ANALYSIS OF THE SPO0A(A257V) MUTANT OF BACILLUS SUBTILIS

by

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B.Sc., University of British Columbia, 2001

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Microbiology and Immunology)

THE UNIVERSITY OF BRITISH COLUMBIA

April, 2006

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ABSTRACT

In response to a deteriorating environment, *Bacillus subtilis* cells are capable of several alternate survival strategies including motility, competence development, secretion of proteases and surfactants, and sporulation. Members of the response regulator family of proteins play key roles regulating entry into these alternate states. In the case of sporulation, the master regulator is Spo0A. Spo0A initiates the onset of sporulation by direct or indirect activation or repression of transcription of over 500 genes within the Spo0A regulon.

The A257V mutation within Spo0A was previously identified as a mutation which abolishes the ability of *B. subtilis* cells to sporulate. *In vivo*, the mutation prevents transcription activation at both the $\sigma^A$-dependent *spoIIIG* operon promoter and the $\sigma^H$-dependent *spolIA* operon promoter, yet does not affect the ability of Spo0A to repress transcription at the *abrB* promoter. In this thesis I investigated the biochemical properties of Spo0A(A257V) to determine how the A257V mutation uncouples transcription activation from repression to lead to a sporulation negative phenotype. I demonstrated that the protein is phosphorylated efficiently by a reconstituted phosphorelay *in vitro*. I showed that Spo0A(A257V) can both repress transcription and activate $\sigma^A$-dependent transcription *in vitro*, although at a reduced level compared to wild type Spo0A. I showed that the A257V mutation did not affect the ability of Spo0A to recognize and bind specific sequences within promoter DNA, but rather that the reduction in transcription activation and repression *in vitro* could be attributed to a modest decrease in the apparent binding affinity of the mutant protein. While the reduction in apparent binding affinity could explain the *in vitro* results, it did not account for the complete lack of sporulation in *spo0A(A257V)* *B. subtilis* cells. Analysis of Spo0A expression in wild type and mutant strains indicated that Spo0A(A257V) expression was decreased as compared to Spo0A. To discriminate between mechanisms controlling the amount of Spo0A *in vivo*, strains were constructed which overexpressed wild type or mutant Spo0A proteins and used to test activation of $\sigma^A$ and $\sigma^H$-dependent promoters *in vivo*. Results from these experiments were inconclusive; the levels of induced protein may have been insufficient to activate stage II sporulation genes.
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# LIST OF ABBREVIATIONS AND SYMBOLS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CAP</td>
<td>Catabolite activator protein</td>
</tr>
<tr>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Chloramphenicol resistance</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>Erm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Erythromycin resistance</td>
</tr>
<tr>
<td>Eσ</td>
<td>RNA polymerase with associated sigma factor (&quot;holoenzyme&quot;)</td>
</tr>
<tr>
<td>HTH</td>
<td>Helix-turn-helix motif</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Kanamycin resistance</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>0A box</td>
<td>Specific Spo0A binding site encoded within DNA (5’TGNCGAA3’)</td>
</tr>
<tr>
<td>RNAP</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>RP&lt;sub&gt;C1&lt;/sub&gt;</td>
<td>Initial closed RNA polymerase-promoter complex</td>
</tr>
<tr>
<td>RP&lt;sub&gt;C2&lt;/sub&gt;</td>
<td>Final closed RNA polymerase-promoter complex</td>
</tr>
<tr>
<td>RP&lt;sub&gt;O&lt;/sub&gt;</td>
<td>Open RNA polymerase-promoter complex</td>
</tr>
<tr>
<td>RP&lt;sub&gt;init&lt;/sub&gt;</td>
<td>Initiated RNA polymerase-promoter complex</td>
</tr>
<tr>
<td>SAAR</td>
<td>Sigma-A-activating region within Spo0A</td>
</tr>
<tr>
<td>Spo0A~P</td>
<td>Activated, phosphorylated form of Spo0A</td>
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<td>Spo0A&lt;sup&gt;N&lt;/sup&gt;</td>
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<tr>
<td>σ</td>
<td>Sigma factor</td>
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ACKNOWLEDGEMENTS

When I began writing this thesis I expected that I would complete it before one of the greatest adventures of my life began. It didn’t happen. Instead, I’ve been busy working on a different kind of experiment, which (if I may say) is no where near as predictable as the experiments I’ve done as a student thus far.

I’d like to thank George for having me in his lab as an undergraduate student, employee, and graduate student. You’ve been a great mentor and I value your guidance and the experience I’ve had in your lab. I’d like to thank members of my committee, Rachel Fernandez and Lindsay Eltis, for direction and help throughout the duration of my work and for patiently waiting whilst I finish the never-ending-thesis. I’d like to thank members of the lab (past and present): Martin Richer for teaching me the basics, Brett McLeod for many discussions over coffee, and lastly my Spo0A-co-conspirator, Steve Seredick, for helping me in countless ways and indulging my chocolate, ice cream, and coffee addictions. I’d like to thank members of the Fernandez lab (past and present), especially Dave Oliver, who was always willing to answer questions from across the hall.

Lastly, I’d like to thank my family, especially a little monkey named Markus.
1. INTRODUCTION

1.1. Sporulation in Bacillus subtilis
In bacteria, regulation of gene expression facilitates adaptation to changes in the environment so that growth and division may be optimized (Lodish, 1999). Bacillus subtilis cells are capable of undergoing several alternate survival strategies in response to an environment with changing nutrients. These include production and secretion of antibiotics, motility and chemotaxis, the development of competence, and endospore formation.

The process of endospore formation is possibly the best understood example of cellular development and differentiation today. Endospores are specialized cell types formed by members of the genus Clostridium and Bacillus in response to prolonged environmental conditions that prevent continued division. Endospores are highly resistant forms that enable the cell to survive environmental stressors such as ionizing radiation, chemical solvents, and hydrolytic enzymes (Nicholson et al., 2000).

1.1.1. Endospore development
Following exhaustion of alternate survival strategies and in response to high cell density and nutrient deprivation, B. subtilis cells will enter the sporulation pathway (Freese, 1981; Grossman and Losick, 1988; Hilbert and Piggot, 2004; Sonenshein, 1989). The master regulator of the onset of this process is the response regulator SpoOA. SpoOA integrates information from a complex network of signals from both internal and external environments to initiate a genetic program that divides the cell asymmetrically and ultimately builds the endospore.

1.1.2. Sporulation morphology
The development of the endospore can be classified into seven stages based upon cell morphology (Figure 1) (Losick et al., 1986; Ryter, 1965). The first stage is characterized by condensation of duplicate copies of the chromosome and elongation to form an ‘axial filament’ of nucleoprotein that stretches longitudinally across the cell (Ben-Yehuda et al.,...
Figure 1. Sporulation morphology. The development of the endospore in *B. subtilis* can be classified into seven stages (0-VII) based upon cell morphology. Vegetative growth is classified as stage "0". Sporulation begins with condensation of the chromosome to form an axial filament along the longitudinal axis of the cell (not shown). Formation of an asymmetric septum in stage II captures one third of the chromosome in the forespore compartment. The forespore is engulfed by the mother cell during stage II. The presence of the forespore as a membrane bound vesicle in the mother cell cytoplasm marks stage III. The spore cortex and proteinaceous spore coat are deposited outside the forespore membrane during stages IV and V. Sporulation is completed upon spore maturation and mother cell lysis (stages VI and VII), releasing the endospore into the environment. Sporulation genes are named according to the stage at which mutants stall sporulation (*spoO*-*spoIV*). This figure is adapted from Seredick, 2005.
The second morphological event is an atypical cell division in which the cell divides asymmetrically to form a mother cell and forespore. During normal vegetative growth, the cell divides medially to produce two-equally sized daughter cells by forming a structure called a ‘Z-ring’ at the mid-cell. The Z-ring and associated cell division machinery constrict to invaginate the cytoplasmic membrane while at the same time direct synthesis of new cell wall material in the space between the membranes (Errington, 2003). During sporulation, the formation of a single medial Z-ring is inhibited and two Z-rings form, one at each pole (Ben-Yehuda and Losick, 2002). Constriction of one of the two Z-rings and disassembly of the other leads to formation of a polar septum at one end of the cell, creating the mother cell and forespore (Errington, 2003).

Formation of the polar septum captures approximately one-third of the chromosome in the forespore; the remainder of the chromosome is transferred through the polar septum to the forespore by the DNA transporter protein SpoIIIE (Errington, 2003). In the second half of stage II the forespore is engulfed by the mother cell. During this process the cell wall material in the septum is degraded and the septal membranes migrate around the forespore cytosol, enveloping the forespore in two membranes, one with reversed polarity (Losick et al., 1986). Fusion of septal membranes at the cell apex releases the forespore as a protoplast into a membrane-enclosed vesicle within the mother cell cytoplasm (Errington, 2003), marking stage III of endospore development.

The spore cortex and “primordial cell wall”, composed of peptidoglycan, are produced and deposited between the two membranes in stage IV (Losick et al., 1986). Endospore development continues with production of the proteinaceous spore coat from deposition of mother cell structural proteins outside the cortex, creating a tough outer shell (Losick et al., 1986). Throughout maturation the spore acquires properties that confer resistance to several environmental stressors. For example, the chromosome is secured from damage by a coating of protective low-molecular weight proteins, and the spore is protected from heat by dehydration and mineralization through uptake of large amounts of dipicolinic acid and calcium ions from the mother cell (Errington, 2003). In the last stage of development the
fully developed spore is released into the environment by lysis of the mother cell (Losick et al., 1986). When the spore is again located in a nutrient-rich environment it will germinate and outgrow into a vegetative cell (Paidhungat and Setlow, 2002).

1.1.3. The sigma factor cascade

Expression of genes required for sporulation is regulated by the sequential production of five sporulation-specific sigma factors (Errington, 1993; Piggot and Losick, 2002). The activated, phosphorylated form of Spo0A, Spo0A-P, initiates this “sigma factor cascade” through indirect regulation of transcription of the first “alternate” sigma factor, $\sigma^H$. During vegetative growth, transcription of spoOH, the gene encoding $\sigma^H$, is inhibited by the transition state regulator, AbrB (Weir et al., 1991). At the onset of sporulation, transcription of abrB is repressed by Spo0A, facilitating expression of $\sigma^H$ and expression of over 87 genes within the $\sigma^H$ regulon (Britton et al., 2002; Hahn et al., 1995). The next sigma factors produced, $\sigma^E$ and $\sigma^F$, are responsible for initiating a specific program of gene expression in the mother cell and forespore, respectively (Dworkin, 2003).

Transcription of the spoIIG and spoIIA operons, encoding $\sigma^E$ and $\sigma^F$, is activated by Spo0A prior to polar septum formation (Gholamhoseinian and Piggot, 1989), although the proteins are held in an inactive state until septum formation is complete (Errington, 2003). The temporal and spatial aspects of $\sigma^E$ and $\sigma^F$ activation are regulated. Activation of $\sigma^F$ is regulated through the activity of an inhibitory protein that binds to $\sigma^F$ whereas activation of $\sigma^E$ is regulated by post-translation processing of a pro- $\sigma^E$ form (Errington, 2003). Activation of each sigma factor takes place only in the appropriate compartment.

Engulfment of the forespore by the mother cell at the end of stage II leads to activation of a third sporulation-specific sigma factor, $\sigma^G$ (Sun et al., 1989). Expression of $\sigma^G$ is limited to the forespore as transcription of the gene encoding $\sigma^G$, spoIIG, is dependent upon RNA polymerase containing $\sigma^F$ (RNAP-$\sigma^F$) (Partridge and Errington, 1993). Activity of $\sigma^G$ is regulated post-translationally, possibly by an anti-sigma factor, and inhibition is not relieved until engulfment is completed (Partridge and Errington, 1993). Engulfment of the forespore also triggers activation of the last sporulation-specific sigma factor in the cascade, $\sigma^K$. This
sigma factor is produced in the mother cell and its activity is regulated at multiple levels. Transcription of $\text{sigK}$ occurs specifically in the mother cell under direction of RNAP-$\sigma^E$ (Kunkel et al., 1988) after excision of a prophage that is integrated at the $\text{sigK}$ locus and interrupts the coding region of the $\text{sigK}$ gene (Kunkel et al., 1990). $\sigma^K$ activity is also regulated post-transcriptionally as it remains inactive until it has been processed proteolytically to remove a short inhibitory N-terminal sequence (Kroos et al., 1989).

1.2. Regulation of sporulation
In prokaryotes, detection and response to changes in the surrounding environment is often controlled by the activity of “two-component signal transduction systems”. Hundreds of two-component systems have been identified in archaea and prokaryotes (Lohrmann and Harter, 2002; Stock et al., 2000). There are 34 two-component systems in *Bacillus subtilis* (Kunst et al., 1997) controlling such diverse processes as phosphate assimilation (Hulett, 1996), secretion of degradative enzymes, the development of competence and endospore formation (Msadek, 1999).

The simplest two-component system is composed of a sensor kinase, responsible for detection of extracellular stimuli, and a response regulator which facilitates an appropriate response (Figure 2). Communication between the two proteins occurs using a conserved mechanism involving phosphoryl group transfer. In response to external stimuli, the sensor kinase undergoes ATP-dependent autophosphorylation of a conserved histidine residue within the sensor kinase core. The response regulator catalyzes transfer of the phosphoryl group from the sensor kinase to a conserved aspartate within the response regulator. This prototypical system may be expanded to a “phosphorelay” that includes intermediate phosphotransfer proteins that serve as additional points for regulation.

Spo0A is the terminal member of the phosphorelay signal transduction system that governs the commitment of *B. subtilis* cells to sporulation (Grossman, 1995; Hoch, 1993; Stragier and Losick, 1996). Phosphoryl transfer to activate Spo0A occurs via an elaborate phosphorelay (Figure 3) whose complexity is indicative of the diversity of signals that must be integrated (Burbulys et al., 1991; Perego et al., 1994). There are five histidine kinases (KinA, B, C, D,
Figure 2. A typical two-component signal transduction system. The simplest two component signal transduction system is composed of a sensor kinase and response regulator. The sensor kinase is usually composed of an N-terminal 'sensing' domain and a C-terminal kinase core. In response to external stimuli, the sensor kinase autophosphorylates by forming a dimer (shown here as a monomer) in which one monomer phosphorylates the other monomer at a conserved histidine residue within the kinase core. The response regulator is able to facilitate a response to the stimuli upon phosphoryl transfer to a conserved aspartate residue within the receiver domain of the response regulator.
Figure 3. Activation and regulation of Spo0A. Nutrient deprivation, high cell density, and progression of the cell cycle initiate the sporulation pathway. Phosphate is transferred to Spo0A from a sensor kinase (e.g. KinA), and two phosphotransfer proteins (Spo0F and Spo0B) in a phosphorelay signal transduction system (indicated by curved arrows). Upon activation, Spo0A~P represses transcription from target promoters such as abrB and spo0Av, and activates transcription from spoIIGp, spoIIAp, and spo0Aps. Transfer of phosphate from KinA~P and Spo0F~P, and the activity of Spo0A~P, are all subject to dephosphorylation by phosphatases. Inhibition/transcription repression are indicated by \( \longrightarrow \) and activation/transcription stimulation are indicated by \( \longrightarrow \).
8
and E) capable of initiating the sporulation phosphorelay (Fabret et al., 1999; Jiang et al., 1999; LeDeaux and Grossman, 1995; Trach et al., 1991). In the case of at least KinA, B, and C, the target of each kinase is the phosphotransferase protein, SpoOF, itself a response regulator lacking an output domain (Burbulys et al., 1991; Jiang et al., 2000b; Perego et al., 1989; Stock et al., 1989a; Trach and Hoch, 1993). The phosphoryl group is transferred from SpoOF to another phosphotransfer protein, SpoOB, before reaching the final phosphoacceptor, SpoOA (Burbulys et al., 1991). Once phosphorylated, SpoOA~P initiates a genetic network which culminates in sporulation by direct or indirect regulation of the expression of over 500 genes within the SpoOA regulon (Molle et al., 2003a).

1.2.1. SpoOA structure
SpoOA shares a common architecture with other members of the response regulator family of proteins. Response regulators generally contain two domains joined by a linker of variable length; an N-terminal receiver domain, and a C-terminal output domain, although some members, such as SpoOF (Stock et al., 1989b), lack an output domain altogether. While the receiver domains of response regulators share a common fold (Stock et al., 1989a), the structures of the output domains vary. With the exception of several members involved in chemotaxis, most response regulators have DNA-binding output domains and function as activators or repressors of transcription initiation (Stock et al., 2000).

1.2.1.1. The receiver domain of SpoOA
The crystal structure of the receiver domain of SpoOA, in an unphosphorylated or phosphorylated monomer form, or in an unphosphorylated dimer form, has been determined (Lewis et al., 1999; Lewis et al., 2000b; Muchova et al., 1998). Like other response regulators it has a three-layer (αβα) sandwich architecture in a Rossman fold, with five central parallel β-sheets surrounded by five α-helices (Lewis et al., 1999; Muchova et al., 1998) (Figure 4). The site of phosphorylation is located within a pocket located at the C-terminal end of strand β3 (Lewis et al., 1999; Muchova et al., 1998). Phosphorylation of the receiver domain of SpoOA results in a concerted and conserved rearrangement of the receiver domain as in other response regulators (Birck et al., 1999; Kern et al., 1999; Lee et al., 2001; Lewis et al., 1999), which in several cases has been shown to result in N-terminal
Figure 4. Structure of the receiver domain of Spo0A. The chain is coloured from the N-terminus (dark blue) to the C-terminus (red) and the secondary structure elements are numbered (α1-α5, β1-β5). The site of phosphorylation (D56) is found at the C-terminal end of β3 (shown with arrow). The structure shown is from Bacillus stearothermophilus, differing from the receiver domain of B.subtilis Spo0A by 24 conservative substitutions (98 conserved residues). The figure is based on PDB file 1QMP deposited by Lewis et al., 1999 in the RCSB protein data bank (http://pdbbeta.rcsb.org/pdb/Welcome.do) and was constructed using PyMOL (Delano Scientific). This figure is adapted from Seredick, 2005.
dimerization (Birck et al., 2003; Da Re et al., 1999; Fiedler and Weiss, 1995; McCleary, 1996). Unlike the response regulators PhoB, NtrC, and FixJ (Birck et al., 2003; Da Re et al., 1999; Fiedler and Weiss, 1995; McCleary, 1996), the structure of the dimer form of SpoOA indicates that a dimer can be formed by unphosphorylated receiver domains through an exchange of helix α5 (Lewis et al., 2000b). However, it is suspected that this unusual dimer form may be a consequence of the low pH conditions used to promote crystallization (Lewis et al., 2000b). It is believed that N-terminal interactions after phosphorylation drive dimerization (Lewis et al., 2002).

1.2.1.2. The DNA-binding domain of Spo0A
The crystal structure of the C-terminal DNA binding domain of Spo0A, alone or in complex with DNA, has also been determined (Lewis et al., 2000a; Zhao et al., 2002) (Figure 5). Unlike the receiver domain, the DNA-binding domain is conserved only among Spo0A homologues from endospore-forming bacteria (Brown et al., 1994). The crystal structure indicates the DNA-binding domain is composed of six α-helices joined by short segments of polypeptide and contains a helix-turn-helix (HTH) motif, commonly found in DNA binding proteins (Lewis et al., 2000a). The αC and αD helices form the HTH motif used for recognition of Spo0A binding sites ("0A boxes") while helix αE composes the SAAR, or "sigma A-activating region" (Lewis et al., 2000a), shown through genetic analysis to be required for activation of σA-dependent promoters (Buckner et al., 1998; Hatt and Youngman, 1998). The crystal structure of the DNA-binding domain in complex with a consensus Spo0A-binding site (from the site of repression of the abrB gene) indicates that Spo0A DNA-binding domains bind tandem 0A boxes by forming a head-to-tail dimer (Zhao et al., 2002) (Figure 6). Hydrophobic interactions, salt bridges, and hydrogen bonds hold the monomers together in the dimer, burying 10% of the surface area of each monomer in the dimer interface (Zhao et al., 2002). The dimer interface is formed by helix αF of the upstream Spo0A monomer and helix αB of the downstream Spo0A monomer (Zhao et al., 2002). The recognition helix, αD, fits perpendicularly into the major groove of the DNA to make three base-specific contacts within the 0A box (Zhao et al., 2002). Other contacts with the DNA are non-specific interactions with the DNA backbone (Zhao et al., 2002).
Figure 5. Structure of the DNA binding domain of Spo0A. The C-terminal DNA binding domain of Spo0A contains six α-helices connected by short segments of polypeptide and a helix-turn-helix (HTH) motif (helices αC and αD). Helix αE contains the SAAR, important for activation of σA-dependent promoters. The chain is coloured from the N-terminus (dark blue) to the C-terminus (red) and the helices are labeled (αA-αF). The structure shown is from Bacillus stearothermophilus, differing from the DNA binding domain of B.subtilis by 6 conservative substitutions (62 conserved residues). The figure is based on PDB file 1FC3 deposited by Lewis et al., 2000 in the RCSB protein data bank (http://pdbbeta.rcsb.org/pdb/Welcome.do) and was constructed using PyMOL (Delano Scientific). This figure is adapted from Seredick, 2005.
Figure 6. Spo0A DNA-binding domains bind DNA as a head-to-tail dimer. Tandem molecules of the Spo0A DNA-binding domain bind the consensus Spo0A binding sequence (5'-TGNCGAA-3') as a head-to-tail dimer. The DNA is shown 5' to 3' and the structure is rotated 90° to indicate the dimer interface. The dimer interface is formed between helices αB and αF. Salt bridges, hydrogen bonds and hydrophobic interactions hold the molecules together as a dimer. The recognition helix αD fits perpendicularly into the major groove of the DNA. The SAAR (helix αE) of each monomer is located on the same side of the dimer. The molecules contact both the phosphate backbone and specific base pairs. Helices: αA, blue; αB, cyan; αC, green; αD, yellow; αE, gold. The structure is based on PDB file 1LQ1 deposited by Zhao and Varughese, 2002 in the RCSB protein data bank (http://pdbbeta.rcsb.org/pdb/Welcome.do). Figure constructed using PyMOL (Delano Scientific). This figure is adapted from Seredick, 2005.
1.2.2. Sporulation initiation signals

The initiation of sporulation involves integration of signals from both external and internal environments concerning the nutrient status of the environment, cell density, and progression of the cell cycle (Burkholder and Grossman, 2000; Perego and Hoch, 2002; Trach and Hoch, 1993).

1.2.2.1. Nutrient deprivation

One of the main signals for sporulation initiation is nutrient deprivation (Burkholder and Grossman, 2000). The nutrient status of the environment is detected by the transition state regulator CodY (Serror and Sonenshein, 1996). CodY senses intracellular levels of guanine nucleotides and branched chain amino acids; when bound to GTP or when stimulated by valine, isoleucine or leucine, CodY activity is stimulated and represses transcription of genes involved in sporulation, motility, competence, and genes required for utilization of alternate energy sources (Ratnayake-Lecamwasam et al., 2001; Serror and Sonenshein, 1996). When GTP levels decrease at the onset of stationary phase, repression of sporulation genes, such as phrA, phrE, and kinB, by CodY is alleviated (Molle et al., 2003b; Ratnayake-Lecamwasam et al., 2001). The net effect is increased transfer of phosphoryl groups to Spo0A, thus facilitating sporulation initiation.

1.2.2.2. High cell density

A second requirement for the initiation of sporulation is high-cell density (Perego and Hoch, 2002). The cell-density of the environment is detected in a mechanism reminiscent of quorum-sensing and involves the secretion and import of short peptides. The peptides are produced from proteolytic processing of the products of the phrA and phrE genes, transcribed with the gene encoding the phosphatases which they inhibit, RapA and RapE (Perego and Hoch, 1996). At low cell density, the RapA and RapE phosphatases dephosphorylate Spo0F\(\sim P\) (Jiang et al., 2000a; Perego et al., 1994). At the same time, the Phr peptide precursors are exported from the cell and processed into short peptides (Jiang et al., 2000a; Perego et al., 1991a; Rudner et al., 1991). The peptides are re-internalized by means of the oligopeptide permease (Opp) transport system to inhibit activity of the Rap
phosphatases (Bongiorni et al., 2005; Perego et al., 1994), thus allowing phosphoryl transfer to Spo0A and facilitating the onset of sporulation.

### 1.2.2.3. Cell-cycle progression

Sporulation initiation is also dependent upon cell cycle progression. If the chromosome is damaged or if there is a block in DNA replication, sporulation will not commence due to inhibition of KinA autophosphorylation by the Sda protein (Ireton and Grossman, 1992, 1994; Lemon et al., 2000; Rowland et al., 2004). Normally, transcription of the sda gene is repressed by the DnaA protein; however, sda repression is alleviated when replication is blocked or DNA is damaged, respectively (Burkholder et al., 2001).

### 1.2.3. Regulation of Spo0A activation

The modular nature of the phosphorelay facilitates many points for regulation of Spo0A activation (Figure 3). The existence of different sensor kinases permits transfer of varying levels of phosphate through the system and permit response to a variety of inputs. Although the activity of KinC and KinD produce a level of Spo0A~P sufficient for regulating abrB transcription, the activity of KinA has proved most critical during sporulation (Antoniewski et al., 1990). Autophosphorylation of KinA is inhibited by the Kipl protein, itself negatively regulated by KipA (Wang et al., 1997).

Phosphorylation of SpoOF is negatively regulated by three phosphatases, RapA, B, and E (Jiang et al., 2000a) and the activity of each of these is subject to negative regulation by small peptides (Jiang et al., 2000a; Perego, 1999). Spo0A is dephosphorylated by the Spo0E, Yisl and YnzD phosphatases (Ohlsen et al., 1994; Perego, 2001). Transcription of the kinA, spo0F, spo0A, and rapA genes is activated by Spo0A~P (Errington, 2003; Hilbert and Piggot, 2004; Molle et al., 2003a; Piggot and Losick, 2002). In addition, the genes encoding the protein precursors (phr) of the peptides required to inactivate Rap phosphatase activity are transcribed by RNAP-σH (McQuade et al., 2001), and transcription of the gene encoding σH is indirectly dependent upon activation by Spo0A~P (McQuade et al., 2001).
1.2.4. The sporulation regulatory network

Activation of Spo0A results in the modulation of expression of a complex genetic network as shown in Figure 7. During vegetative growth, intracellular levels of Spo0A~P are low due to constitutive expression of spo0A from the σ^A-dependent spo0Apv promoter, Spo0A-mediated repression of the higher-activity spo0Aps promoter (Strauch et al., 1992), and reduced phosphorylation of Spo0A by regulation of the phosphorelay as described previously. Upon detection of nutrient deprivation, high cell density, and normal cell cycle progression, there is an increase in transfer of phosphate through the phosphorelay to activate Spo0A. Activated Spo0A initiates two programs of gene expression, one specific for the forespore, the other specific to the mother cell.

1.2.4.1. Repression of abrB

An important consequence of Spo0A activation is repression of the global regulatory protein, AbrB (Strauch et al., 1990). During vegetative growth, AbrB regulates the expression of genes encoding other regulatory proteins, such as ScoC (Perego and Hoch, 1988) and σ^H (Britton et al., 2002), and sporulation genes such as spo0E (Perego and Hoch, 1991; Strauch et al., 1989), and sinI (Shafikhani et al., 2002). AbrB also represses synthesis of enzymes needed to search for alternate carbon sources (Strauch and Hoch, 1993), thus AbrB is usually thought of as a transition state regulator. Repression of abrB also permits expression of the Spo0E phosphatase (Perego and Hoch, 1991; Strauch et al., 1989), which might limit the level of Spo0A~P during the time when alternate energy sources are investigated (Phillips and Strauch, 2002). Spo0A~P-mediated repression of abrB inhibits the activity of ScoC, itself a global regulator. ScoC represses transcription of sinI (Kallio et al., 1991) and the opp operon (Koide et al., 1999; Perego and Hoch, 1988). Continued repression of abrB indirectly leads to increased levels of Spo0A~P and thus entry into stationary phase.

1.2.4.2. Alleviation of SinR repression

An additional effect of abrB repression is expression of SinI, an antagonist of a negative regulator of sporulation, SinR (Bai et al., 1993). The two proteins are encoded in the sinIR operon (Gaur et al., 1988). SinR is expressed from an internal promoter (Gaur et al., 1988)
Figure 7. Sporulation regulatory network. A complex genetic network regulated by Spo0A–P governs creation the forespore and mother cell. Genes expressed during vegetative growth are indicated at the top of the diagram and those expressed during later stages of sporulation are indicated progressively from the top. The regulatory inputs affecting transcription of each gene are indicated; positive regulation is indicated by arrow heads while short horizontal bars indicate negative regulation. This figure is adapted from Seredick, 2005.
and inhibits transcription from the spo0Aps, spoIIG, spoIIA, and spoIIE promoters (Louie et al., 1992; Mandic-Mulec et al., 1992; Mandic-Mulec et al., 1995). At the onset of sporulation, Spo0A~P repression of abrB allows synthesis of $\sigma^H$ and the subsequent transcription of sinI (Shafikhani et al., 2002). This permits expression of SinI which interacts with SinR to relieve SinR inhibition of key sporulation gene promoters (Bai et al., 1993).

1.2.4.3. Antagonism by a negative regulator of sporulation, Soj

Another key regulator of the spo0Aps, spoIIG, spoIIA, and spoIIE promoters is Soj (Cervin et al., 1998; Marston and Errington, 1999; Quisel et al., 1999; Quisel and Grossman, 2000). Soj, and another protein SpoOJ, are thought to play a role in linking chromosome partitioning with sporulation. During chromosome segregation, SpoOJ binds near the oriC and is required for proper chromosome segregation (Lin and Grossman, 1998). However, in the absence of SpoOJ, Soj binds to the spo0Aps, spoIIG, spoIIA, and spoIIE promoters to repress transcription (Cervin et al., 1998; Marston and Errington, 1999; Quisel et al., 1999; Quisel and Grossman, 2000). Repression is alleviated by SpoOJ, which may antagonize Soj by retaining Soj at the poles of cell (Marston and Errington, 1999; Quisel et al., 1999).

1.2.4.4. Induction of $\sigma^H$ expression

Of particular importance for the initiation of sporulation is de-repression of the spo0H gene which encodes the sigma factor, $\sigma^H$ (Weir et al., 1991). Expression of spo0H and post-transcriptional stimulation of $\sigma^H$-directed transcription by the ClpX protease (Liu et al., 1999; Liu and Zuber, 2000) permits expression of sporulation genes such as spoVG, required for spore coat synthesis (Rosenbluh et al., 1981), transcription of genes encoding the sensor kinases KinA and KinE (Britton et al., 2002; Predich et al., 1992), and genes encoding the phr peptide precursors required for repression of the Rap phosphatases (Britton et al., 2002). In addition, transcription of the spo0F, and fitsAZ genes is increased due to promoter switching to the $\sigma^H$-dependent promoters of these genes (Britton et al., 2002; Predich et al., 1992).
The most significant effect of $\sigma^H$ expression is induction of a positive-feedback loop that leads to increased transcription of $spoOA$ from the $\sigma^H$-dependent, Spo0A-dependent promoter of $spoOA$ ($spo0Aps$). Together, the accumulative effects of $abrB$ repression, alleviation of SinR and soj repression, and $\sigma^H$-dependent transcription are to increase intracellular levels of phosphorylated Spo0A. This increase in Spo0A~P is required for regulation of "high-threshold Spo0A" genes (Fujita et al., 2005), such as those encoding other sporulation-specific sigma factors, $spoIIA$ and $spoIIG$ (Errington, 1993; Hilbert and Piggot, 2004; Piggot and Losick, 2002), and $spoIIE$ (York et al., 1992), encoding a protein responsible for $\sigma^F$ activation (Arigoni et al., 1996; Duncan et al., 1995; Feucht et al., 1996). Expression of the $spoIIA$ and $spoIIG$ operons and subsequent activation of the $\sigma^F$ and $\sigma^E$ sigma factors directs compartmentalized gene expression in the forespore and mother cell, respectively (Errington, 1993; Hilbert and Piggot, 2004; Piggot and Losick, 2002), committing the cell to endospore formation. Activation of $\sigma^E$ permits transcription of $sigK$ and $spoIIDD$, which in turn activate transcription of the final mother cell-specific transcription factor, $gerE$. Similarly, transcription of the final forespore-specific transcription factor, $spoVT$, is activated following $\sigma^F$-directed transcription of $spoIIG$, encoding the $\sigma^G$ transcription factor, required for $spoVT$ transcription.

1.3. Transcription

The ability to adapt to cellular circumstance through differential gene expression is essential for free-living organisms, whether single- or multi-cellular. In fact, changes in the patterns of gene regulation, in addition to the evolution of new genes, may have played a significant role in generating much of the biological diversity observed today (Ptashne and Gann, 2002). In principle, regulation of gene expression may affect any step leading to a functional gene product. There are four levels of regulation common to both eukaryotes and prokaryotes. These are transcription initiation (how often, when or where a gene is transcribed into mRNA), translation (how efficiently mRNA is translated into a protein), mRNA degradation (how long each molecule of mRNA is functional), and protein degradation (how long each protein molecule is functional, if the functional gene product is protein and not RNA). Although each regulatory level impacts the amount of functional gene product ultimately produced, to initiate new metabolic or developmental activities requires induction of
transcription of new genes. Thus much of the control over regulation, from bacteria to higher eukaryotes, involves the control of initiation of transcription by RNAP (Ptashne and Gann, 2002).

1.3.1. RNA polymerase
RNAP is a multi-subunit enzyme responsible for RNA synthesis. In prokaryotes, the transcriptionally-competent core of RNAP is a stable non-covalent assembly of four subunits: two $\alpha$ subunits, one $\beta$ subunit and one $\beta'$ subunit (Darst, 2001). In vitro, the core will bind and transcribe template DNA, but in a non-specific manner (Borukhov and Nudler, 2003; Murakami and Darst, 2003; Record et al., 1996). Specificity of initiation is provided by a $\sigma$ subunit which associates more loosely with the core enzyme to form the holoenzyme (Ec) (Ptashne and Gann, 2002). The high-resolution crystal structure of core RNAP from *Thermus aquaticus* has revealed that the RNAP is reminiscent of a crab claw, with an internal channel running along its length (Borukhov and Nudler, 2003; Darst, 2001; Zhang et al., 1999). One pincer of the claw is formed primarily by the $\beta$ subunit while the $\beta'$ subunit comprises most of the other pincer. The two $\alpha$ subunits are relatively far removed from the active site, consistent with their roles in promoter recognition and as a scaffold upon which RNAP assembles (Darst, 2001). Organisms such as *Escherichia coli* and *Bacillus subtilis* produce multiple sigma factors which recognize different sequences, allowing expression of entire batteries of genes (Gross et al., 1998; Gruber and Gross, 2003; Helmann and Moran, 2002; Paget and Helmann, 2003). In *E.coli*, $\sigma^{70}$ is produced during exponential growth, whereas alternate $\sigma$-factors such as $\sigma^{38}$ and $\sigma^{54}$ are activated during stationary phase and in response to nitrogen limitation, respectively. Although sigma factors confer the ability of RNAP to recognize different classes of promoters, they can be categorized into one of two families, the $\sigma^{70}$ or $\sigma^{54}$ families (Gruber and Gross, 2003; Paget and Helmann, 2003).

1.3.2. Promoter elements
As the primary control point for transcription in prokaryotes, the process of initiation has been extensively studied. The basic elements of the transcription cycle have been elucidated through study of *E.coli* and involve RNAP complexed with the major vegetative sigma factor, $\sigma^{70}$, leading to the mechanism described below. The transcription cycle begins with
location of the promoter. The sigma subunit bound to RNAP recognizes two conserved 6-base pair sequences centered approximately 10 and 33 base pairs upstream from the transcription start site (+1), called the "-10" and "-35" hexamers (Record et al., 1996). The regions within $\sigma^{70}$ which contact the conserved sequences have been identified from mutational studies and contain HTH motifs responsible for DNA binding (Arthur et al., 2000; Gruber and Gross, 2003; Paget and Helmann, 2003; Zhang et al., 1999). Flanking sequences upstream of the -35 hexamer also contribute to promoter binding. AT-rich sequences ("UP elements"), found 40 to 60 base pairs upstream of the transcription start site, stabilize RNAP at the promoter by binding the C-terminal domain of at least one of the $\alpha$ subunits of RNAP (Estrem et al., 1998; Estrem et al., 1999; Record et al., 1996). In the absence of regulatory factors, promoter strength, the efficiency at which a given promoter is utilized by RNAP, is a function of core promoter sequence and spacing relative to an idealized consensus promoter. Deviations from this ideal reduce the amount of transcription initiation (Record et al., 1996).

1.3.3. The transcription initiation cycle
The initiation process for *B. subtilis* RNAP is generally believed to be similar to that for *E.coli* (Helmann and Moran, 2002). Structural and kinetic data for initiation of RNA synthesis in *E.coli* has been recently reviewed (Browning and Busby, 2004; Murakami and Darst, 2003; Record et al., 1996). The basic pathway presented below and the following description is based on these reviews.

$$R + P \leftrightarrow RP_{C1} \leftrightarrow RP_{C2} \leftrightarrow RP_{O1} \leftrightarrow RP_{O2} \leftrightarrow RP_{\text{init}}$$

In the case of *E.coli* the initial complex formed by RNAP at a strong promoter such as $P_{\text{lacUV5}}$ is referred to as an initial "closed" complex ($RP_{C1}$). In this complex, Ec$\sigma^{70}$ is bound to only one face of the double helix and the DNA near the start site of transcription is double-stranded or "closed". The initial closed complex is then converted to a secondary closed complex, $RP_{C2}$, following major conformational changes in RNAP and promoter DNA. In this intermediate form, RNAP makes more extensive contact with the promoter by binding additional DNA upstream and downstream of the transcription start site. Downstream of the
-10 element polymerase contacts both strands indicating that the DNA is enveloped by RNAP.

Without further changes in the extent of the RNAP-DNA interface, $\text{RP}_C$ is then reversibly converted to an initial "open" complex ($\text{RP}_{01}$) in which the DNA strands extending from the -10 element region to immediately adjacent to the start site region (-1) are opened in a 'transcription bubble'. The transcriptionally-competent, or final open complex ($\text{RP}_{02}$), is formed when the denatured region extends past the transcription start site (+1). Formation of this complex is not observed at all promoters and has been shown to be Mg$^{2+}$ and/or temperature dependent at $E. coli$ Ec$^{70}$ promoters. The mechanism behind DNA melting remains unknown despite years of research.

Once the final open complex is formed, the template strand is accessible to initiating nucleotide triphosphates (NTPs), and is primed to complete transcription initiation. The NTP complementary to the first base pair of the template DNA strand binds reversibly to the stable open complex to form the first in a series of ternary initiation complexes ($\text{RP}_{\text{init}}$). The ternary initiation complex advances by binding subsequent NTPs, the $\beta$ subunit catalyzing covalent bond formation. RNAP characteristically undergoes a process of "abortive initiation" in which short transcripts are continually synthesized and released to reform $\text{RP}_C$.

The transcription cleavage factors GreA and GreB (Borukhov et al., 1993; Sparkowski and Das, 1990) are involved in increasing the efficiency of transcription elongation. The two proteins promote cleavage of RNA in elongating complexes that are stalled (Borukhov et al., 1993; Feng et al., 1994; Izban and Luse, 1992; Komissarova and Kashlev, 1997a, b; Lee et al., 1994; Nudler et al., 1997; Reines, 1992a; Reines, 1992b). This allows RNAP to reinitiate to create a productive elongation complex (Toulme et al., 2000). Upon formation of a 7-12 nucleotide-long RNA chain, RNAP undergoes "promoter clearance", characterized by release of the $\sigma$-subunit from the core and synthesis of full-length transcripts; at this point no specific contacts between RNAP and the promoter remain.
1.4. Regulation of transcription initiation

Regulation of transcription is facilitated by proteins that either activate or repress transcription initiation by binding to specific sites on DNA and having a direct effect on the kinetics or equilibrium of the individual steps of initiation (Record et al., 1996). These key regulatory proteins, “activators” and “repressors”, underlie the ability of cells to turn genes on and off (Lodish, 1999).

1.4.1. Transcriptional activators

In prokaryotes, there are four known mechanisms whereby activators stimulate transcription from specific promoters. In the first mechanism, activators regulate recruitment of σ70 to specific promoters. In this scenario the holoenzyme is in a ‘constitutively active’ state and cooperative binding of holoenzyme with the transcriptional activator, such as CAP, leads to transcription initiation (Busby and Ebright, 1999; Lawson et al., 2004). In the second mechanism, activators such as SpoOA (Bird et al., 1996) and cl (Jain et al., 2004; Nickels et al., 2002) stimulate a conformational change in RNAP after binding which converts the RNAP-DNA complex from a closed to open complex. Conversely, some holoenzymes, such as σ54, are not constitutively active. In this case the activator, such as NtrC, activates a holoenzyme that is pre-bound to the DNA by inducing a conformational change in σ54 such that it becomes capable of initiating transcription (Ptashne and Gann, 2002; Zhang et al., 2002). A fourth proposed mechanism of transcriptional activation involves binding of an activator, such as MerR, to induce a conformational change in the conformation of promoter DNA, thus leading to initiation of transcription (Heldwein and Brennan, 2001). In this mechanism it is unclear whether MerR binds prior to σ70 or after the DNA occupied by a pre-bound, inactive σ70.

1.4.2. Transcriptional repressors

As in activation, there are multiple mechanisms whereby transcriptional repressors can reduce the frequency of transcription initiation. In the simplest case, repressors may physically exclude RNAP from binding to target promoter sequences. For example, in the bacteriophage λ, the repressor λcl and RNAP compete for binding to overlapping sites at the
promoter (Ptashne and Gann, 2002). In cases where repressor DNA-binding sites are adjacent to promoter DNA, cooperative repressor binding causes intervening regions of DNA to loop out, again excluding RNAP from binding to promoter sequences (Ptashne and Gann, 2002). Repressors can also inhibit an activator, either by preventing the activator from binding to operator sequences, as the repressor CytR inhibits CAP binding (Gerlach et al., 1990), or repressors can interfere with activator-RNAP interaction (Ptashne and Gann, 2002). Lastly, transcriptional repressors, such as the Gal repressor and AbrB, inhibit DNA-bound RNAP by inhibiting the conversion from closed to open complex (Choy et al., 1995; Choy et al., 1997).

1.5. Mechanism of activation by positive regulators

1.5.1. Catabolite activator protein (CAP)

The mechanism of activation of transcription initiation has been extensively studied in *E. coli* using the transcriptional activator, CAP. CAP is responsible for activating transcription of over one-hundred genes in the presence of the allosteric effector cAMP by binding as a dimer to specific DNA sites within target promoters and enabling RNAP to initiate transcription (Busby and Ebright, 1999; Lawson et al., 2004). There are three mechanisms whereby CAP stimulates transcription, distinguished by the position of the CAP binding site within the target promoter and whether that promoter requires additional regulatory proteins for activation. As a "class I activator", CAP can stimulate transcription initiation by simple recruitment of RNAP to promoter DNA. In this mechanism, the binding site for the CAP dimer is located upstream of the conserved promoter elements (typically located between -62 and -93), such as at the *lac* promoter (Busby and Ebright, 1999). Interactions between the DNA-bound CAP dimer and the C-terminal domain (CTD) of one of the α subunits of RNAP facilitates binding of αCTD and the remainder of RNAP to the promoter, thereby permitting formation of an initial closed promoter complex (RPCI) and stimulation of transcription initiation (Busby and Ebright, 1999). CAP can also activate transcription from promoters in which the CAP binding site overlaps the conserved -35 promoter element, as in the case at the *galP1* promoter (Busby and Ebright, 1999). In this mechanism, CAP functions as a "class II activator" and both recruits RNAP to the promoter and stimulates a post-recruitment isomerization of RNAP leading to formation of an open promoter complex (RP0) by making
three interactions with RNAP (Busby and Ebright, 1999). An interaction between the upstream CAP monomer and αCTD functions to recruit RNAP to the promoter while additional interactions between the downstream CAP monomer and the N-terminal domain (NTD) of α and σ70 facilitate isomerization from a closed to open promoter complex (Busby and Ebright, 1999). Lastly, as a “class III activator” CAP can activate transcription at promoters which encode multiple CAP binding sites within the promoter DNA sequence. In this mechanism, CAP may interact with additional regulatory proteins to recruit RNAP to the promoter, or recruit RNAP to the promoter and stimulate closed to open complex isomerization, or facilitate interaction between the additional regulatory proteins and RNAP by bending promoter DNA (Busby and Ebright, 1999).

1.5.2. Regulation of transcription by Spo0A
As a transcription factor, the activated form of Spo0A (Spo0A~P) exerts its regulatory function by binding to specific DNA sequences termed “0A boxes” via its C-terminal domain (Spiegelman et al., 1995; Strauch et al., 1990). At promoters activated by Spo0A, binding of Spo0A to 0A boxes 5’ of the transcription start site increases the rate of transcription initiation (Bird et al., 1996; Rowe-Magnus and Spiegelman, 1998). Promoters activated by Spo0A include the spoIIA, and spoIIIG operons, which encode the forespore-specific sigma factor σF, and the mother cell specific transcription factor σE (Hoch and Silhavy, 1995). In contrast, binding of Spo0A to specific DNA sequences 3’ of the transcription start site causes repression of transcription (Spiegelman et al., 1995; Strauch et al., 1990). Promoters repressed by Spo0A include the abrB, spo0F and spo0A promoters, which encode the transition state regulator AbrB, the response regulator Spo0F, and Spo0A itself (Bai et al., 1990; Chibazakura et al., 1991; Hoch and Silhavy, 1995; Strauch et al., 1990; Strauch et al., 1992).

Spo0A is a particularly compelling response regulator in that it likely activates transcription by two distinct mechanisms. At the spoIIA promoter, Spo0A interacts with RNAP containing σH while at the spoIIIG promoter, Spo0A interacts with another sigma factor, σA (Buckner et al., 1998; Hatt and Youngman, 1998; Kumar et al., 2004). At spoIIIG, Spo0A appears to compensate for overlong spacing between the conserved consensus elements to
which RNA polymerase binds (McLeod and Spiegelman, 2005; Seredick and Spiegelman, 2004). The binding sites for Spo0A overlap the upstream -35 element for RNA polymerase. Mutations in both Spo0A and σ^A specifically affecting transcription from the spoIIIG promoter have been defined (Baldus et al., 1995; Buckner et al., 1998; Hatt and Youngman, 1998; Schyns et al., 1997). The majority of the mutations within Spo0A that effect activation are located on the SAAR (Lewis et al., 2000a). In contrast, the role of Spo0A in transcription activation of σ^H-dependent promoters such as spoIIA is not understood.

1.6. Experimental rationale

A large number of mutations within Spo0A have been identified and tested. One Spo0A mutant which contains a substitution of valine for alanine at position 257 within the C-terminal domain, Spo0A(A257V), is particularly unusual (Ferrari et al., 1985; Perego et al., 1991b). Previous in vivo studies indicate that this mutant is incapable of activating transcription initiation at either the spoIIA promoter or the spoIIIG promoter, yet proficiently represses the abrB promoter (Perego et al., 1991b; Rowe-Magnus et al., 2000). These data suggest that Spo0A(A257V) binds DNA normally, but is somehow defective in stimulating transcription, perhaps due to a faulty interaction between RNAP and Spo0A(A257V). Previous determination of the crystal structure of the isolated CTD (Lewis et al., 2000a) has determined that this mutation lies in helix αF. A more recent structure of the isolated CTD complexed with DNA revealed that when the CTD alone is bound at 0A boxes such as those at the abrB gene the A257V mutation would likely indirectly weaken intermolecular contacts between Spo0A monomers (Zhao et al., 2002). The location of two suppressor mutations of Spo0A(A257V) within the dimer structure support this hypothesis. The suv4 (H162R on helix αA) and suv3 (L174F on helix αB) (Perego et al., 1991b) mutations have been proposed to suppress the A257V mutation by strengthening intermolecular interactions between two molecules of Spo0A complexed at promoter DNA (Zhao et al., 2002).

However, since Spo0A(A257V) represses transcription of the abrB gene normally the explanation that the A257V mutation disrupts dimer binding seems improbable. Adding to the confusion is the fact that the 0A boxes presumed to be involved do not always occur in pairs, such as at the Spo0A-activated spoIIA and spo0Aps promoters (Seredick and
Spiegelman, 2001; Spiegelman et al., 1995) or with the same spacing, such as at the spoIIG promoter. In addition, the flexibility in the linker region between the receiver and DNA-binding domains permits the possibility of formation of several orientations upon DNA binding. If Spo0A does activate transcription as a dimer, a recent model of Spo0A activation suggests that dimerization is mediated by interactions between the receiver domains and not by interactions between DNA-binding domains (Lewis et al., 2002), possibly minimizing the role of the A257V mutation in disruption of crucial interactions within a dimer.

Another possible explanation for the effect of the A257V mutation on Spo0A function is that it disrupts an interaction with $\sigma^H$ required for transcription activation. Rowe-Magnus et al. proposed that the C-terminal region of Spo0A (containing A257V) is a second area required for transcription stimulation (Rowe-Magnus et al., 2000). Mutations in this region might reduce interaction with $\sigma^H$. Since interaction with $\sigma^H$ is needed to increase levels of Spo0A-P which is required for stimulation of stage II sporulation genes such as spoIIA and spoIIG (Fujita et al., 2005), it seemed possible that Spo0A(A257V) was not defective in transcription initiation of $\sigma^A$ dependent genes. To determine if the A257V mutation specifically effected $\sigma^H$-dependent transcription initiation, I have conducted an in vitro characterization of A257V.

Here I have demonstrated that Spo0A(A257V) is phosphorylated normally, can bind DNA, and is able to activate and repress transcription initiation in vitro. However, the mutant protein is expressed at reduced levels as compared to Spo0A. In order to distinguish whether the reduction in Spo0A(A257V) expression was due to a defective interaction between Spo0A and $\sigma^H$ or between Spo0A dimers I analyzed activation of a $\sigma^A$- and a $\sigma^H$-dependent promoter in vivo when wild type or mutant proteins were overexpressed. Although the results from these experiments were inconclusive and require further investigation, I suggest that the A257V mutation uncouples a positive feedback loop, preventing the accumulation of Spo0A to levels sufficient to activate transcription of stage II genes required for sporulation.
2. EXPERIMENTAL PROCEDURES

2.1. Bacterial strains and media

Bacterial strains, plasmids, and oligonucleotides used in this study are indicated in Tables 1, 2, and 3. *E. coli* cultures were grown in Luria Bertani (LB) media supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol where necessary for plasmid selection. *B. subtilis* cultures were grown in LB (Sambrook *et al.*, 1989) or Schaeffer’s sporulation media (SSM; (Schaeffer *et al.*, 1965), First Growth Period Supplemented MG (Hoch, 1991), or Second Growth Period Supplemented MG (Hoch, 1991) with the addition of tryptophan and phenylalanine (10 μg/ml each), and the antibiotics erythromycin (0.3 μg/ml), chloramphenicol (5 μg/ml), or kanamycin (5 μg/ml) when necessary. Standard genetic techniques, enzymatic reactions and DNA manipulations were performed as described (Sambrook *et al.*, 1989) or as recommended by the manufacturer. *Bacillus* transformations were performed as described (Hoch, 1991).

2.2. Synthesis and cloning of *spoOA*(A257V)

Chromosomal DNA was isolated from *B. subtilis* strain JH695 as described previously (Hoch, 1991). The *spoOA*(A257V) gene was amplified by PCR using the upstream primer 0A-5 and the downstream primer 0A-4 (Table 3). The resulting 848 bp fragment was directly ligated into the pGEM-T cloning vector, creating plasmid pGEMA9V, and transformed into *E. coli* DH5α. The A257V substitution was confirmed by sequencing completed by the Nucleic Acid and Protein Service Unit, University of British Columbia. To create a plasmid construct for protein expression, pGEMA9V was digested with *Nco*I and *BamHI* to yield an 848 bp fragment encoding the full length *spoOA*(A257V) sequence, including the *spoOA*(A257V) stop codon and transcription terminator. This fragment was ligated into a *NcoI/BamHI* digested pET16b (Novagen) expression vector creating plasmid pET16bA9V. pET16bA9V was transformed into *E. coli* BL21(DE3)pLysS, creating strain BTA9V.
Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>[hsdR17(rl−,m−+) supE44 Thi-1 recA1 gyrA (Nal') relA1 Δ(lacZYA-argF)U169 (φ80lacZΔM15)]</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>BL21(λDE3)pLysS</td>
<td>F' ompT hsdSB (F' mB) gal dcm (DE3) pLysS(CmR)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>BTA9V</td>
<td>BL21(λDE3)pLysS pET16bA9V</td>
<td>This study</td>
</tr>
<tr>
<td>MC0A</td>
<td>BL21(λDE3)pLysS pET16b0A</td>
<td>Lab Stock</td>
</tr>
<tr>
<td>B. subtilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JH642</td>
<td>trpC2 phe-1</td>
<td>J. Hoch, Scripps Research Institute La Jolla California USA</td>
</tr>
<tr>
<td>JH695</td>
<td>trpC2 phe-1 spo0A(A257V)</td>
<td>Ferrari et al, 1985</td>
</tr>
<tr>
<td>JH16124</td>
<td>trpC2 phe-1 amyE::(spoIIA-lacZ), CmR</td>
<td>M. Perego, Scripps Research Institute La Jolla California USA</td>
</tr>
<tr>
<td>JH16304</td>
<td>trpC2 phe-1 amyE::(spoIG-lacZ), KanR</td>
<td>M. Perego, Scripps Research Institute La Jolla California USA</td>
</tr>
<tr>
<td>BT2001</td>
<td>JH642 spo0A::(pMNSpo0AN), ErmR</td>
<td>This study</td>
</tr>
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<td>BT2002</td>
<td>JH695 spo0A::(pMNSpo0AN), ErmR</td>
<td>This study</td>
</tr>
<tr>
<td>BT2003</td>
<td>BT2001 amyE::(spoIIA-lacZ), CmR</td>
<td>This study</td>
</tr>
<tr>
<td>BT2004</td>
<td>BT2001 amyE::(spoIIG-lacZ), KanR</td>
<td>This study</td>
</tr>
<tr>
<td>BT2005</td>
<td>BT2002 amyE::(spoIIA-lacZ), CmR</td>
<td>This study</td>
</tr>
<tr>
<td>BT2006</td>
<td>BT2002 amyE::(spoIIG-lacZ), KanR</td>
<td>This study</td>
</tr>
</tbody>
</table>

*a* spoIIA-lacZ and spoIIG-lacZ are integrated in the amyE gene; pMNSpo0AN is integrated into the spo0A gene; CmR, chloramphenicol resistance; KanR, kanamycin resistance; ErmR, erythromycin resistance.

*b* all *B. subtilis* strains were derived from the parent JH642 and contain the trpC2 and phe-1 mutations.
Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM-T</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt; vector used for cloning PCR products</td>
<td>Promega</td>
</tr>
<tr>
<td>pGEM-A9V</td>
<td>pGEM-T with a 848 bp insert bearing the spo0A(A257V) coding sequence, start and stop codons and transcription terminator.</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-Spo0A</td>
<td>pGEM-T with an 831 bp insert bearing the spo0A ribosome binding site and coding sequence up to E263.</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-0AN</td>
<td>pGEM-T with a 505 bp insert bearing the spo0A ribosome binding site and coding sequence up to P146.</td>
<td>This study</td>
</tr>
<tr>
<td>pET16b</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt; vector used for protein expression</td>
<td>Novagen</td>
</tr>
<tr>
<td>pET16b-0A</td>
<td>pET16b with a 848 bp Ncol-BamHI insert bearing the spo0A coding sequence, start and stop codons and transcription terminator.</td>
<td>Lab stock</td>
</tr>
<tr>
<td>pET16b-A9V</td>
<td>pET16b with a 848 bp Ncol-BamHI insert bearing the spo0A(A257V) coding sequence, start and stop codons and transcription terminator.</td>
<td>This study</td>
</tr>
<tr>
<td>pMUTIN-4</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, Erm&lt;sup&gt;R&lt;/sup&gt; integration vector used for systematic inactivation of coding sequences in <em>B. subtilis</em>. Features a spo0V-lacZ translational fusion, the LacI-repressed/IPTG-inducible Pspac promoter, lacI, ermAM, bla, and T0, T1, and T3 transcription terminators.</td>
<td>Vagner et al., 1998</td>
</tr>
<tr>
<td>pMNSpo0A</td>
<td>pMUTIN-4 with a 2025 bp deletion (<em>Aspo0V-lacZ</em>&lt;sup&gt;1-1934&lt;/sup&gt;) and a 874 bp insert bearing the spo0A ribosome binding site and coding sequence up to E263.</td>
<td>This study</td>
</tr>
<tr>
<td>pMN0AN</td>
<td>pMNSpo0A with a 855 bp deletion (<em>Aspo0A</em>) and a 481 bp insert bearing the spo0A ribosome binding site and coding sequence up to P146.</td>
<td>This study</td>
</tr>
<tr>
<td>pUCII-GtrpA</td>
<td>pUC19 with a 240 bp HindIII to BamHI DNA fragment bearing the spo0IIG promoter and 100 bp downstream of the transcription start site encoding the trpA terminator.</td>
<td>Satola et al., 1991</td>
</tr>
<tr>
<td>pJM5134</td>
<td>Cloning vector with an 814 bp DNA fragment bearing the -703 to +37 region of abrB, relative to the P2 promoter start site. Includes two abrB transcription initiation sites (P1, P2) and two downstream and one upstream 0A box.</td>
<td>Perego et al., 1988</td>
</tr>
<tr>
<td>pUCII-GtrpA&lt;sub&gt;down&lt;/sub&gt; 1</td>
<td>pUCII-GtrpA with mutation of the site 2.1 0A box</td>
<td>Seredick et al., unpublished</td>
</tr>
<tr>
<td>pUCII-GtrpA&lt;sub&gt;down&lt;/sub&gt; 2</td>
<td>pUCII-GtrpA with mutation of the site 2.2 0A box</td>
<td>Seredick et al., unpublished</td>
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Table 3. Oligonucleotides used in this study

<table>
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<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>OA-5</td>
<td>5’-CGCCATGGGAGAAATTAAAGTTTGTGGT-3’</td>
</tr>
<tr>
<td>OA-4</td>
<td>5’-CGGGATCCCAAAGACGTTTTGAT-3’</td>
</tr>
<tr>
<td>abrB-F</td>
<td>5’-AAGGATTTTGTCAATAATGACGAA-3’</td>
</tr>
<tr>
<td>abrB-R</td>
<td>5’-TCTTCGTCATTATTCGACAAATCC-3’</td>
</tr>
<tr>
<td>IIG 2X</td>
<td>5’-GGGGATCCCTCGAGGTCA-3’</td>
</tr>
<tr>
<td>M13R</td>
<td>5’-CAGGAAACAGCTATGACC-3’</td>
</tr>
<tr>
<td>BT0ARBS</td>
<td>5’-AAGCTTGGTTGAATCCTGTTA-3’</td>
</tr>
<tr>
<td>0AEco</td>
<td>5’-TCTAACCTCGCTATTCCCGC-3’</td>
</tr>
<tr>
<td>BT0Alinker</td>
<td>5’-GTGACAGGCTGGCTGCTGCTATAAT-3’</td>
</tr>
</tbody>
</table>

2.3. Expression and purification of Spo0A(A257V)

Spo0A and Spo0A(A257V) were expressed and purified from strains MC0A and BTA9V as described previously (Seredick et al., 2003) up to and including concentration of protein following heparin-agarose affinity purification. Concentrated protein was dialyzed overnight at 4°C against 2 L of buffer C (20mM sodium phosphate (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF)) + 50mM NaCl. Following dialysis the protein was loaded directly onto a 30 ml DNA-cellulose column equilibrated with buffer C + 50 mM NaCl and eluted with a 150 ml linear gradient of 50-850 mM NaCl in buffer C. Samples were analyzed by SDS-PAGE and those containing Spo0A or Spo0A(A257V) were pooled and concentrated by placing in dialysis tubing and surrounding with PEG20,000 at 4°C for approximately 4 hours. The concentrated protein was dialyzed overnight at 4°C against 2 L of buffer C containing 150 mM NaCl, 0.1 mM dithiothreitol (DTT) and 30% glycerol. Purified Spo0A and Spo0A(A257V) were aliquoted and stored at -20°C. The final products were approximately 95-98% pure as determined by spot densitometry of silver stained SDS-PAGE gels and was used directly for in vitro studies.

2.4. In Vitro phosphorylation reactions

2.4.1. In vitro phosphorylation at equilibrium

Spo0A and Spo0A(A257V) were activated as previously described (Bird et al., 1993). Briefly, 4 μM Spo0A and Spo0A(A257V) were incubated with 1.56 μM KinA, 0.1 μM
SpoOB, 2 μM SpoOF, 10 μM ATP in 1x transcription buffer (Bird et al., 1993) for 2 hours at 25°C. The phosphorelay proteins used in activation of wild type and mutant Spo0A proteins were isolated as described previously (Grimshaw et al., 1998; Zapf et al., 1996; Zhou et al., 1997).

In vitro phosphorylation of Spo0A and Spo0A(A257V) was compared by incubating 0.5, 1.0, or 2.0 μM Spo0A or Spo0A(A257V) with 0.75 μM KinA, 0.1 μM Spo0B, 0.5 μM Spo0F, 25 μM ATP, and 30 μCi of [γ-32P]ATP (6000Ci/mM; Amersham Biosciences) in 1x transcription buffer in a final volume of 10 μl for 4.5 hours at 25°C. Labeled proteins were separated by electrophoresis through 15% SDS-PAGE and detected using a Molecular Dynamics PhosphorImager SI. The level of protein phosphorylation was quantified using ImageQuant 5.2 software.

2.4.2. Rate of in vitro phosphorylation
The in vitro phosphorylation rates of wild type and mutant Spo0A proteins were determined by incubating a large reaction (140 μl) of phosphorelay components (0.75 μM KinA, 0.1 μM Spo0B, 0.5 μM Spo0F, 25 μM ATP and 25 μCi of [γ-32P]ATP (6000Ci/mM; Amersham Biosciences) in 1x transcription buffer at 25°C. Aliquots (10 μl) were removed at 10 seconds and 1, 2, 4, 6, 8, 10, and 12 minutes following addition of 0.5 μM Spo0A or Spo0A(A257V) and were added directly to 5 μl of 2x SDS-PAGE buffer (100 mM Tris-Cl, pH 6.8, 4 % SDS, 0.2 % bromophenol blue, 20% glycerol, 2 mM β-mercaptoethanol). Labeled proteins were separated by electrophoresis through 15% SDS-PAGE and detected using a Molecular Dynamics PhosphorImager SI system. The incorporation of radioactive phosphorus was quantified using ImageQuant 5.2 software.

2.5. In vitro transcription reactions
2.5.1. Preparation of template DNA
A 600 bp DNA fragment generated from PvuII digestion of either pUCIIGtrpA, pUCIIG2.1down, or pUCIIG2.2down plasmids were used as DNA templates for spoIIIG in vitro transcription reactions. The PvuII fragments were separated by electrophoresis though an 8% polyacrylamide gel in 1x TBE buffer and recovered using a QIAquick Gel Extraction
Kit (Qiagen Inc, Mississauga Ontario). DNA template encoding the *abrB* promoter used for *in vitro* transcription reactions was a 804 bp fragment recovered as described above from *EcoRI/HindIII* digest of pJM5134 (Greene and Spiegelman, 1996; Perego et al., 1988). This fragment includes both *abrB* transcription initiation sites (P1, P2) in addition to a pair of downstream 0A boxes and one upstream 0A box, but lacks a transcription terminator.

### 2.5.2. *In vitro* transcription reactions

*In vitro* transcription assays to test the stimulatory or inhibitory effects of different forms of Spo0A were completed as described previously (Bird et al., 1993; Greene and Spiegelman, 1996). For *spoIIG* transcription, template DNA (4 nM) and 0-1200 nM unphosphorylated or phosphorylated Spo0A and Spo0A(A257V) were incubated with the initiating nucleotides, ATP (0.4 nM) and GTP (5 μM), in 1x transcription buffer for two minutes at 37°C. RNAP containing σ^A^ (Dobinson and Spiegelman, 1987) (20nM) was added to the reaction to permit transcription initiation. After two minutes (for equilibrium assays) or 0-90 seconds (for kinetic assays), UTP, CTP (0.4 nM each) and heparin (5 μg/ml final concentration) were added to allow elongation of initiated complexes. For *abrB* transcription, template DNA (4 nM) and unphosphorylated or phosphorylated Spo0A and Spo0A(A257V) was incubated with ATP, GTP and UTP in 1x transcription buffer for three minutes at 37°C. RNAP-σ^A^ (20nM) was added to the reaction and the mixture allowed to equilibrate for 2 minutes before the addition of CTP and heparin. The reactions were terminated after five minutes by the addition of 5 μl of stop buffer (7 M urea, 0.1% bromophenol blue, and 0.1% xylene cyanol in 0.5x TBE (Sambrook et al., 1989). Transcripts were separated by electrophoresis through an 8% polyacrylamide gel containing 7 M urea and detected using a Molecular Dynamics PhosphorImager SI system. The amount of transcripts produced was quantified using ImageQuant 5.2 software.

### 2.6. *In Vitro* DNase I footprinting assay

#### 2.6.1. Preparation of template DNA

Forty micrograms of pUC119gstA plasmid DNA was incubated with 30 U *BamH*I to linearize the vector 135 bp downstream of the +1 transcription start site of the *spoIIG* promoter. The linearized vector was dephosphorylated by two sequential 45 minute
incubations with 20 U of calf intestinal alkaline phosphatase (CIAP). The dephosphorylation reaction was terminated with the addition of 0.1% SDS and 20 mM ethyleneglycol-bis(β-aminoethyl)-N,N',N'-tetraacetic Acid (EGTA). CIAP was removed from the reaction by phenol-chloroform extraction and the DNA was ethanol precipitated. Dephosphorylated template DNA was end-labeled with \([\gamma^{32}\text{P}]\text{ATP}\) by resuspension of the precipitated DNA in 1x forward kinase buffer (0.5 M Tris-HCl, pH 7.6; 0.1 M MgCl\(_2\); 50 mM DTT; 1 mM spermidine; 1 mM EDTA, pH 8.0) and incubation with 1333 μCi \([\gamma^{32}\text{P}]\text{ATP}\) (6000 Ci/mmol) (ICN Biomedicals) and 12 U T4 kinase (Invitrogen) at 37°C. Following 45 minutes incubation, the reaction was stopped by incubation at 60°C for 15 minutes and the end-labeled DNA was treated with 80 U \(Pvu\)I for 3 hours to generate 111 bp, 411 bp and 2394 bp fragments from cleavage of \(pUC119\) at sites within the vector backbone. The DNA fragments were separated by electrophoresis through a 5% non-denaturing gel and located by autoradiography. The piece of the acrylamide gel containing the 411 bp DNA fragment with the \(spoIIG\) promoter was excised from the gel and incubated in 800 μl of passive elution buffer (500 mM NH\(_4\)Ac; 10 mM MgAc; 10 mM Tris, pH 7.9; 1 mM EDTA, pH 8.0; 0.1 % SDS) at room temperature. After 12 hours incubation, the labeled DNA fragment was recovered by ethanol precipitation and the total radioactivity in the re-dissolved DNA sample was determined by measuring the Cerenkov radiation in an aliquot.

2.6.2. *In vitro* DNasel footprinting reactions

DNase I footprinting assays to determine the location of Spo0A−P and Spo0A(A257V)−P binding were carried out as described previously (Bird *et al.*, 1996). Briefly, 2.0 \(\times 10^5\) CPM \([\gamma^{32}\text{P}]\text{ATP}\) end-labeled \(spoIIG\) promoter DNA was incubated with 0-600 nM Spo0A−P or Spo0A(A257V)−P in 1x T2 transcription buffer (40 mM Hepes, pH 8.0; 10 mM MgAc; 1 mM EDTA, pH 8.0; 1 mM DTT; 1 mg/ml acetylated BSA) at 37°C. Control reactions contained only \(spoIIG\) promoter DNA in 1x T2 transcription buffer. After two minutes incubation, DNasel was added to the reactions at a final concentration of 4 μg/ml and allowed to cleave the DNA for 10 seconds before the reaction was terminated with the addition of 75 μl of DNase stop buffer (0.1 % SDS; 4 mM EDTA, pH 8.0; 270 mM NaCl; 40 μg/ml salmon sperm DNA). The reactions were ethanol precipitated and resuspended in 5 μl of formamide loading buffer (80 % formamide; 10 mM EDTA, pH 8.0; 1 mg/ml xylene
cyanol; 1 mg/ml bromophenol blue) and the activity of labeled DNA in each reaction was estimated from Cerenkov radiation in the sample. Approximately 1.13 x 10^5 CPM of labeled fragments from each reaction was separated by electrophoresis through a 6% sequencing polyacrylamide gel. Following 3 hours electrophoresis at 2000 V, the gel was dried at 80°C for 1 hour and the DNasel protection patterns of Spo0A~P and Spo0A(A257V)~P were detected by autoradiography.

2.7. Electrophoretic mobility shift assay (EMSA)

2.7.1. Preparation of template DNA
To assess binding of phosphorylated and unphosphorylated Spo0A and Spo0A(A257V) to consensus 0A boxes, template DNA was prepared by annealing complementary oligonucleotides abrB-F and abrB-R (Table 3) (Alpha DNA, Montreal Quebec) to form a 23 bp duplex DNA with two-base pair overhangs at both 5’ and 3’ ends. The duplex sequence matched that of abrB gene from +3 to +29 and contained two 0A boxes which exactly matched the consensus 0A binding site. Duplex DNA (10 pmol) was labeled by filling in the two base overhangs by incubation with 0.016 U Klenow Fragment (Invitrogen), 16.6 μM dGTP, 16.6 μM dATP, 20 μM dTTP (Amersham Biosciences), and 30 μCi [α-32P]dTTP (3000Ci/mmol) in 1x React 2 buffer (Invitrogen) in a final volume of 30 μl for 30 minutes at 25°C. The labeled oligonucleotide duplex was precipitated, re-suspended in 10 mM Tris, 0.1 mM EDTA (pH 7.6) and the amount of incorporated radioactivity was estimated from the Cerenkov radiation in an aliquot.

2.7.2. Electrophoretic mobility shift assay
Electrophoretic mobility shift assays were performed as described previously (Seredick et al., 2003). To test binding to consensus 0A boxes encoded within abrB DNA, unphosphorylated and phosphorylated Spo0A and Spo0A(A257) were incubated with 5 x 10^4 CPM (8 nM) of labeled duplex DNA in 1x transcription buffer at 37°C. After two minutes, 3.3 μl of loading buffer (0.3 mg/ml sonicated calf thymus DNA and 20% glycerol in 1x transcription buffer) was added to each sample and the reactions were loaded onto a running 8% polyacrylamide gel (40% acrylamide:1.38% bisacrylamide) in 0.8x TAE (Sambrook et al., 1989) containing 2% glycerol. Protein-DNA complexes were separated from unbound
duplex DNA by electrophoresis at 12 V cm⁻¹ for 1.5 hr and detected using a Molecular Dynamics PhosphorImager SI system. The fractional saturation of DNA binding was determined at each protein concentration. The dissociation constant (K_d) for binding of phosphorylated and unphosphorylated Spo0A and Spo0A(A257V) was determined by fitting the normalized percent duplex DNA bound into a one-site ligand binding model (y = B_max * x/kd + x) using the program SigmaPlot version 8.0 (SPSS). This calculation assumes that Spo0A binds as a dimer to the two 0A boxes which act as a single site on the duplex.

2.8. Cloning of Pspac-spo0A(1-146)
Plasmid pMNOAN is the integrative vector used to create strains of B. subtilis which encode a copy of spo0A or spo0A(A257V) under control of the LacI/IPTG-inducible Pspac promoter. This plasmid was created in a two-step process which involved removing most of the spoVG-lacZ translational fusion from the vector pMUTIN4 in the first step and sub-cloning the N-terminal sequence of spo0A into this construct in the second step.

pMUTIN4 is an integrative Bacillus vector designed to separate a coding sequence from its natural regulatory regions (Figure 8A). The plasmid contains a multiple cloning site upstream of the Pspac promoter, an origin of replication for growth and maintenance in E.coli (oriE.coli), selectable markers for both E.coli and Bacillus (bla and ermAM), a lacI cassette, a spoVG-lacZ translational fusion to follow expression via β-galactosidase activity, and the transcription termination sequences T₀, T₁, and T₂. To remove the spoVG ribosome binding site and create a truncated, non-functional copy of the lacZ gene (Welply et al., 1980), pMUTIN4 was treated with HindIII and ScaI to remove 2025 bp from the vector. The 6585 bp vector backbone was ligated directly with an 874 bp fragment generated from a similarly digested plasmid, pGEMSpoOA, to create the integrative vector pMNSpo0A (Figure 8B, C). The plasmid pGEMSpo0A had been created by ligation of the cloning vector pGEM-T with an 831 bp PCR product generated by amplification of the spo0A locus from isolated B. subtilis JH642 chromosomal DNA using primers BT0ARBS and 0AEco (Table 3). pGEMSpo0A encodes the first 263 amino acids of the spo0A coding sequence and 37 bp of sequence upstream of the GTG start codon which encodes the spo0A ribosome binding site.
Figure 8. Construction of $P_{\text{spac}}$-*spo0A*(1-146). (A) pMUTIN4 is an integrative Bacillus vector designed to separate a coding sequence from its natural regulatory regions. The plasmid contains a multiple cloning site upstream of the LacI-repressed/IPTG-inducible $P_{\text{spac}}$ promoter, an origin of replication ($\text{ori}_{E.\text{coli}}$) for growth and maintenance in *E. coli*, selectable markers for both *E. coli* and *Bacillus* (bla and *ermAM*), a lacI cassette, a $spoVG$-*lacZ* translational fusion to follow expression via $\beta$-galactosidase activity, and the transcription termination sequences $T_0$, $T_1$ and $T_2$. The $HindIII$-$SacI$ region of pGEMspo0A (B) was inserted into $HindIII$-$SacI$ digested pMUTIN4 to create pMNSpo0A (C), replacing the $spoVG$-*lacZ* translational fusion in the parent vector with sequences encoding a truncated *spo0A* gene and the *spo0A* ribosome binding site in pMNSpo0A. To construct the $P_{\text{spac}}$-*spo0A* strains, the $HindIII$-$SalI$ region of pMNSpo0A was replaced with the $HindIII$-$SalI$ region of pGEM0AN (D) to create pMN0AN (E). This plasmid contains the same elements as pMUTIN4 but the $spoVG$-*lacZ* translational fusion has been replaced by sequences encoding the N-terminal domain of *spo0A* (amino acids 1-146) and the *spo0A* ribosome binding site.
To create an integrative vector construct that encoded only the N-terminal domain of spoOA under the control of Pspac, JH642 chromosomal DNA was used to amplify the first 146 amino acids of spoOA using the primers BT0ARBS and BT0Alinker (Table 3). The 481 bp PCR product was ligated directly into pGEM-T to create pGEM0AN. HindIII/SalI digest of pGEM0AN (Figure 8D) generated a DNA fragment encoding the N-terminal domain of spoOA. This fragment was ligated with the 6604 bp HindIII-SalI fragment from pMNSpoOA. The resulting construct, pMN0AN (Figure 8E), encodes all the same elements as the parent vector pMUTIN4 but has the spoVG-lacZ translational fusion replaced by the spoOA ribosome binding site and sequences encoding the N-terminal domain of spoOA (amino acids 1-146). The sequence of the spoOA gene was confirmed to be free of errors by sequencing completed by the Nucleic Acid and Protein Service Unit, University of British Columbia.

2.9. Construction of Pspac-spoOA and Pspac-spoOA(A257V) B. subtilis strains

To create strains of B. subtilis which encoded a copy of spoOA or spoOA(A257V) under control of the LacI-repressed/IPTG-inducible Pspac promoter, JH642 and JH695 were transformed with pMN0AN as described previously (Hoch, 1991). Briefly, JH642 and JH695 were grown in First Growth Period Supplemented MG media (Hoch, 1991) until stationary phase. The cultures were induced to develop competence by causing amino acid starvation through dilution of the cultures with Second Growth Period Supplemented MG media (Hoch, 1991) and incubation with 2.5 μg pMN0AN. Transformants were selected by erythromycin resistance (0.3 μg/ml) and screened by western blot for the ability to express Spo0A or Spo0A(A257V) in response to IPTG induction using 0, 1, and 4 mM IPTG.

2.10. Determination of sporulation frequency

Sporulation frequency was determined as described previously (Hoch, 1991). Briefly, cultures of B. subtilis were grown at 37°C in SSM supplemented with 10 μg/ml tryptophan and phenylalanine, the appropriate antibiotic, and IPTG when required. After 28 hours growth the cultures were sampled and the total number of viable cells per ml of culture was determined by serial dilution and plating on LB agar supplemented with antibiotic (if required). The number of spores was determined by treating each dilution with 0.1 volume of chloroform and plating onto LB agar containing the appropriate antibiotic (if required).
After 24 hours incubation, the number of colonies on plates containing between 30 and 300 colonies were counted and the sporulation frequency determined. Sporulation frequency is defined as the number of colony forming units before and after chloroform treatment.

2.11. Immunoblot analysis

*B. subtilis* cultures were grown in SSM at 37°C and 10 ml samples were collected at selected times and harvested by centrifugation. The cell pellets were rinsed with wash buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 10% glycerol; 1M KCl; 1.7 mM PMSF) and resuspended in 1 ml of lysis buffer (20 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0; 300 mM KCl; 100 mM MgCl₂; 1.7 mM PMSF; 0.1 mM DTT) prior to breaking open the cells by sonication (3 x 30 sec, level 4, Sonicator® Ultrasonic Liquid Processor Model XL2020, Misonix Inc). The protein concentration of whole-cell extracts was determined by absorbance at 595 nm in a Bradford Assay (BioRad Inc) using bovine serum albumin as a standard. Purified recombinant Spo0A or Spo0A(A257V) (11-50 ng used as standards) and equivalent amounts of extracts (10-25 μl) were separated by electrophoresis through 12% SDS-PAGE and transferred onto nitrocellulose membranes (BioRad) by electrophoresis. The membranes were blocked overnight at 4°C in phosphate-buffered saline (PBS) containing 0.1 % Tween-20 (PBST) and 5 % skim milk. The membranes were washed four times in PBST for 8 minutes each before incubation with a 1:5000 dilution of rabbit-anti-Spo0A antiserum in PBST containing 2% skim milk at room temperature with gentle agitation. The anti-Spo0A antiserum was generously provided by the lab of C. P. Moran, Jr (Emory University, Atlanta, Georgia). After 1 hour incubation, unbound anti-Spo0A antibodies were removed by washing the membranes four times in PBST for 8 minutes each. The membranes were then probed with a 1:10 000 dilution of secondary antibody (donkey anti-rabbit IgG Horseradish Peroxidase-linked whole antibody, GE Healthcare) in PBST containing 2 % skim milk for 30 minutes at room temperature with gentle agitation. Unbound antibodies were removed by washing twice with PBST and twice with PBS for 8 minutes each. The Spo0A and Spo0A(A257V) proteins were then detected using ECL Western blotting detection reagents (GE Healthcare) as described by the manufacturer and exposure to X-ray film.
2.12. β-Galactosidase assay

2.12.1. Construction of LacZ reporter strains

The spoIIG and spoIIA promoter fusions were used as reporters of Spo0A and Spo0A(A257V) activity in strains encoding mutant or wild type proteins under transcriptional control of the Pspac promoter. To create reporter strains, chromosomal DNA was isolated from B. subtilis strains 16124 and 16304 containing the spoIIG-lacZ and spoIIA-lacZ fusions, respectively (Perego et al., 1991b; Rowe-Magnus et al., 2000). BT2001 and BT2002 were transformed with 2.5 ng chromosomal DNA as described previously and clones containing the fusions were selected for by plating on LB supplemented with erythromycin (0.3 μg/ml) and 5 μg/ml kanamycin (for spoIIG-lacZ) or chloramphenicol (for spoIIA-lacZ). Transformants were screened by sporulation assay and by western blot for the ability to express Spo0A or Spo0A(A257V) in response to IPTG induction.

2.12.2. β-Galactosidase activity

To assay for β-galactosidase activity, 50 ml of SSM supplemented with the appropriate antibiotic was inoculated with a 1/100 dilution of an overnight culture of the appropriate strain. Four millimolar IPTG was either added to the cultures at the time of inoculation or was added to the cultures at various time points during growth, as indicated. The growth of each culture at 37°C was followed by measurement of optical density at 525 nm. At various times during logarithmic growth and stationary phase, 1 ml samples of each culture were collected in triplicate and harvested by centrifugation. The cell pellets were frozen at -20°C until assayed for β-galactosidase activity. Cell pellets were thawed by resuspension in 730 μl of Z-buffer (60 mM Na₂HPO₄; 40 mM NaH₂PO₄; 10 mM KCl; 1 mM MgSO₄; 50 mM β-Mercaptoethanol). Cells were permeabilized by addition of 0.1 % Triton X following 5 minutes incubation with 0.1 mg/ml lysozyme. O-nitrophenol-β-D-galactoside (ONPG), a chromogenic β-galactosidase substrate, was added to a final concentration of 0.45 mg/ml and incubated with the permeabilized cells at 28°C. After 15 minutes incubation the reactions were terminated with the addition of Na₂CO₃ to 180 mM. The absorbance at 420 nm was measured for each reaction and the enzyme specific activity was determined using the calculation: specific activity (Miller Units) = (A₄₂₀ nm x 66.7)/OD₅₂₅nm of the culture.
3. RESULTS

In this study I have investigated the in vitro and in vivo characteristics of a substitution mutant of the response regulator SpoOA. I was interested in how changes in the DNA binding domain affect the ability of SpoOA to activate transcription initiation. It was previously reported that a single amino acid substitution made within the DNA binding domain of SpoOA, A257V, resulted in a sporulation-deficient phenotype (Ferrari et al., 1985). Although the substitution was associated with loss of activation of transcription initiation in vivo from both σ^A- and σ^H-dependent promoters by SpoOA (Perego et al., 1991b; Rowe-Magnus et al., 2000), it did not affect inhibition of transcription initiation by SpoOA at the abrB promoter (Perego et al., 1991b; Rowe-Magnus et al., 2000), shown to depend on the DNA binding properties of SpoOA. In this thesis I have investigated the uncoupling of the ability of SpoOA to activate and repress transcription initiation in vitro by testing for potential defects in SpoOA(A257V). First, did the A257V mutation interfere with the ability of SpoOA to be phosphorylated? Second, did the mutation affect the ability to repress transcription initiation? Third, did the mutation specifically eliminate the ability to stimulate transcription initiation at a σ^A-dependent promoter or bind DNA from that promoter? Finally, did the A257V mutation interfere with a positive feedback mechanism in vivo which leads to upregulation of spoOA transcription?

3.1. Examination of the effect of the A257V mutation on SpoOA phosphorylation in vitro

The ability of SpoOA to stimulate transcription is dramatically increased by transfer of a phosphoryl group through a phosphorelay signal transduction system to D56 located within the N-terminal domain of SpoOA (Bird et al., 1993). Although the A257 residue is distant from the site of phosphorylation and is located in the CTD of SpoOA, it was possible that the valine substitution could alter the structure of the protein such that it became a poor substrate for the phosphotransferase protein SpoOB, and that decreased phosphorylation could explain the lack of transcription activation observed in vivo (Perego et al., 1991b; Rowe-Magnus et al., 2000). I expressed and purified wild type and mutant SpoOA proteins and compared both the rate and level of protein phosphorylation using an in vitro phosphorylation assay composed of reconstituted phosphorelay components. The rate of phosphorylation was
determined by incubation of Spo0A and Spo0A(A257V) with phosphorelay components KinA, Spo0F, Spo0B and [γ³²P]ATP for various times prior to separation by SDS-PAGE and quantification of phosphorylation by PhosphorImager analysis (Figure 9A). The initial rates of phosphorylation of wild type and mutant Spo0A proteins were the same (Figure 9B). Phosphorylation of Spo0A and Spo0A(A257V) increased linearly until maximum phosphorylation was reached after six minutes incubation. This suggested that the A257V mutation did not influence phosphotransfer from Spo0B. As a second test to compare phosphorylation, excess concentrations of wild type and mutant proteins were incubated in a phosphorylation reaction for 4.5 hours prior to SDS-PAGE separation and quantification by PhosphorImager analysis (Figure 10A). Both Spo0A and Spo0A(A257V) were phosphorylated to the same level at each concentration tested (Figure 10B), suggesting that decreased phosphorylation of Spo0A(A257V) could not explain the lack of transcription activation in vivo in the mutant strain (Perego et al., 1991b; Rowe-Magnus et al., 2000).

3.2. Investigation of the effect of the A257V substitution on in vitro repression of the abrB promoter

Spo0A represses transcription of the abrB promoter by preventing RNA polymerase from inducing DNA strand separation during transcription initiation (Greene and Spiegelman, 1996). Previous studies have shown that both Spo0A and Spo0A(A257V) are capable of repressing abrB transcription to similar levels in vivo (Perego et al., 1991b; Rowe-Magnus et al., 2000). In order to validate these results in vitro, I compared the abilities of both phosphorylated and unphosphorylated wild type and mutant proteins to repress abrB transcription in an in vitro transcription assay.

Unphosphorylated and phosphorylated wild type and mutant proteins were incubated with a linear DNA fragment encoding both the P1 and P2 transcription initiation sites of the abrB promoter (abrBp), initiating nucleotides (ATP, UTP and GTP), and RNAP-σ⁸. After two minutes incubation, transcripts were permitted to elongate with the addition of CTP and heparin. Transcripts resulting from a single round of transcription were separated by electrophoresis and quantified by PhosphorImager analysis (Figure 11A).
Figure 9. Time course of *in vitro* phosphorylation of Spo0A and Spo0A(A257V). The phosphorelay components KinA, SpoOF and SpoOB were incubated with [γ\(^{32}\)P]ATP prior to addition of 0.5 μM Spo0A or Spo0A(A257V). Samples were removed at various intervals and the transfer reaction was terminated by addition of SDS-PAGE loading buffer. Following separation of phosphorylated proteins by 15% SDS-PAGE, \(^{32}\)P labeled Spo0A or Spo0A(A257V) were detected by exposure to a phosphor screen (A) and the level of phosphorylation was quantified by PhosphorImager analysis (B). Symbols: filled circles, Spo0A~P; filled squares, Spo0A(A257V)~P. A representative PhosphorImage is shown and values are representative of multiple experiments.
Figure 10. Phosphorylation of varying amounts of Spo0A and Spo0A(A257V) in vitro. Spo0A and Spo0A(A257V) were incubated with the phosphorelay components KinA, Spo0F and Spo0B, and [\gamma^{32}P]ATP for 4.5 hours before the transfer reaction was terminated by the addition of SDS-PAGE loading buffer. The radiolabeled components were separated by 15% SDS-PAGE and (A) $^{32}$P labeled Spo0A or Spo0A(A257V) were detected by exposure to a phosphor screen. The extent of phosphorylation was determined by PhosphorImager analysis (B). White bars, Spo0A~P; grey bars, Spo0A(A257V). A representative PhosphorImage is shown. The values reflect the average of three independent experiments and their standard deviations.
Figure 11. Repression of the $abrB$ promoter by Spo0A and Spo0A(A257V) in vitro.
Phosphorylated or unphosphorylated mutant and wild type Spo0A proteins were incubated with initiating nucleotides ATP, UTP, and GTP and a linear DNA fragment encoding both the P1 and P2 transcription initiation sites of the $abrB$ promoter ($abrB_p$). CTP and heparin were added three minutes after addition of $\sigma^A$-RNA polymerase to permit transcript elongation. The reactions were terminated after five-minutes and the transcripts were separated by electrophoresis through an 8% denaturing polyacrylamide gel. (A) $^{32}$P-labeled $abrB$ transcripts were detected by autoradiography and (B) the level of transcripts produced from the $abrB$ promoter was determined by PhosphorImager analysis. Symbols: open circles, Spo0A; filled circles, Spo0A−P; open squares, Spo0A(A257V); filled squares, Spo0A(A257V)−P. Representative films are shown. Values reflect the average of three independent experiments and their standard deviations.
Consistent with earlier results, increasing amounts of Spo0A reduced the amount of run-off transcript produced from the *abrB* promoter, and Spo0A(A257V) was capable of repressing transcription from *abrB* (Figure 11B). As measured by the decrease in transcript levels, Spo0A(A257V)~P did not inhibit transcription as effectively as Spo0A~P; at 800 nM, repression by Spo0A~P was 2-fold more effective and at 1200 nM, repression by Spo0A~P was 5-fold more effective. Phosphorylation enhanced the ability of Spo0A to repress *abrB* transcription by 3.3-fold, whereas phosphorylation of Spo0A(A257V) only enhanced repression by 1.3-fold. This may be a consequence of the unexpected effectiveness of unphosphorylated Spo0A(A257V) in repressing transcription. The significance of the apparently effective repression by Spo0A(A257V) is uncertain as *in vivo* experiments have indicated that the temporal pattern of repression of the *abrB* gene in a strain with Spo0A(A257V) mimics that in the wild type strain (Perego *et al.*, 1991b). Critically, these data confirmed that the A257V substitution did not substantially affect the ability of Spo0A to repress *abrB* transcription.

3.3. Investigation of the effect of the A257V substitution on *in vitro* stimulation of the *spolIG* promoter

Gene expression during sporulation is directed by five different sigma factors produced at various times during endospore formation. Among the first sigma factors produced are the $\sigma^E$ and $\sigma^F$ sigma factors which direct gene expression in the mother cell and forespore, respectively (Dworkin, 2003). The $\sigma^E$ sigma factor is transcribed from the $\sigma^A$-dependent *spolIG* operon whereas $\sigma^F$ is transcribed from the $\sigma^H$-dependent *spolIIA* operon. Both the *spolIG* and *spolIIA* operons are dependent upon activation by Spo0A~P. The *spolIIA* promoter is directly dependent on $\sigma^H$ since it is transcribed by RNA polymerase containing this sigma factor. The *spolIG* promoter is indirectly dependent on $\sigma^H$ as it is known to be transcribed by RNA polymerase containing $\sigma^A$, but is not transcribed in a *spo0H* mutant. For both the *spolIG* and *spolIIA* operons, transcription requires that Spo0A~P reaches a threshold sufficient for activation (Fujita *et al.*, 2005). This increase in concentration of phosphorylated Spo0A appears to require increased transcription of *spo0A* and the phosphorelay components *kinA* and *spo0F*. 
Previous studies have indicated that the A257V eliminates activation of transcription initiation from the *spolIG* promoter *in vivo*. However, because activation of transcription initiation at this promoter *in vivo* is not only dependent upon Spo0A~P, but also upon a $\sigma^H$-dependent increase in concentration of Spo0A~P, it was possible that the A257V mutation did not change the ability of the protein to stimulate $\sigma^A$-dependent transcription initiation. I tested whether the A257V mutation affected the ability of Spo0A to activate transcription initiation *in vitro* testing both the initial rate of transcription and the effect of increasing Spo0A~P concentrations (Figures 12, 13).

Phosphorylated and unphosphorylated Spo0A and Spo0A(A257V) were incubated with template DNA and the initiating nucleotides ATP and GTP. RNAP-$\sigma^A$ was added to the reactions and the proteins were allowed to form initiated complexes for 0-120 seconds before elongation was permitted with the addition of heparin and the remaining nucleotides. Phosphorylated Spo0A was able to stimulate the rate of *spolIG* transcription initiation to a greater extent than the other proteins tested (Figure 12A, B). The number of transcripts increased rapidly in the presence of Spo0A~P and reached a maximum after 90 seconds incubation. As noted in previous results (Rowe-Magnus and Spiegelman, 1998), both unphosphorylated and phosphorylated Spo0A were capable of stimulation of *spolIG* transcription initiation, although the unphosphorylated protein stimulates the rate at a much reduced level. Similarly, in the presence of phosphorylated Spo0A(A257V) there was an increase in the number of *spolIG* transcripts, although at a rate approximately four times lower than that found in the presence of Spo0A~P. In the presence of unphosphorylated mutant protein the rate of transcription initiation was approximately ten times slower than stimulation by Spo0A~P.

The effect of varying the concentration of unphosphorylated and phosphorylated wild type and mutant proteins on *spolIG* transcription was determined (Figure 13). Unphosphorylated and phosphorylated wild type and mutant proteins were incubated with promoter DNA and RNAP-$\sigma^A$ for two minutes to allow formation of initiated complexes. Transcription was limited to a single-round from initiated complexes by the addition of the CTP, UTP and heparin (Figure 13A).
Figure 12. Time course of \textit{in vitro} transcription initiation stimulated by Spo0A and Spo0A(A257V). Phosphorylated or unphosphorylated Spo0A or Spo0A(A257V) (800 nM) were incubated with a linear DNA fragment encoding the \textit{spolIG} operon promoter (\textit{spolIG_p}) and initiating nucleotides ATP and GTP. \(\sigma^A\)-RNAP was added to the mixture at varying times, the remaining nucleotides and heparin were added. Reactions were terminated by the addition of transcription stop buffer at the times indicated. Elongated \(^{32}\)P-labelled \textit{spolIG} transcripts were separated by electrophoresis through an 8% denaturing polyacrylamide gel and (A) detected by autoradiography. The level of transcripts produced in a given time interval from the \textit{spolIG_p} promoter were determined by PhosphorImager analysis (B). Symbols: open circles, Spo0A; filled circles, Spo0A~P; open squares, Spo0A(A257V); filled squares, Spo0A(A257V)~P. Representative films are shown. Values reflect the averages of three independent experiments and their standard deviations.
Figure 13. Effect of SpoOA or Spo0A(A257V) protein concentration on stimulation of spoIIG promoter activity in vitro. Phosphorylated or unphosphorylated SpoOA or Spo0A(A257V) were incubated with a linear DNA fragment encoding the spoIIG operon promoter (spoIIG_p) and initiating nucleotides ATP and GTP. σ^A-RNAP was added to the mixture and allowed to bind DNA for 2 minutes prior to addition of the remaining nucleotides and heparin. Following a five-minute incubation, elongated transcripts were separated by electrophoresis through an 8% denaturing polyacrylamide gel. (A) ^32P-labeled spoIIG transcripts were detected by autoradiography and (B) the level of transcripts produced from the spoIIG promoter were determined by PhosphorImager analysis. Symbols: open circles, SpoOA; filled circles, Spo0A~P; open squares, Spo0A(A257V); filled squares, Spo0A(A257V)~P. Representative films are shown. Values reflect the average of three independent experiments and their standard deviations.
Increasing amounts of Spo0A–P led to increasing amounts of transcription over the range of 200 to 800 nM protein at which point the level of transcription was maximal (Figure 13B). Overall Spo0A–P stimulated transcription 23-fold relative to the number of transcripts produced in the absence of Spo0A. Both phosphorylated and unphosphorylated Spo0A(A257V) were capable of stimulating transcription initiation, but only to levels approximately 50% of those stimulated by wild type Spo0A. Over the range of concentration at which Spo0A(A257V) was tested, transcription increased linearly and at 1200 nM the amount of transcription was 14-fold greater than that obtained in the absence of Spo0A. In addition, both Spo0A and Spo0A(A257V) exhibited the same fold-increase in transcription stimulation upon phosphorylation at each protein concentration tested. For example, at 800 nM the phosphorylated form of both proteins was four-fold more effective in stimulating transcription than was the unphosphorylated protein. These data demonstrate that Spo0A(A257V) was capable of stimulating transcription from a σA-dependent promoter in vitro.

3.4. Examination of the effect of the A257V mutation of Spo0A on binding to 0A boxes encoded within the spoIIG promoter

Spo0A binds to specific sites (0A boxes) located upstream or downstream of the transcription start site at promoters activated or repressed by Spo0A. At these sites Spo0A makes both base-specific contacts and contacts with the DNA backbone (Zhao et al., 2002). At the spoIIG promoter there are four 0A boxes at two different sites (Baldus et al., 1994). The site 1 0A boxes, centered 90 bp upstream of the +1 transcription start site, were previously shown to be high affinity Spo0A binding sites (Baldus et al., 1994). In contrast the site 2 0A boxes, centered approximately 45 bp upstream of the +1 transcription start site, are low-affinity Spo0A binding sites and are bound only by phosphorylated Spo0A (Baldus et al., 1994). I tested whether the two fold reduction in spoIIG transcription stimulation observed in this study was due to lack of binding or inappropriate binding of Spo0A(A257V) to 0A boxes encoded within promoter DNA using an in vitro DNasel footprinting assay.

Phosphorylated Spo0A and Spo0A(A257V) were incubated with a [γ^{32}P]ATP end-labeled fragment of the spoIIG promoter and partially digested with DNasel. The labeled fragments
were separated by electrophoresis and the protection patterns were detected by autoradiography (Figure 14). Consistent with previous findings, phosphorylated Spo0A bound to both site 1 and site 2 0A boxes protecting a large region of the promoter (Baldus et al., 1994). Phosphorylated Spo0A(A257V)~P also bound to site 1 and site 2 0A boxes within promoter DNA, although with lower affinity than Spo0A~P. The pattern of protected sites was not changed as far as could be observed. These data suggested that the A257V mutation reduced spoIIIG binding to some degree but did not eliminate it. The reduction could be the cause of lower transcription stimulation.

3.5. In vitro examination of the binding affinity of Spo0A(A257V) for consensus 0A boxes

Although the A257V mutation did not cause inappropriate binding to the spoIIIG promoter, results from the DNaseI footprinting assay suggested that the mutation did reduce the affinity of the protein for its DNA binding sites. I tested this possibility using a different approach by measuring the ability of both phosphorylated and unphosphorylated Spo0A and Spo0A(A257V) to bind DNA fragments containing 0A boxes in an electrophoretic mobility shift assay (EMSA).

Various concentrations of unphosphorylated and phosphorylated wild type and mutant proteins were incubated with [α\(^{32}\)P]NTP end-labeled duplex DNA encoding the two 0A boxes found at the abrB promoter. After two minutes incubation, binding reactions were loaded onto a running polyacrylamide gel and complexes were separated by electrophoresis. The extent of binding was determined using PhosphorImager analysis (Figure 15A). Both Spo0A(A257V) and Spo0A(A257V)~P were able to bind to the duplex DNA (Figure 15B). A single complex was observed at all protein concentrations for both protein forms.

Dissociation constants were determined for both phosphorylated and unphosphorylated proteins using the Program SigmaPlot 8.0 (SPSS) as described in Experimental Procedures. Phosphorylated Spo0A bound with approximately 2-fold greater affinity than phosphorylated Spo0A(A257V) and had an apparent dissociation constant of 1 x 10\(^{-7}\) M (standard error = 0.22) compared to an apparent dissociation constant of 3 x 10\(^{-7}\) M (standard error = 0.44) for Spo0A(A257V)~P. Unphosphorylated Spo0A bound DNA with a reduced affinity (about
Figure 14. DNase footprint of SpoOA and SpoOA(A257V) at the spoIIG promoter. Phosphorylated SpoOA and SpoOA(A257V) (200, 400, 600 nM) were incubated with a 454 bp PvuII-BamHI DNA fragment from the spoIIG operon promoter (spoIIGp) end labeled with [γ-32P]ATP. Control reactions contained only spoIIGp DNA. After two minutes, DNase I was added and allowed to cleave the DNA for 10 seconds before terminating cleavage with addition of DNase stop buffer. 32P Labeled fragments were separated by electrophoresis through an 6% denaturing polyacrylamide gel and (A) the DNaseI protection patterns were detected by autoradiography. The positions of the nucleotides relative to the +1 start site are indicated to the left of diagram. (B) Location of the site 1 and site 2 OA boxes relative to the conserved -10 and -35 elements of the spoIIG promoter.
Figure 15. EMSA of binding of Spo0A and Spo0A(A257V). Phosphorylated and unphosphorylated Spo0A and Spo0A(A257V) were incubated with double-stranded $\alpha^{32}$P-labeled oligonucleotides that contain two consensus OA boxes (5'-TGACGAA-3') and had the same sequences as the +3 to +29 region of the abrB gene. The proteins were allowed to bind DNA for two minutes prior to challenge with calf thymus DNA in glycerol and loading on a running 8% non-denaturing polyacrylamide gel. (A) $^{32}$P labeled DNA was detected by autoradiography. (B) The percentage of free and bound DNA was quantified by PhosphorImager analysis. Symbols: open circles, Spo0A; filled circles, Spo0A~P; open squares, Spo0A(A257V); filled squares, Spo0A(A257V)~P. Representative films are shown. Values reflect the average of three independent experiments and their standard deviations.
one-third the amount bound by Spo0A~P at 800 nM) and had an apparent dissociation constant of 1 \times 10^6 \text{ M}. The binding of Spo0A(A257V) to the duplex DNA was negligible, precluding an accurate assessment of its dissociation constant. These data indicated that Spo0A(A257V)~P was able to bind to duplex DNA with approximately half the apparent affinity of Spo0A~P and suggested that the two-fold reduction in \textit{in vitro} transcription activation and repression described in this study could be explained by a reduction in the binding activity of the mutant protein.

3.6. Effect of mutations in the 0A boxes encoded within the \textit{spolIG} promoter on stimulation of transcription by Spo0A~P and Spo0A(A257V)~P

A recent model of Spo0A activation suggests that the primary consequence of NTD phosphorylation is to facilitate interactions between the domains leading to dimerization (Lewis \textit{et al.}, 2002). The implication of this model is that the active form of Spo0A is a dimer. The crystal structures of the DNA binding domain alone and in complex with DNA containing tandem 0A box sequences derived from the \textit{abrB} promoter show the two domains in a head-to-tail orientation with each member making specific DNA backbone and base contacts within the 0A box sequence (Lewis \textit{et al.}, 2000a; Zhao \textit{et al.}, 2002).

At the \textit{spolIG} promoter the site 2 0A boxes are found between positions -53 to -37 bp with the downstream 0A box (site 2.2, positions -43 to -37) overlapping the -35 sequence of the \textit{spolIG} promoter. When the site 2 0A boxes are occupied by a dimer of Spo0A~P, only the downstream monomer is expected to be close to the sigma subunit of the polymerase. Genetic evidence (Baldus \textit{et al.}, 1995; Buckner \textit{et al.}, 1998; Hatt and Youngman, 1998; Schyns \textit{et al.}, 1997) and recent structural modeling (Kumar \textit{et al.}, 2004) have indicated that Spo0A contacts the sigma subunit to activate transcription at the \textit{spolIG}. While A257 is distinct from the predicted $\sigma^A$ activation surface, the helix within which the A257 residue is located (helix $\alpha_F$) forms part of the interface between the two Spo0A monomers in the crystal structures. Furthermore, the structures predict that the A257V mutation would prevent appropriate packing between residues forming this interface by influencing the flexibility and orientation of $\alpha_F$ (Zhao \textit{et al.}, 2002).
It seems likely that the strength of binding of a Spo0A~P dimer to the tandem 0A boxes in its DNA binding site would reflect both the strength of interaction between each monomer and one 0A box and the strength of interaction between the two monomers. If dimer-binding conditions were compromised, so that simultaneous contact of the monomers and 0A boxes was altered, then potentially one of the monomers would be able to make favorable contacts with the DNA while the second protein might not be unable to make the expected contacts. This situation might well explain why the Spo0A(A257V) bound DNA with less affinity and was both less effective at repressing *abrB* transcription and at stimulating *spoIIG* transcription. Since the interaction of Spo0A~P with the sigma subunit is critical to transcription activation at the *spoIIG* promoter, it should be more affected by binding of the downstream monomer of the Spo0A dimer than binding of the upstream monomer. To test this idea I altered the *spoIIG* promoter through mutation of each of the site 2 0A boxes separately and tested whether the effects of these mutations would be exaggerated for Spo0A(A257V). In the case of wild type Spo0A, where the protein-protein interactions are most favorable, mutation of either 0A box should have roughly the same effect, reducing the sum of contacts stabilizing dimer binding to the DNA. On the other hand, in the case of Spo0A(A257V), potentially only one of the binding domains could make the appropriate contacts with the DNA at any one time since dimer packing may be disrupted. If contacts with the downstream 2.2 0A box are essential to position Spo0A for subsequent sigma contacts, the mutation of the downstream 2.2 0A box might have a more deleterious effect on transcription than mutation of the upstream 2.1 0A box. Thus I measured the effects of mutations in each of the two 0A boxes on transcription activation by wild type Spo0A and Spo0A(A257V).

Two mutant promoter DNA templates were used in this experiment that encoded versions of the site 2 0A boxes with decreased similarity to the consensus 0A box sequence (Figure 16A). The “2.1 down” template encodes a mutant 2.1 0A box in which the sequence 5’-CTCAACA-3’ was changed to 5’-CAGAAGA-3’, eliminating a single base-specific DNA contact for Spo0A on both the template and non-template strands that have been identified from the crystal structure (Zhao *et al.*, 2002). To mutate the 2.2 0A box (creating the “2.2-down” template), the sequence was changed from 5’-ATTGACA-3’ to 5’-ACTGAGA-3’,
Figure 16. Wild type and mutant SpoOA stimulation of transcription from mutant spoIIG promoters in vitro. Phosphorylated or unphosphorylated SpoOA or SpoOA(A257V) were incubated with a linear DNA fragment encoding either wild type or mutant spoIIG operon promoters (spoIIG, 2.1 down, 2.2 down) and the initiating nucleotides ATP and GTP. σA-RNA polymerase was added to the mixture and allowed to bind DNA for 2 minutes prior to addition of the remaining nucleotides and heparin. Following a five-minute incubation, elongated transcripts were separated by electrophoresis through an 8% denaturing polyacrylamide gel. (A) The -34 to -56 region of the spoIIG promoter. SpoOA binding sites (OA boxes, consensus sequence 5'-TGACGAA-3') are underlined. Nucleotides thought to be specifically contacted by SpoOA are shown in bold and those expected to be contacted by the σA subunit of RNA polymerase are italicized. Mutated bases are shown in lower case. (B) 32P-labeled transcripts were detected by autoradiography and (C) the level of transcripts produced were determined by PhosphorImager analysis. Symbols: black circles, Spo0A~P (spoIIGp); black squares, Spo0A(A257V)~P (spoIIGp); grey circles, Spo0A~P (promoter templates: 2.1 down, 2.2 down); grey squares, Spo0A(A257V)~P (promoter templates: 2.1 down, 2.2 down). Representative films are shown. Values reflect the average of three independent experiments and their standard deviations.
mutating one base contacted for both Spo0A and the σ subunit of RNAP and eliminating one σ-specific contact, as predicted from the crystal structure (Campbell et al., 2002). The ability of Spo0A~P and Spo0A(A257V)~P to stimulate transcription initiation from wild type and mutant promoter templates was determined using an in vitro transcription assay (Figure 16B). Mutation of either the 2.1 or 2.2 0A boxes decreased, but did not abolish, the ability of phosphorylated Spo0A or phosphorylated Spo0A(A257V) to stimulate transcription (Figure 16C). Overall the pattern of transcription stimulation as a function of activator input was similar to that seen with the wild type spoIIG promoter; transcription in the presence of Spo0A~P reached a maximum at 800 nM whereas stimulation by Spo0A(A257V)~P increased in a roughly linear manner over the range tested (up to 1200 nM).

The 2.1 0A box mutation led to a decrease in the maximum level of transcription stimulation by both phosphorylated Spo0A and Spo0A(A257V) as would be expected if the binding affinity of the dimer was reduced by eliminating critical contacts. For both Spo0A~P and Spo0A(A257V)~P there was a 3-fold reduction in transcription from the mutant 2.1 down promoter compared to transcription from the wild type spoIIG promoter. The reduction was similar to the decrease in Spo0A~P binding in vitro and transcription in vivo observed when the guanine and thymine residues at positions -47 and -51, relative to the +1 transcription start site, are mutated independently (Baldus et al., 1994; Satola et al., 1991; Satola et al., 1992). In the case of the 2.2 down template, stimulation by Spo0A~P was reduced 3-fold and stimulation by Spo0A(A257V)~P was reduced 4.5-fold. Thus it appeared that the A257V mutation did make transcription stimulation slightly more sensitive to changes in the 2.2 0A box. While the difference between the effects of the 0A box mutations on stimulation by the two types of activator proteins was not dramatic, the combination of the A257V mutation and loss of one of the 0A boxes lead to very low levels of transcription.

3.7. Spo0A(A257V) protein expression in the sporulation negative B. subtilis strain JH695

Previous studies have indicated that genes within the Spo0A regulon respond differentially to high and low doses of Spo0A and that sporulation requires a high level of Spo0A (Chung et
Genes which require low-levels of Spo0A for repression ('low-threshold repressed genes') include the gene for the transition state regulator AbrB (abrB) and the gene for the positive regulator of competence, ComK (comK) (Chung et al., 1994; Fujita et al., 2005). Other genes, such as the spoIIE and sinI genes and the spoIIIG and spoIIA operons, require a higher level of Spo0A for activation and are referred to as 'high-threshold activated genes' of the Spo0A regulon (Chung et al., 1994; Fujita et al., 2005).

One possible explanation for the uncoupling of transcription stimulation from transcription repression in B. subtilis JH695 is that Spo0A(A257V) is expressed to lower levels than Spo0A. Reduced expression of the mutant protein would be sufficient to repress low-threshold genes such as abrB, but would not be of sufficient threshold to activate high-threshold genes such as spoIIIG and spoIIA operons nor be of sufficient concentration to permit sporulation.

To test this idea, we observed expression of wild type and mutant Spo0A proteins over time in an immunoblot analysis. Lysates of whole cell-extracts were collected from wild type and spo0A(A257V) B. subtilis strains JH642 and JH695 at 1 hour intervals during late-logarithmic growth through stationary phase. The protein samples were subject to immunoblot analysis and probed for expression of Spo0A and Spo0A(A257V) using anti-Spo0A antibodies (Figure 17A). Expression of both wild type and mutant Spo0A proteins increased over time and reached a maximum level approximately two hours after the onset of stationary phase (T+2). However, whereas previous reports suggested that similar amounts of the Spo0A protein were observed in spo0A+ and spo0A(A257V) B. subtilis strains (Perego et al., 1991b), Spo0A(A257V) expression was estimated to be two-thirds that of Spo0A expression (35 ng Spo0A(A257V) vs. 52 ng Spo0A in samples taken at T+2) (Figure 17A).

To correlate the reduction in mutant Spo0A expression with the ability to sporulate, strains JH642 and JH695 were tested for the ability to sporulate (Figure 17B). As expected, the wild type strain (JH642) had a sporulation frequency of 5.2 x 10⁻¹, whereas the mutant strain (JH695) did not sporulate at all. This correlation suggested that reduced protein expression and lower transcription stimulation efficiency could account for the lack of sporulation in spo0A(A257V) B. subtilis strains.
Figure 17. Wild type and mutant Spo0A protein expression *B. subtilis* JH642 and JH695. *B. subtilis* strains JH642 and JH695 were grown in SSM and samples were collected and harvested at 1 hour intervals from the end of exponential growth (T-1) into stationary phase (T+1 to T+4). T0 indicates the end of exponential growth. Samples containing 40.5 µg of total protein from whole cell extracts and samples containing 11 and 33 ng of purified recombinant Spo0A and Spo0A(A257V) were separated by electrophoresis through 12% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and (A) subject to immunoblot analysis using anti-Spo0A polyclonal antibodies. (B) After 28 hours of growth, the strains were assayed for sporulation frequency. Sporulation frequency is defined as the number of colony forming units before and after chloroform treatment.
3.8. Construction of *B. subtilis* strains which over-express wild type and mutant Spo0A proteins

During the onset of sporulation, both Spo0A synthesis and activity are ultimately dependent upon transcription by RNAP-σ^H_. The *spoOA* gene can be transcribed from one of two promoters, *spoOApv* or *spoOAps* (Strauch et al., 1992). During vegetative growth, transcription from the σ^A-dependent vegetative promoter (*spoOApv*) results in low-level expression of Spo0A. Increased expression of Spo0A required for sporulation occurs during the onset of sporulation as a consequence of a positive feedback mechanism in which Spo0A stimulates transcription of its own gene from the σ^H-dependent, sporulation specific promoter (*spoOAps*). Similarly, an increase in Spo0A activity could be achieved by increased phosphorylation as a result of greater synthesis of the phosphorelay components *spoOF* and *kinA* whose transcription also depends on σ^H_ (Britton et al., 2002; Predich et al., 1992).

One possible explanation for the reduction in Spo0A protein expression observed in *B. subtilis* strain JH695 is that A257 or the region around it is required for specific interaction with σ^H-RNA polymerase. Strains containing the *spo0A(A257V)* mutation would not only be unable to sporulate because of lack of activation of Spo0A-dependent, σ^H-dependent promoters but also because the threshold level of Spo0A would never increase sufficiently to permit activation of σ^H-independent, high threshold Spo0A activated promoters, such as the σ^A-dependent *spoIIIG* promoter. One way to test this hypothesis *in vivo* was to create *B. subtilis* strains which over-expressed either wild type or mutant Spo0A proteins and monitor transcription activation of both a σ^H-dependent and a σ^A-dependent promoter *in vivo*.

To test this hypothesis I created strains of *B. subtilis* in which expression of wild type or mutant Spo0A protein was placed under the control of the LacI-repressed/IPTG-inducible Pspac promoter (Vagner et al., 1998). The plasmid construct used to create these strains, pMN0AN (Figure 8), encodes the first 146 amino acids of the *spo0A* gene downstream of Pspac and was created from the integrative *B. subtilis* vector pMUTIN-4 as outlined in *Experimental Procedures*. pMN0AN was transformed into *B. subtilis* strains JH642 and JH695 and integrated into the *spo0A* locus by a single recombination event (Figure 18A).
Figure 18. Construction of *B. subtilis* strains which overexpress wild type or mutant Spo0A. Construction of *B. subtilis* strains encoding P<sub>spac</sub>-spo0A and P<sub>spac</sub>-spo0A(A257V). Plasmid pMN0AN encodes the spo0A ribosome-binding site and the N-terminal receiver domain (amino acids 1-146) of spo0A downstream of the LacI-repressed/IPTG-inducible P<sub>spac</sub> promoter. The plasmid contains an origin of replication (ori<sub>E.coli</sub>) for growth and maintenance in *E.coli*, selectable markers for both *E.coli* and *Bacillus* (bla and ermAM), a lacI cassette, sequences encoding the terminal 644-1019 amino acids of the lacZ gene, and the transcription termination sequences T<sub>0</sub>, T<sub>1</sub> and T<sub>2</sub>. pMN0AN was transformed into *B. subtilis* strains JH642 and JH695 and integrated into the spo0A locus of the chromosome by a single recombination event. The resulting strains, BT2001 and BT2002, had the genotypes JH642 spo0A::(pMNSpo0AN ermAM) and JH695 spo0A::(pMNSpo0AN ermAM), as shown in the schematic in panel B. Both recombinant strains encode the N-terminal receiver domain of Spo0A transcribed from the native sporulation and vegetative promoters spo0A<sub>pv</sub> and spo0A<sub>ps</sub>, in addition to either the full length spo0A or spo0A(A257V) gene transcribed from the P<sub>spac</sub> promoter.
The strains created, BT2001 and BT2002, encoded both a full length copy of either the wild type or mutant \( spoOA \) gene under control of the Pspac promoter in addition to a truncated \( spoOA \) gene encoding the first 146 amino acids of the N-terminal domain downstream of the two \( spoOA \) promoters (\( spo0Apv \) and \( spo0Aps \)) (Figure 18B).

3.9. Sporulation frequencies of the Pspac-\( spoOA \) \( B. \ subtilis \) strains

As an initial experiment, strains BT2001 and BT2002 were assessed for the ability to produce wild type or mutant Spo0A proteins and sporulate in the presence of inducer. Strains BT2001 and BT2002 were grown in SSM supplemented with 0, 1 or 4 mM IPTG. After 16 hours of growth a sample of each culture was removed and tested for expression of wild type or mutant Spo0A proteins using an immunoblot analysis (Figure 19A). Lysates of whole-cell extracts collected from cultures of BT2001 and BT2002 were probed for presence of mutant or wild type Spo0A proteins using anti-Spo0A antibodies. As expected, the cultures grown in the absence of inducer did not produce detectable amounts of wild type or mutant Spo0A whereas both BT2001 and BT2002 expressed protein reacting with the \( \alpha \)-Spo0A antibody in response to IPTG. Under these conditions it was estimated that both samples contained approximately the same amount of mutant and wild type protein: 36 ng Spo0A in BT2001 and 32 ng Spo0A(A257V) in BT2002 when either strain was induced with 1 mM IPTG.

After 28 hours of growth strains BT2001 and BT2002 were tested for the ability to sporulate. Only strain BT2001, which encodes the wild type \( spoOA \) gene under the control of Pspac (Pspac-\( spoOA \)), was able to sporulate, albeit poorly, with the addition of IPTG (Figure 19B). Strain BT2002, which encodes Pspac-\( spoOA(A257V) \), was unable to sporulate at both IPTG concentrations tested. Strain BT2001 was also able to sporulate at an extremely low frequency (3.4 \( \times \) 10\(^{-6} \) spores/ml) in the absence of IPTG. Addition of 1 mM IPTG increased the ability of BT2001 to sporulate by a factor of 100 while addition of 4 mM IPTG caused a 1000-fold increase in sporulation frequency. However, while higher levels of IPTG caused an increase in the ability of strain BT2001 to sporulate, there was no obvious difference in expression of mutant or wild type proteins from Pspac at 4 mM IPTG (Figure 19A). Moreover, although IPTG induction of Pspac resulted in an increased ability of strain
Figure 19. Wild type and mutant Spo0A protein expression and sporulation in *B. subtilis* strains BT2001 and BT2002 in response to IPTG. *B. subtilis* strains BT2001 and BT2002 were grown in SSM supplemented with 0, or 4 mM IPTG. After 16 hrs of growth at 37°C, a portion of each culture was harvested and samples of whole cell extracts containing 50 μg of total protein were separated by electrophoresis through 12% SDS-PAGE. A 50 ng sample of purified recombinant Spo0A was also separated by electrophoresis to serve as a control. The protein samples were transferred onto a nitrocellulose membrane and (A) subject to immunoblot analysis using anti-Spo0A polyclonal antibodies. After 28 hours of growth, the remaining culture grown in each condition was assayed for sporulation frequency (B). Sporulation frequency is defined as the number of colony forming units before and after chloroform treatment.

<table>
<thead>
<tr>
<th>Strain</th>
<th>[IPTG] (mM)</th>
<th>No. CFU (ml⁻¹)</th>
<th>No. Spores (ml⁻¹)</th>
<th>Sporulation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT2001</td>
<td>0</td>
<td>3.5 x 10⁸</td>
<td>1.2 x 10³</td>
<td>3.4 x 10⁻⁶</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>7.9 x 10⁸</td>
<td>5.6 x 10⁵</td>
<td>7.0 x 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.1 x 10⁸</td>
<td>1.5 x 10⁶</td>
<td>2.0 x 10⁻³</td>
</tr>
<tr>
<td>BT2002</td>
<td>0</td>
<td>1.5 x 10⁸</td>
<td>0</td>
<td>&lt;1.0 x 10⁻⁸</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6.3 x 10⁸</td>
<td>0</td>
<td>&lt;1.0 x 10⁻⁸</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5.7 x 10⁸</td>
<td>0</td>
<td>&lt;1.0 x 10⁻⁸</td>
</tr>
</tbody>
</table>
BT2001 to sporulate, the maximum sporulation frequency observed was 100-fold lower than the sporulation frequency typically observed in wild type *B. subtilis* strains.

### 3.10. Investigation of the effect of varying IPTG concentration on expression from Pspac-spo0A(A257V)

The goal of constructing the inducible *B. subtilis* strains was to alleviate the requirement of Spo0A-σ^H interaction for increased expression of Spo0A. However, it appeared that protein expression from the Pspac promoter was decreased in strains BT2001 and BT2002 as compared to protein expression from the endogenous spo0Aps promoter in JH642 (Figures 17 and 19). Thus it was possible that the lack of sporulation in strain BT2002 relative to strain BT2001 was due to decreased expression from the Pspac promoter and not due to the A257V mutation. I tested this hypothesis by monitoring Spo0A(A257V) expression and the ability to sporulate in response to increasing concentrations of IPTG. Cultures were grown in SSM supplemented with 0-16 mM IPTG for 16 hours prior to harvest for immunoblot analysis or for 28 hours before sporulation assay. Lysates of whole cell extracts were probed for Spo0A(A257V) using anti-Spo0A antibodies. In the absence of any IPTG, no protein reacting with the anti-Spo0A antibody was detected (Figure 20A, lane 2). Addition of 1 mM IPTG induced expression of Spo0A(A257V) (Figure 20A, lane 3) and increasing concentrations did not appear to cause an additional increase in protein accumulation (Figure 20A, lanes 4-7). However, although Spo0A(A257V) was expressed in strain BT2002, no concentration of IPTG lead to induction of sporulation (Figure 20B).

### 3.11. Construction of *B. subtilis* spoIIA-lacZ and spoIIG-lacZ reporter strains which overexpress wild type and mutant Spo0A

While the data presented in this study have suggested that the A257V mutation does not inhibit the ability of the mutant protein to be phosphorylated, the mutation did alter binding to 0A boxes, transcription repression and activation of σ^A-dependent transcription. An unanswered question is the effect of spo0A(A257V) transcription directed by σ^H. One way to test this interaction is to compare the ability of strains expressing equal levels of wild type or mutant Spo0A to activate σ^A-dependent or σ^H-dependent transcription in vivo.
Figure 20. Spo0A(A257V) expression in BT2002 as a function of IPTG induction. Samples of B. subtilis strain BT2002 were collected and harvested from cultures grown in SSM supplemented with or without IPTG supplementation (0-16 mM) for 16 hours at 37°C. Purified recombinant Spo0A(A257V) and samples containing 50 µg of total protein from whole cell extracts were separated by electrophoresis through 12 % SDS-PAGE. The protein samples were transferred onto a nitrocellulose membrane and (A) subject to immunoblot analysis using anti-Spo0A polyclonal antibodies. Lane 1: 33 ng purified recombinant Spo0A(A257V); lane 2-7: lysates from BT2002 cultures supplemented with 0, 1, 2, 4, 8, or 16 mM IPTG. (B) After 28 hours of growth, the remaining culture grown in each condition was assayed for sporulation frequency. Sporulation frequency is defined as the number of colony forming units before and after chloroform treatment.
To create *B. subtilis* strains encoding σ^A^-dependent and σ^H^-dependent lacZ reporter constructs, strains BT2001 and BT2002 were transformed with chromosomal DNA encoding either a spoIIA-lacZ or a spoIIG-lacZ translational fusion integrated in the amyE locus of the chromosome. A double recombination event resulted in integration of the lacZ translational reporter into the amyE locus of the chromosome of strain BT2001 and BT2002, creating strains BT2003, BT2004, BT2005, and BT2006. I first tested the ability of the strains to sporulate in the absence and presence of inducer (Figure 21A). Cultures were grown in SSM supplemented with 0 or 4 mM IPTG for 28 hours prior to sampling for sporulation assays. As expected, strains BT2003 and BT2004 were able to sporulate to extremely low levels in the absence of IPTG and the addition of 4 mM IPTG increased sporulation frequency by at least 100-fold, although this frequency was still 100-fold lower than wild type *B. subtilis* strain JH642. In contrast, strains BT2005 and BT2006, encoding the spoIIA-lacZ and spoIIG-lacZ fusions in a ?spac-spoOA(A257V) background, did not sporulate with or without IPTG. Analysis of protein expression in strains BT2003, BT2004, BT2005 and BT2006 with and without IPTG revealed that all strains expressed either the mutant or wild type proteins to similar levels (Figure 21B).

### 3.12. Measurement of spoIIA-lacZ induction in strains encoding inducible wild type and mutant Spo0A

To determine whether the A257V mutation caused a faulty interaction between σ^H^ and Spo0A, we tested the ability of strains encoding an IPTG-inducible spo0A or spo0A(A257V) gene to activate spoIIA-lacZ transcription with and without induction. Strains JH16124, BT2003 and BT2005 were grown in SSM supplemented with 0 or 4 mM IPTG at the time of inoculation. Samples were collected from each culture over half-hour intervals from mid-log growth through stationary phase and tested for β-galactosidase activity (Figure 22). As expected, spoIIA-lacZ activation in strain JH16124, which encodes a spoIIA-lacZ fusion in a wild type *B. subtilis* background, increased linearly beginning 1.5 hours before the onset of stationary phase (T−1.5) and reached a maximum three hours later (T+1.5). In contrast, activation of spoIIA-lacZ transcription in strains BT2003 and BT2005 was negligible, even with the addition of 4 mM IPTG. In general, there was little or no difference in the β-galactosidase activity measured in strains grown with or IPTG, although the activities
Figure 21. Protein expression and sporulation in *B. subtilis* strains which overexpress wild type or mutant Spo0A and encode *lacZ* translational fusions. *B. subtilis* strains BT2003 and BT2004 encode Pspac-spo0A and strains BT2005 and BT2006 encode Pspac-spo0A(A257V). Samples of *B. subtilis* strains BT2003-BT2006 were collected and harvested from cultures grown in SSM supplemented with or without IPTG supplementation for 16 hours at 37°C. Purified recombinant Spo0A and samples containing 50 μg of total protein from whole cell extracts were separated by electrophoresis through 12 % SDS-PAGE. The protein samples were transferred onto a nitrocellulose membrane and (A) subject to immunoblot analysis using anti-Spo0A polyclonal antibodies. Lane 1: 50 ng purified recombinant Spo0A; lane 2: BT2003; lane 3: BT2003 + 4 mM IPTG; lane 4: BT2005; lane 5: BT2005 + 4 mM IPTG; lane 6: BT2004; lane 7: BT2004 + 4 mM IPTG; lane 8: BT2006; lane 9: BT2006 + 4 mM IPTG. (B) After 28 hours of growth, the remaining culture grown in each condition was assayed for sporulation frequency. Sporulation frequency is defined as the number of colony forming units before and after chloroform treatment.
Figure 22. PspoIIA: lacZ activity in B. subtilis strains BT2003 and BT2005. B. subtilis strains JH16124, BT2003 and BT2005 were grown in SSM +/- 4mM IPTG and samples were collected in half-hour intervals from mid exponential growth and assayed for β-galactosidase activity. Time '0' indicates the end of exponential growth. Symbols: closed triangles, JH16124, open circles, BT2003 (no IPTG); filled circles, BT2003 + 4mM IPTG; open squares, BT2005 (no IPTG); filled squares, BT2005 + 4mM IPTG. Values reflect the average of three independent experiments and their standard deviations.
measured in strain BT2003, which encode Pspac-spo0A, were marginally greater at all time points than the β-galactosidase activity measured in strain BT2005. At maximum, spoIIA-lacZ activation in strains BT2003 and BT2005, with or without induction, was over 10-fold lower than the maximum level reached in strain JH16124.

It was possible that the inability of strains BT2003 and BT2005 to activate spoIIA-lacZ transcription was due to inappropriate timing of induction. If spo0A or spo0A(A257V) were expressed early in growth and if IPTG induction decreased over time, then it would be possible that the protein produced could have been degraded prior to entry into sporulation. As a result, the concentration of wild type or mutant Spo0A would be below the threshold required for activation of stage II sporulation genes such as spoIIA and spoIIG. To address this potential problem I attempted to optimize the timing of IPTG induction in strains harboring either spoIIA-lacZ or spoIIG-lacZ fusions in Pspac-spo0A and Pspac-spo0A(A257V) backgrounds. Cultures were grown in SSM and were supplemented with 4 mM IPTG at half hour intervals during exponential growth in hopes of inducing wild type and mutant Spo0A protein expression coordinately with an increase in phosphorelay component expression. Samples of each culture were harvested 3 hours after the onset of stationary phase and tested for β-galactosidase activity (Table 4).

Altering the time of IPTG induction did not lead to a significant increase in the β-galactosidase activity measured in induced cultures of BT2003, BT2004, BT2005 and BT2006. Addition of IPTG at different time points during exponential growth had little effect upon activation of spoIIG-lacZ transcription in strains BT2004 and BT2006. The greatest β-galactosidase activity measured for both of these strains was only 2-fold greater than that of the uninduced cultures and occurred when IPTG was added 1.5 hours prior to the onset of stationary phase. Activation of spoIIA-lacZ transcription was greatest upon IPTG addition during exponential growth approximately 2 to 2.5 hours before the onset of stationary phase in strain BT2003. In these cultures, β-galactosidase activity was increased 5-fold over the uninduced culture. Similarly, spoIIA-lacZ transcription was greatest when strain BT2005 was induced between 1.5 and 2 hours before the onset of stationary phase, although the maximum β-galactosidase activity measured for this strain was only 2-fold
Table 4. Activation of spoIIA-lacZ and spoIIG-lacZ transcription in *B. subtilis* strains which overexpress wild type or mutant Spo0A.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Induction Time (hr)</th>
<th>[IPTG] (mM)</th>
<th>β-galactosidase Activity (Miller Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>spoIIA-lacZ</em></td>
</tr>
<tr>
<td>JH642 spo0A::(pMNspoOANermAM)</td>
<td>uninduced</td>
<td>0</td>
<td>4.05</td>
</tr>
<tr>
<td></td>
<td>T-2.5</td>
<td>4</td>
<td>20.18</td>
</tr>
<tr>
<td></td>
<td>T-2.0</td>
<td>4</td>
<td>21.00</td>
</tr>
<tr>
<td></td>
<td>T-1.5</td>
<td>4</td>
<td>15.47</td>
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<td>T-1.0</td>
<td>4</td>
<td>8.76</td>
</tr>
<tr>
<td></td>
<td>T0</td>
<td>4</td>
<td>6.7</td>
</tr>
<tr>
<td>JH695 spo0A::(pMNspoOANermAM)</td>
<td>uninduced</td>
<td>0</td>
<td>4.13</td>
</tr>
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*a* *B. subtilis* strains used in this experiment also encode *amyE::(spoIIA-lacZ)* Cm<sup>R</sup> or *amyE::(spoIIG-lacZ)* Kan<sup>R</sup>.

*b* *B. subtilis* strains BT2003-BT2006 were grown in SSM and supplemented with 4 mM IPTG at half hour intervals during exponential growth through the onset of stationary phase (T-2.5 to T0). Samples of each culture were harvested 3 hours after the onset of stationary phase and tested for β-galactosidase activity. Values shown are the average of three independent experiments.

*c* A culture of each *B. subtilis* strain was grown in SSM without addition of IPTG. Samples of each culture were harvested and tested for β-galactosidase activity similarly to the test cultures. Values shown are the average of three independent experiments.
greater than the uninduced culture. However, although there were marginal increases in \textit{spoIIA} and \textit{spoIIG} promoter activities when induced at different times during exponential growth, the $\beta$-galactosidase activities measured in each condition were well below the levels normally reported in the literature (Perego \textit{et al.}, 1991b; Rowe-Magnus \textit{et al.}, 2000). This indicates that both the \textit{spoIIA-lacZ} and \textit{spoIIG-lacZ} promoters are not activated in strains encoding Pspac-\textit{spoOA} or Pspac-\textit{spoOA(A257V)} and suggest that the concentration of phosphorylated wild type or mutant protein is not of sufficient threshold concentration to activate transcription from either promoter. As a consequence, the data from this experiment did not provide insight into the specific question of whether the A257V mutation causes a faulty interaction with $o^H$. 
4. DISCUSSION

4.1. Spo0A is the master regulator of the onset of sporulation
In response to nutrient deprivation and high cell density, *B. subtilis* cells will undergo the developmental process of sporulation. Sporulation is a tightly regulated process and initiation of sporulation is ultimately controlled by the response regulator Spo0A. Spo0A directly controls the expression of 121 genes by repressing the expression of genes required for alternate stationary phase phenomena and activating genes required for sporulation (Molle *et al.*, 2003a). An early step in the process begins with the Spo0A-dependent repression of the *abrB* gene which encodes a transition state regulator. During vegetative growth, AbrB represses transcription of many stationary phase genes and some genes required for sporulation (Strauch and Hoch, 1993). Repression of *abrB* permits expression of the *spo0H* gene, encoding **H**, an alternate σ factor that together with Spo0A regulates transcription during the onset of sporulation. Together the two proteins cooperate to increase transcription of the *spo0A* gene itself as the result of a positive feedback mechanism. **H** is also responsible for increasing the concentration of activated, phosphorylated Spo0A by increasing transcription of the genes of the phosphorelay components, *kinA* and *spo0F*.

4.2. The A257V mutation of Spo0A uncouples transcription activation and repression
I was interested in how changes in the DNA-binding domain of Spo0A affect the ability of Spo0A to activate transcription. One substitution mutation in Spo0A, A257V, was found to differentially affect the ability of Spo0A to activate and repress transcription. The A257V mutation abolishes the ability of *B. subtilis* cells to sporulate but does not affect the ability of Spo0A to repress transcription at the *abrB* promoter. Instead, the mutation eliminates the ability of Spo0A to activate both σA-dependent transcription of the *spoIIG* operon promoter and **H**-dependent transcription of the *spoIIA* operon promoter (Perego *et al.*, 1991b).

4.3. Spo0A can activate and repress transcription initiation

4.3.1. Repression of transcription by Spo0A
The mechanism by which Spo0A represses transcription initiation has been investigated at the *abrB* promoter. The *abrB* promoter contains two transcription initiation start sites, P1 and P2 (Perego *et al*., 1988), and two consensus 0A boxes located downstream of the second transcription start site (Strauch *et al*., 1990). Spo0A-P binds to the 0A boxes at *abrB* by interacting with only one side of the DNA by binding across the minor groove (Greene and Spiegelman, 1996; Strauch *et al*., 1990). Spo0A-P represses transcription initiation at P2 by preventing DNA strand separation without blocking the initial interaction of RNAP with the *abrB* promoter (Greene and Spiegelman, 1996). It has been presumed, although not directly proven, that the formation of a blocked ternary RNAP-Spo0A-P-*abrB* P2 complex inhibits transcription from *abrB* P1 by steric occlusion (Greene and Spiegelman, 1996).

### 4.3.2. Activation of σ^A^-dependent transcription by Spo0A

How Spo0A stimulates transcription is best understood at the σ^A^-dependent *spoIIG* promoter. Previous work has demonstrated that RNAP can bind to the *spoIIG* promoter but is transcriptionally inactive (Bird *et al*., 1993, 1996). At *spoIIG*, Spo0A functions as a class II transcriptional activator (Busby and Ebright, 1997, 1999), binding to a pair of 0A boxes lying immediately upstream of and overlapping with the −35 element, placing it in a position to interact with the 4.1 region of σ^A^. Importantly, the distance separating the conserved -35 and -10 elements of this promoter and another Spo0A-dependent, σ^A^-dependent promoter, *spoIIE*, are 22 and 21 base pairs respectively, instead of the consensus 17 base pairs (Spiegelman *et al*., 1995). Recently it has been demonstrated that reducing the spacer length at *spoIIG* eliminates the requirement for Spo0A during transcription initiation, suggesting that Spo0A stimulates transcription by compensating for the overlong spacing of these promoters (McLeod and Spiegelman, 2005). The requirement for Spo0A-P during transcription initiation at *spoIIG* can also be bypassed by artificially denaturing the −10 element (Seredick and Spiegelman, 2004; Seredick, 2005), demonstrating that activated Spo0A and RNAP cooperate to separate the DNA strands around the −10 element. A recent study has also shown that Spo0A-P bound to the 0A box overlapping with the −35 element appears to re-position RNAP to facilitate the interaction of the σ^A^ subunit of RNAP with the non-template strand of the -10 region of heteroduplex *spoIIG* promoters (Seredick and Spiegelman, 2004). From these data, one would predict that Spo0A re-positions σ^A^ for recognition of the double-stranded −10 element and for nucleation of promoter melting.
Thus, transcription initiation at spoIIG involves at least three steps: formation of an inactive RNAP-spoIIG complex; activation of the RNAP-spoIIG complex by Spo0A, including open complex formation; and initiation of transcription and promoter clearance. However, it is not clear whether the binding of Spo0A–P to 0A boxes overlapping the -35 element and the contact with the σ^A subunit stimulates release of σ^A contacts at the -35 element while tethering RNAP close to the -10 element, or whether Spo0A–P passively retains RNAP that has diffused downstream (Seredick and Spiegelman, 2004). Recent work suggests that RNAP maintains contact with the upstream promoter and recruits Spo0A–P as the downstream 0A boxes are exposed when σ slides off the –35 element (Seredick & Spiegelman, unpublished data).

4.3.3. Activation of σ^H-dependent transcription by Spo0A

Little is known of the role Spo0A plays in transcription initiation at promoters transcribed by RNAP-σ^H. Genetic studies suggest that Spo0A contacts homologous regions in σ^H and σ^A (Baldus et al., 1995). However, the arrangement and orientation of 0A boxes and the spacing of the –35 and –10 elements at σ^H-dependent promoters differs from those at σ^A-dependent promoters (Spiegelman et al., 1995) (Figure 23), suggesting that Spo0A uses a different surface to contact σ^H and a different mechanism to activate transcription (Seredick and Spiegelman, 2001). Studies investigating Spo0A-dependent transcription activation of the spoIIA promoter indicate that deletion of sequences 64 bases 5’ to the transcription initiation site completely abolish promoter activity in vivo (Wu et al., 1991), suggesting that the 0A box located upstream is critical for activation. While a σ^A contact surface on Spo0A has been defined (Buckner et al., 1998; Hatt and Youngman, 1998; Kumar et al., 2004; Schyns et al., 1997), the region responsible for interaction with σ^H has not been identified. Genetic screens designed to identify the σ^H contact surface (Hatt and Youngman, 1998) may have been unsuccessful because of the complexity of the genetic regulatory network controlling Spo0A synthesis and activation, or for other technical reasons.

4.4. Objective of thesis

The objective of this study was to resolve how a single amino acid substitution within the DNA binding domain of Spo0A could prevent sporulation while differentially affecting the
Figure 23. Location of 0A boxes within promoters activated and repressed by Spo0A. Spo0A binds to 0A boxes (5'-TGNCGAA-3') encoded upstream and downstream of the +1 transcription start site of target promoters to activate and repress transcription, respectively. Examples of promoters activated by Spo0A ('+', to right of promoter) include the spoIIG, spoIIA promoters and the sporulation promoter of spo0A. Two promoters repressed by Spo0A ('-', to right of promoter) include the vegetative promoter of spo0A and the abrB promoter. The number, location (bp relative to +1 indicated above promoters) and orientation of 0A boxes within target promoters is not conserved. 0A boxes are indicated by arrows, and the direction of the arrow indicates orientation. Promoters recognized by RNAP complexed with $\sigma^A$ and $\sigma^H$ are indicated.
ability of the protein to activate and repress transcription (Perego et al., 1991b; Rowe-Magnus et al., 2000). I used biochemical methods to explore how the A257V mutation affected Spo0A function to prevent activation without compromising abrB repression.

4.5. In vitro characterization of Spo0A(A257V)

Spo0A(A257V) was phosphorylated at the same rate and to the same extent as Spo0A (Figures 9, 10), indicating that the mutation did not alter the structure of the protein to compromise it as a substrate for the phosphotransferase protein Spo0B. This indicated that insufficient phosphorylation of Spo0A(A257V) could not account for the absence of sporulation in vivo. Similarly, Spo0A(A257V) was able to repress transcription from the abrB promoter in vitro, although to levels approximately half those achieved by the wild type protein (Figure 11). This suggested that the A257V mutation did affect DNA binding. Previous research determined that the A257V mutation does not affect abrB repression in vivo (Perego et al., 1991b), indicating that in vivo the lowered binding efficiency is not crucial for repression.

I showed for the first time that the A257V mutation did not abolish the ability of Spo0A to activate $\sigma^A$-dependent transcription in vitro. Spo0A(A257V) stimulated spoIIG transcription, although at a reduced rate (Figure 12) and to levels approximately half those observed for Spo0A (Figure 13). This finding was novel since in vivo research had indicated that the A257V mutation rendered Spo0A unable to activate both the $\sigma^A$-dependent spoIIG promoter and the $\sigma^H$-dependent spoIIA promoter (Rowe-Magnus et al., 2000). This indicated that the A257V mutation did not obliterate the ability of Spo0A to stimulate transcription activation but suggested the mutation could possibly represent a defect in activation of $\sigma^H$-dependent transcription.

One possible explanation for the reduced ability of Spo0A(A257V) to activate spoIIG and repress abrB in vitro was that the A257V mutation may have caused Spo0A to bind inappropriately to 0A boxes located at these promoters. However, DNacl footprinting analysis revealed that Spo0A(A257V) interacted with both site 1 and site 2 boxes of the spoIIG promoter in a seemingly identical fashion, although with reduced affinity relative to
the wild type protein (Figure 14). This indicated that the A257V mutation of Spo0A did not alter the specificity of binding or alter the interaction of the mutant protein with DNA bases or the DNA backbone.

To quantitatively measure the effect of the A257V mutation on the binding affinity of Spo0A for 0A boxes I used an EMSA to compare the ability of wild type and mutant proteins to bind consensus 0A boxes encoded within duplex DNA. Analysis of DNA binding revealed that the affinity of Spo0A(A257V) for a consensus binding site was reduced relative to Spo0A (Figure 15). The observed 2-fold reduction in binding correlated with the 2-fold reduction in transcription stimulation and repression. Thus a common modest defect in DNA binding likely underlies the modest reduction observed for in vitro transcription activation and repression. However, the twofold reduction in DNA binding is associated with a 10^7 fold reduction in sporulation in spo0A(A257V) B. subtilis strains (Perego et al., 1991b). Previous research has indicated that mutations within Spo0A at residues crucial for DNA contact (E213, R214) render Spo0A incapable of stimulation of both σ^A- and σ^H-dependent promoters in vivo and either decrease or eliminate DNA binding in vitro (Hatt and Youngman, 2000). Unlike the A257V substitution, mutation of these residues compromises the ability of Spo0A to repress abrB in vivo as well, arguing against the possibility that A257 might play a direct role in DNA binding.

To further investigate how the A257V mutation decreased the apparent DNA binding affinity of Spo0A for 0A boxes, I assessed whether the binding interactions by two Spo0A monomers in the dimer was equivalent. I found that the A257V mutation had a minor effect on dimer packing at the spoIIG promoter as assessed by productive interactions with RNAP leading to transcription (Figure 16), suggesting that the binding interactions with DNA by the downstream monomer are more critical than the binding interactions of the upstream monomer. This indicated that altered packing of the DNA binding domain, as a consequence of the A257V substitution, reduced the affinity of the mutant proteins for the 0A boxes. However, this effect was relatively modest and seemed unlikely to be able to account for the loss of sporulation in vivo. In sum, these results indicated that the A257V mutation did not significantly effect on the ability of Spo0A to stimulate transcription activation. The lack of
a dramatic effect on transcription activation by RNAP-σA points to the possibility that the mutation could possibly represent a defect in activation of σH-dependent transcription.

4.6. In vivo investigation of the effect of the A257V mutation of Spo0A

Results from this study have revealed that the A257V substitution reduced but did not eliminate the ability of the Spo0A to activate σA-dependent transcription. Spo0A(A257V) was able to both activate and repress transcription in vitro, albeit to levels approximately half that achieved by wild type Spo0A. Spo0A(A257V) did not impair phosphorylation, or cause inappropriate binding to 0A boxes within the spoIIG promoter. The mutation modestly reduced the affinity of Spo0A for 0A boxes and appeared to have a negligible effect on the packing of Spo0A dimers against RNAP. The data from this in vitro investigation indicated noticeable but slight effects of the A257V mutation contrasted with the dramatic changes in the phenotype in vivo.

Studies have indicated that genes within the Spo0A regulon respond differentially to high and low concentrations of Spo0A (Chung et al., 1994; Fujita et al., 2005). One possible explanation for the lack of sporulation observed in vivo in the spo0A(A257V) mutant was that the mutant protein was not expressed to the same level as the wild type protein. As a consequence, genes that are activated by high levels of Spo0A and required for sporulation, such as the spoIIA and spoIIIG operons would not be transcribed and the cells would not sporulate. I tested this idea and found that the mutant Spo0A protein was not expressed to the same level as wild type Spo0A (Figure 17). This result was unexpected since previous work had suggested that both wild type and mutant proteins were expressed to the same extent (Perego et al., 1991b). This suggested that the lack of sporulation in B. subtilis strains encoding spo0A(A257V) could be attributed to insufficient mutant Spo0A protein expression.

Spo0A expression increases at the onset of stationary phase as a consequence of a positive feedback mechanism in which transcription of the spo0A gene is increased due to activation of a second promoter for the spo0A gene transcribed by RNAP-σH. A similar regulatory mechanism exists controlling expression of the master regulator of competence in B. subtilis, comK. Like Spo0A, there are multiple regulatory inputs affecting transcription from the
comK promoter that determine the threshold level of comK expression (Hahn et al., 1996; Smits et al., 2005; van Sinderen and Venema, 1994). At some point, the concentration of ComK becomes sufficient to activate an auto-stimulatory loop (Maamar and Dubnau, 2005; Smits et al., 2005). In competence development, this auto-stimulatory loop is essential and sufficient to establish the “bistable response” typical of biological systems such as competence and sporulation (Hofer et al., 2002; Smits et al., 2005).

The reduction in expression of Spo0A(A257V) observed in this study could be attributed to a deficiency in the positive feedback loop leading to upregulation of spo0A(A257V) expression. There are two possible explanations to explain this defect. Firstly, the A257V mutation could interfere with a specific interaction between Spo0A and σH leading to lack of, or inefficient, expression from spo0Apv. Alternatively, since Spo0A activates transcription as a dimer, small changes in dimer properties could lead to low levels of Spo0A protein and failure to activate essential promoters. In order to differentiate between these two possibilities in vivo, I attempted to create a system in which the expression of wild type and mutant proteins was inducible and then tested the ability of cells over-expressing wild type or mutant protein to activate transcription from both a σA-dependent and a σH-dependent promoter in vivo. I predicted that if the A257V mutation caused a defective interaction between Spo0A and σH, cells expressing the mutant protein would activate σA-dependent promoters, but not σH-dependent promoters whereas cells expressing the wild type protein would be able to activate both σA-dependent and σH-dependent transcription. If the A257V mutation caused a defective interaction between Spo0A monomers leading to low levels of Spo0A in vivo, over-expression of Spo0A(A257V) would overcome the defective interaction and permit activation of both σA- and σH-dependent transcription.

Although wild type and mutant Spo0A proteins were expressed in response to IPTG induction, the levels of expression of either protein were low and were insufficient to permit wild type levels of sporulation or drive activation of either σA- or σH-dependent transcription. Increasing the concentration of IPTG used to induce expression revealed that expression from the Pspac promoter construct could not be increased. Similarly, induction at various cell densities eliminated the possibility that the timing of Spo0A induction was not
synchronized with the expression of other proteins required for sporulation as induction at various points during growth did not activate either $\sigma^A$-dependent or $\sigma^H$-dependent transcription.

There are two possible explanations for the decreased levels of sporulation and lack of activation of both spoIIG and spoIIA promoters in the inducible-Spo0A B. subtilis strains used in this study. Fujita and Losick (2005) induced a constitutively active form of Spo0A during mid-exponential growth and demonstrated that sudden high level expression of Spo0A exerts a dominant-negative effect on sporulation. From their study, the authors postulated that a gradual increase in the threshold concentration of activated Spo0A is required for sporulation. Alternatively, decreased levels of sporulation and lack of activation of stage II promoters in the inducible-Spo0A B. subtilis strains used in this study may have been observed because there were insufficient levels of phosphorylated wild type or mutant protein available for stimulation of transcription and subsequent sporulation. The low concentration of phosphorylated protein available may have been a consequence of the genotype of the strains created. For example, the strains created here encode a wild type or mutant spo0A gene under transcriptional control of Pspac and one or more copies of the N-terminal receiver domain of Spo0A (Spo0AN) transcribed under the control of the native vegetative- and sporulation-specific promoters of spo0A. The spo0A gene duplication arose as a result of the recombination event that inserted the Pspac-controlled spo0A allele into the chromosomal spo0A locus. It is conceivable that if Spo0AN was expressed it would compete for phosphorylation by the phosphorelay in vivo. Furthermore, if multiple copies of pMNSpo0AN were integrated into the spo0A locus, there would be many duplications of the truncated spo0A gene encoding Spo0AN and only a single copy of the full length wild type or mutant spo0A under Pspac transcriptional control. In this genetic background there is an even greater possibility for competition for phosphorylation by the phosphorelay between Spo0AN and the full length wild type or mutant Spo0A proteins. This may be sufficient to reduce the concentration of phosphorylated full length wild type or mutant Spo0A below the level required for activation and repression of high-threshold Spo0A-dependent promoters and prevent sporulation. The hypothesis has some support from previous research that has shown that Spo0AN can successfully compete with the full length protein for phosphorylation.
in vitro, and when Spo0AN is over-expressed in the mother cell sporulation is impaired prior to polar septum formation (Fujita and Losick, 2003). In Western blot analyses performed in this study a low molecular weight band corresponding to a protein of approximately 14 KDa was observed which could represent Spo0AN (data not shown). However, since the polyclonal antibody used in the analyses reacted with other proteins in cell lysates, it was unclear if this band represented Spo0AN or another cellular protein.

There are several methods by which one might increase the low levels of Spo0A and Spo0A(A257V) expression in the reporter strains. One alternative is to eliminate competition by Spo0AN for phosphorylation. This can be accomplished by reconstructing the Pspac-spo0A and Pspac-spo0A(A257V) strains encoding spoIIA-lacZ and spoIIG-lacZ translational fusions in either a Δspo0A background, or by removing the native spo0A promoters from the existing strains. Alternatively, expression of the full length wild type or mutant Spo0A proteins could be increased in vivo without removing the spo0AN sequence. One method to achieve this is to introduce a second copy of Pspac-spo0A or Pspac-spo0A(A257V) into a site on the chromosome separate from the spo0A locus. Alternatively wild type and mutant Spo0A protein expression could be increased by expressing a σA mutant, crsA, which permits high-level transcription of spo0A from the vegetative promoter (Dixon and Spiegelman, 2002; Yamashita et al., 1989). The latter approaches will increase the concentration of wild type or mutant Spo0A protein but do not eliminate the possibility of competition for phosphorylation by Spo0AN. Repetition of the sporulation and β-galactosidase assays using true over-expression strains should provide a more definitive answer to whether the A257V substitution interferes with the interaction of Spo0A and σH or whether the results are more consistent with the A257V mutation interfering with Spo0A dimerization.

4.7. Effect of residue A257 on Spo0A dimer function

It has been suggested that the effect of the A257V mutation within Spo0A is to weaken or disrupt crucial interactions within a Spo0A dimer (Lewis et al., 2002). The crystal structure of the DNA binding domain of Spo0A complexed with DNA indicates that two CTD bind tandem 0A boxes in a head-to-tail orientation (Zhao et al., 2002). In this orientation two
adjacent DNA binding domains are able to interact with each other through a network of salt bridges, hydrogen bonds and hydrophobic patches. The dimer interface in the crystal structure is formed by helix αF of the upstream Spo0A monomer and helix αB of the downstream Spo0A monomer (Zhao et al., 2002) (Figure 6). Predictions of the effect of substitution of valine for alanine at position 257 within the dimer interface suggest that the mutation would weaken interactions between the monomers by altering the orientation and flexibility of the helix within which A257 is located (Zhao et al., 2002). It has been suggested that two suppressor mutations of spo0A(A257V), H162R and L174F, repress the effects of the A257V mutation by facilitating compensatory interactions between adjacent monomers (Seredick and Spiegelman, 2001; Zhao et al., 2002). Head-to-tail dimerization places the suppressor mutations and the A257V mutation together at the dimer interface as both suppressor mutations are located at the opposite face of the C-terminal domain from residue A257 (Zhao et al., 2002) (Figure 24). This orientation strengthens hydrophobic interactions between monomers and permits formation of a new hydrogen bond between H162R and F236 (Zhao et al., 2002).

Like the A257V mutation, deletion of the last 10 amino acids of Spo0A and the D258V and L260V mutations abolish the ability of Spo0A to activate both σH- and σA-dependent transcription but do not effect the ability of the protein to repress transcription (Rowe-Magnus et al., 2000). These residues are also located at the dimer interface and as such may also affect Spo0A activity because of disrupted or weakened contacts within a Spo0A dimer. Residue D258 in one monomer forms an intermolecular salt bridge with R177 in the other monomer and mutation would eliminate this interaction, weakening dimerization (Zhao et al., 2002). Similarly, the predicted effect of the L260V mutation is similar to that of the A257V mutation in that it likely weakens intermolecular interactions by decreasing the flexibility of helix αF (Zhao et al., 2002).

Similarly to Spo0A, members of the OmpR-PhoB family of response regulators bind DNA in a head-to-tail orientation (Blanco et al., 2002). The C-terminal domains of Spo0A and PhoB lack structural homology outside their DNA binding motifs and consequently the dimer interface differs between the two. Stable interactions between monomers may be crucial in
Figure 24. Suppressor mutations of spoOA(A257V) strengthen intermolecular contacts within the Spo0A dimer. Tandem Spo0A DNA binding domains bind two consensus OA boxes as a head-to-tail dimer. Head-to-tail dimerization places the suppressor mutations (H162R on αA and L174F on αB) and the A257V mutation (αF) together at the dimer interface, strengthening hydrophobic interactions between monomers and permitting formation of a new hydrogen bond between. H162, L174 and A257 are indicated in space filling model. The DNA is shown 5' to 3' and the structure is rotated 90° to indicate the dimer interface. Helices: αA, blue; αB, cyan; αC, green; αD, yellow; αE, gold. The structure is based on PDB file 1LQ1 deposited by Zhao and Varughese, 2002 in the RCSB protein data bank (http://pdbbeta.rcsb.org/pdb/Welcome.do). Figure constructed using PyMOL (Delano Scientific).
sequential monomer binding or cooperative binding of response regulators such as Spo0A and PhoB to DNA. Like Spo0A(A257V), mutations within PhoB have been isolated that map to the dimer interface which reduce or completely abolish DNA binding (Makino et al., 1996). However all known DNA binding sequences of all members of the OmpR-PhoB family are direct repeats with identical orientation with respect to the direction of transcription (Blanco et al., 2002). In contrast, O boxes do not always occur in the same orientation. Dimerization facilitated by interactions between adjacent DNA-binding domains is also suggested from the crystal structure of another response regulator, NarL, although NarL dimers are oriented in an antiparallel orientation (Maris et al., 2002). Like the spo0AΔ10 allele in which the last 10 amino acids (residues 253 to 263) have been removed (Rowe-Magnus et al., 2000), deletion of the last 7 amino acids of the NarL homolog UhpA abolishes the ability of the regulator to activate transcription (Webber and Kadner, 1995). Based on the NarL structure bound to DNA, the last 7 amino acids in UhpA would also be predicted to form part of the dimer interface. While the C-terminal domain structures of Spo0A, PhoB/OmpR and UhpA/NarL are different, the dimer interface of the output domain clearly makes a contribution to response regulator function.

There is evidence in opposition to the hypothesis that the A257V mutation within the C-terminal domain of Spo0A weakens interactions required for dimer stabilization. A recent model suggests that the primary effect of receiver phosphorylation in Spo0A is dimerization (Lewis et al., 2002); that is, the response regulator receiver domains act as inducible dimerization domains (Fiedler and Weiss, 1995). Receiver domain dimerization has been observed in the response regulators NtrC, PhoB, FixJ, and PhoP, and speculated in NarL (Baikalov et al., 1996) in response to phosphorylation (Birck et al., 2003; Da Re et al., 1999; Fiedler and Weiss, 1995; McCleary, 1996). In both FixJ and PhoP mutations have been isolated within the receiver domain which do not dimerize and have decreased DNA binding activity (Chen et al., 2003; Da Re et al., 1999). While the DNA-binding domain interactions revealed from the crystal structures should not be ignored, such interactions would seem to be redundant in light of the ability of the receivers to dimerize (Lewis et al., 2002) and the ability of the Spo0A(A257V) mutant to bind DNA (Figures 14,15). In addition, while A257V does reduce transcription activation by half, repression of the abrB promoter, which
also depends on DNA binding and therefore Spo0A dimerization is unaffected by the spo0A(A257V) mutation \textit{in vivo} (Perego et al., 1991b).

If Spo0A is active as a dimer, it is conceivable that stabilizing interactions play an important role in increasing the local concentration of Spo0A at ‘high-threshold Spo0A-activated’ promoters but not at promoters responding to low concentrations of Spo0A. This prediction is consistent with the lack of activation of the ‘high-threshold Spo0A-activated’ (Fujita et al., 2005) spoIIA and spoIIG operon promoters \textit{in vivo} (Perego et al., 1991b) and repression of the ‘low-threshold Spo0A-repressed’ (Fujita et al., 2005) abrB promoter \textit{in vivo} (Perego et al., 1991b) by Spo0A(A257V). One serious problem for this threshold model is the lack of induction of the ‘low-threshold Spo0A-activated’ (Fujita et al., 2005) \( \sigma^H \)-dependent, sporulation-specific promoter of spo0A (Chibazakura et al., 1995) \textit{in vivo} and the subsequent decrease in Spo0A(A257V) expression observed in this study. This promoter is key to the autostimulatory loop proposed \textit{in vivo}.

One hypothesis that may account for these observations is that at low concentrations of activated protein, Spo0A may dimerize on the DNA instead of in solution. Under these conditions, monomers of Spo0A~P may bind DNA cooperatively with intermolecular interactions between the C-terminal domains playing a crucial role. Following the initial interactions between the Spo0A dimers and the 0A boxes and between the C-terminal domains of the monomers, interaction of the phosphorylated receiver domains would serve to stabilize the Spo0A~P-DNA complex. The stability of the complex would be a function of the sum of interdomain interactions between N-terminal and C-terminal domains, and the interactions of the C-terminal domains with the DNA. If the A257V mutation affected dimerization, then at protein concentrations or conditions too low to allow receiver dimerization in solution Spo0A and Spo0A(A257V) would be expected to bind DNA differently. Specifically, Spo0A would be expected to bind cooperatively, and Spo0A(A257V) would be expected to bind with a lesser degree of cooperativity. None of the binding experiments carried out so far show any indication of cooperativity with either Spo0A~P or Spo0A(A257)~P. This may reflect a technical issue that the phosphorylation reactions were carried out at relatively high concentrations of protein. The choice of protein
concentration was dictated by the desire to keep the conditions for binding similar to those used for transcription analysis. In the transcription analysis it was necessary to add small volumes of activator to the reactions to minimize the effects of ionic and organic solvent (glycerol). Thus, the concentrations of SpoOA or SpoOA(A257) were kept high. However, it is also interesting to note that in the experiments with binding to the oligonucleotide containing the 0A boxes from the \textit{abrB} promoter, no evidence of binding of a single SpoOA monomer to the DNA fragment was ever detected and only a single species with an altered electrophoretic mobility was ever detected.

4.8. Residue A257 as a part of a $\sigma^H$-Spo0A interaction surface

The second possible explanation for the effect of the A257V mutation on Spo0A function is that residue A257 is important for contact with the $\sigma^H$ subunit of RNAP and that the A257V mutation diminishes this interaction. This hypothesis would explain the lack of activation of the $\sigma^H$-dependent \textit{spoIIA} promoter (Perego \textit{et al.}, 1991b) and the lack of transcript from the $\sigma^H$-dependent sporulation-specific promoter of \textit{spo0A} (Chibazakura \textit{et al.}, 1995) observed \textit{in vivo}. Furthermore, this hypothesis accounts for the lack of activation of the 'high-threshold Spo0A-activated' $\sigma^A$-dependent \textit{spoIIG} promoter \textit{in vivo} since increased expression of \textit{spo0A}, which is needed to increase Spo0A levels, is dependent upon successful interaction between Spo0A and $\sigma^H$ during stimulation of \textit{spo0Aps} transcription. This hypothesis could also explain the lack of transcription activation of promoters that do not contain tandem 0A boxes and presumably bind monomeric Spo0A--P, such as \textit{spo0Aps} (Figure 23). Moreover, because the direction of the 0A boxes at $\sigma^H$-dependent promoters (eg. \textit{spoIIA, spo0Aps}) differs from the direction of 0A boxes at $\sigma^A$-dependent promoters (eg. \textit{spoIIG}), the face of the DNA-binding domain of Spo0A(A257V) encoding A257V would be oriented downstream in a position to contact RNAP bound downstream (Figure 23).

Although the hypothesis that A257 and/or the region around it represents that $\sigma^H$ contact region within Spo0A accounts for the \textit{in vivo} and \textit{in vitro} characteristics of Spo0A(A257V) thus far, the structural data available run counter to this hypothesis. The region within Spo0A that contacts $\sigma^A$ during transcription activation (the SAAR) is located within and around the $\alpha E$ helix of the DNA binding domain of Spo0A (Lewis \textit{et al.}, 2000a) (Figure 5).
Head-to-tail binding of a dimer of C-terminal domains of Spo0A to tandem 0A boxes places the αE helix of both monomers on the same side of the dimer in proximity to σ^A during transcription activation (Zhao et al., 2002). In comparison, efforts to locate a similar region within Spo0A that contacts σ^H during stimulation of transcription initiation have been unsuccessful, although previous research have found regions within σ^H contacted by Spo0A during transcription activation (Baldus et al., 1995).

If A257 was located in a region within Spo0A contacted by σ^H, we would expect that suppressor mutations of A257V would be located near this residue. This is not observed as two of three A257V suppressor mutations are located far from A257 on the opposite face of the DNA binding domain of Spo0A. The third A257V suppressor mutation, sof114 (a D92Y substitution), was isolated as a suppressor of a spo0F deletion mutant (Spiegelman et al., 1990). D92 is located in the receiver domain of Spo0A and because the structure of the full length Spo0A protein has yet to be determined, it is possible that the orientation of the two domains places D92Y near A257V. However, a more likely explanation for suppression of A257V by D92Y is that intermolecular contacts between the receiver domains within a Spo0A dimer are strengthened in Spo0A(D92Y, A257V) since previous work demonstrated that the D92Y mutation enhanced stability of Spo0A-RNAP complexes (Cervin and Spiegelman, 1999).

Further research could determine if residue A257 and/or the region around it identifies the σ^H interaction region within Spo0A. A direct test of this hypothesis in vitro would be to include Spo0A(A257V)~P and RNAP-σ^H in an in vitro transcription reaction and assess the stimulation of either the spoIIA or the spo0Aps promoters. From this experiment I would expect that Spo0A(A257V)~P would be unable to stimulate either spoIIA or spo0Aps transcription at any protein concentration whereas Spo0A~P would be able to stimulate transcription activation. Similarly, whether A257 represented a residue within Spo0A contacted by σ^H could be tested in vivo utilizing the experiments attempted in this work involving inducible-Spo0A expression and activation of spoIIA-lacZ and spoIIG-lacZ translational fusions.
REFERENCES


Seredick, S.D. (2005) Spo0A-Stimulated Transcription Initiation at the Bacillus subtilis spoIIG promoter. In Department of Microbiology and Immunology, UBC Vancouver, BC: UBC.


