; the first base corresponds to base 1 of the pBluescript parent vector; the first lower case region corresponds to CatII in figure 5 (CatII= 5'end of *cat* fragement, bps 2881-3519 of Tn*10* ORF); second upper case region corresponds to pBluescript etc.

CTGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTAC

GCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTC

GCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGctctggtatttggactcctgtaaag

aatgacttcaaagagttttatgatttatacctttctgatgtagagaaatataatggttcggggaaattgtttcccaaaacac

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TGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATT

CTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAAT

GAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGCT

TACAATTTCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGAT

CGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGC

TGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGT

TGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCG

AATTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCC

CGGGCTGCAGGAATTCGATAGGATATCCTTATTATCAAGATAAGAAAGA

AAAGGATTTTTCGCTACGCTCAAATCCTTTAAAAAAACACAAAAGACCAC

ATTTTTTAATGTGGTCTTTTATTCTTCAACTAAAGCACCCATTAGTTCAAC

AAACGAAAATTGGATAAAGTGGGATATTTTTAAAATATATATTTATGTTA

CAGTAATATTGACTTTTAAAAAAGGATTGATTCTAATGAAGAAAGCAGAC

AAGTAAGCCTCCTAAATTCACTTTAGATAAAAATTTAGGAGGCATATCAA

ATGAACTTTAATAAAATTGATTTAGACAATTGGAAGAGAAAAGAGATATT

TAATCATTATTTGAACCAACTAGCTATAatcaagcttatcgatACCGTCGACCTCG

AGGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTG

CGCGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTAT

CCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAG

CCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTC

ACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGA

ATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCG

CTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGC

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CGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCT

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AGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCC

CCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTC

CAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAAC

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GGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGC

TCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCC

GGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGC

AGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCT

ACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGG

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CGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGG

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GCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATT

TAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGC

CAC

The complete sequence corresponding to figure 7: pJP17 inserted into Tn*10*, the first base corresponds to base 2016 of Tn*10*, the capital case represents pJP17 sequence.

cccaaagcgtaaccatgtgtgaataaattttgagctagtagggttgcagccacgagtaagtcttcccttgttattgtgtagccagaat

gccgcaaaacttccatgcctaagcgaactgttgagagtacgtttcgatttctgactgtgttagcctggaagtgcttgtcccaaccttg

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CTTTTAAAAAAGGATTGATTCTAATGAAGAAAGCAGACAAGTAAGCCTCCTA

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