THE EFFECTS OF THE crsA MUTATION IN THE MAJOR VEGETATIVE SIGMA FACTOR $\sigma^A$ ON THE REGULATION OF SPORULATION INITIATION IN *Bacillus subtilis*

by

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Abstract

The \textit{crsA} mutation is located within the gene for the major vegetative sigma factor of \textit{Bacillus subtilis}, $\sigma^A$. The presence of this mutation results in alterations in the regulatory events controlling sporulation initiation, such that spore formation proceeds despite the presence of inhibitory concentrations of glucose. In an effort to more fully understand the mechanisms of glucose repression of sporulation, the effects of the \textit{crsA} mutation on sporulation gene expression were examined.

The \textit{in vivo} promoter activity of genes involved in the initial stages of sporulation was examined in the \textit{crsA} mutant using promoter-\textit{lacZ} fusion constructs. The observed patterns of gene expression indicated that key regulatory checkpoints in the sporulation initiation pathway were bypassed in the presence of the mutant $\sigma^A$. The activity of genes encoding phosphorelay proteins was altered, suggesting the inappropriate activity of the sporulation sigma factor, $\sigma^H$; as well, both the expression of the operon encoding the transition state regulator SinR, and the expression of \textit{spo} genes negatively regulated by SinR, were altered.

Analysis of \textit{spoOA} promoter expression suggested that transcription from the vegetative promoter of \textit{spoOA} was increased in \textit{crsA} mutant strains. Analysis of both the expression from and the sporulation frequency of a \textit{spoOA} promoter mutant supported this observation, and implicated altered \textit{spoOA} expression in the glucose resistant sporulation phenotype of the \textit{crsA} mutant. Comparative \textit{in vitro} transcription assays were performed using wild type and \textit{crsA} mutant RNA polymerases, providing evidence that transcription from the $\sigma^A$ dependent \textit{spoOA} promoter by the \textit{crsA} mutant RNA polymerase was increased over that seen with the wild type enzyme.
The data presented herein suggested that the alteration of \textit{spo0A} gene expression was a direct effect of the \textit{crsA} mutation in $\sigma^A$. This increase in \textit{spo0A} expression, combined with inappropriate $\sigma^H$ activity and altered \textit{sin} expression, resulted in changes in the expression patterns of key genes involved in the initiation of sporulation, overcoming regulatory checkpoints at which sporulation would normally be repressed by glucose. These data indicate that prevention of Spo0A accumulation and negative regulation of $\sigma^H$ activity are important in the mechanism of glucose inhibition of sporulation.
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...
## Abbreviations and Symbols

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0A box</td>
<td>nucleotide sequence at which Spo0A~P binds</td>
</tr>
<tr>
<td>abrB</td>
<td>gene encoding transition state regulator AbrB, a negative regulator of sporulation</td>
</tr>
<tr>
<td>alsA</td>
<td>allele of ccpA</td>
</tr>
<tr>
<td>Am&lt;sup&gt;f&lt;/sup&gt;</td>
<td>ampicillin resistance phenotype</td>
</tr>
<tr>
<td>amp</td>
<td>ampicillin resistance gene</td>
</tr>
<tr>
<td>BGSC</td>
<td>Bacillus Genetic Stock Center</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CAC</td>
<td>citric acid cycle</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cat</td>
<td>chloramphenicol resistance gene</td>
</tr>
<tr>
<td>ccp</td>
<td>genes ccpA, ccpB, and ccpC, genes encoding catabolite control proteins CcpA, CcpB, and CcpC</td>
</tr>
<tr>
<td>clp</td>
<td>clpA, clpX, clpC and clpP, genes encoding stress induced chaperonins/ATPases (ClpA and ClpX) and proteases (ClpC and ClpP)</td>
</tr>
<tr>
<td>Cm&lt;sup&gt;f&lt;/sup&gt;</td>
<td>chloramphenicol resistance phenotype</td>
</tr>
<tr>
<td>CR</td>
<td>catabolite repression</td>
</tr>
<tr>
<td>cre</td>
<td>catabolite responsive element</td>
</tr>
<tr>
<td>CRP/CAP</td>
<td>catabolite repressor protein/cAMP activated protein</td>
</tr>
<tr>
<td>crs</td>
<td>catabolite resistant sporulation mutants; crsA allele resides in sigA</td>
</tr>
<tr>
<td>CsCl</td>
<td>cesium chloride</td>
</tr>
<tr>
<td>Δ</td>
<td>gene knockout</td>
</tr>
<tr>
<td>E</td>
<td>RNA polymerase core enzyme</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>FDP</td>
<td>fructose-1,6-diphosphate</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>Hepes</td>
<td>4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid</td>
</tr>
</tbody>
</table>
Gene Hpr encodes the transition state regulator Hpr, a negative regulator of sporulation. HPr is the PTS protein phosphorylated by enzyme I. IPTG is the isopropyl-thio-β-D-galactoside. The kan gene encodes kanamycin resistance. kb refers to kilobase pair. KinA is the gene encoding the major sporulation kinase KinA. The Km' phenotype indicates kanamycin resistance. The lacZ gene encodes β-galactosidase, used as a reporter for promoter activity analysis.

**Techniques and Abbreviations**
- **LB**: Luria broth
- **MCS**: Multiple cloning site
- **MES**: 2-[N-morpholino] ethanesulfonic acid
- **MOPS**: 3-[N-morpholino] propanesulfonic acid
- **nt**: Nucleotide
- **NTP**: Nucleotide triphosphate
- **ONPG**: Orthonitrophenyl pyranogalactoside
- **ori**: Plasmid origin of replication
- **P0A**: Promoter from the spo0A gene of Bacillus
- **PA2**: Promoter from φ29 phage A2 of Bacillus
- **PS**: σ^H specific sporulation promoter of spo0A
- **PV**: σ^A specific vegetative promoter of spo0A
- **PAGE**: Polyacrylamide gel electrophoresis
- **PCR**: Polymerase chain reaction
- **PEP**: Phosphoenolpyruvate
- **PTS**: Phosphotransferase system
- **ptsH**: Gene encoding the PTS protein HPr
- **rbs**: Ribosome binding site
- **σ^A**: Major vegetative sigma factor subunit of RNA polymerase, encoded by sigA
- **σ^A47**: crsA mutant vegetative sigma factor subunit of RNA polymerase
- **σ^H**: Minor abundance stationary phase sigma factor subunit of RNA polymerase
encoded by *spo0H*

**SDS**  sodium lauryl sulfate; sodium dodecyl sulfonate

**sigA**  gene encoding major vegetative sigma factor subunit of RNA polymerase, sigma A (s$^A$)

**sinI**  gene encoding SinI protein that acts to sequester SinR

**sinR**  gene encoding transition state regulator SinR, a negative regulator of sporulation

**spo**  sporulation gene

**Spo0A-P**  phosphorylated form of Spo0A protein

**SSM**  Schaeffer’s spore media

**TBE**  10mM Tris-HCl, pH 7.9, 10 mM boric acid, 1 mM EDTA

**TE**  10mM Tris-HCl, pH 7.9, 1 mM EDTA, pH with HCl

**TfbI**  transformation buffer 1

**TfbII**  transformation buffer 2

**X-gal**  5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Acknowledgements

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To my family, a special thanks for their love, patience, and support, without which this thesis would not have been possible.
Introduction

A. Sporulation in *Bacillus subtilis*.

1. Sporulation as a starvation response.

In natural environments, microbial growth is often limited by the availability of nutrients (Harder and Dijkhuizen, 1983). For the Gram-positive soil bacterium, *Bacillus subtilis*, cells that have ceased to grow vegetatively will differentiate into metabolically inert endospores, a strategy that enables *B. subtilis* to survive in inhospitable conditions, such as prolonged periods of starvation. The mature spore is highly resistant to extremes in dehydration, temperature and pH. When favorable growing conditions are encountered, the spore will germinate, yielding a single cell.

Sporulation is one of the most comprehensively studied examples of cellular differentiation among prokaryotes. This bacterial cell adaptation to nutrient limitation features remarkable changes in cell physiology, morphology and biochemistry, all genetically coordinated in both a temporal and spatial manner (for reviews, see Errington, 1993; Grossman, 1995; Stragier and Losick, 1996; Dunny and Leonard, 1997). Due to the interest of many scientists over many years, *B. subtilis* has been the most intensely studied of the bacterial endospore formers. More than 125 genes essential to the sporulation process (*spo* genes) have been identified (Stragier and Losick, 1996), and the functions of the protein products of these genes are in the process of being examined. The study of sporulation has already provided many insights into the regulatory mechanisms governing the coordinated expression of genes involved in cellular differentiation. As more and more information becomes available about genes, genomes and gene expression, some of the most exciting challenges in developmental biology will be to unravel the details of the regulatory pathways...
and networks that underlie and couple growth, metabolism, differentiation, and development. The advantages of using *B. subtilis* as a model system to study developmental processes include its relatively simple cellular organization, its experimental tractability, and its excellent genetics.

2. The morphology of sporulation.

Spore formation in *B. subtilis* is characterized by a series of morphological changes, the appearance of which has been used to divide the sporulation event into several stages, spo0 through spoVII (Losick *et al.*, 1986; Errington, 1993). The sporulation pathway is entered through a "transition state," in which cells acquire new traits to adapt to changing nutrient availability (Strauch and Hoch, 1993). These include the induction of chemotaxis, motility and competence, secretion of proteases and nucleases, and antibiotic production (Grossman, 1995; Msadek *et al.*, 1998). In response to improving nutrient availability, cells in the transition state will resume vegetative growth. After extended starvation, sporulation will be initiated through a complex series of interactions that ultimately result in commitment to the sporulation pathway.

As shown in Figure 1, the first noticeable structure associated with the sporulation process is the formation of an asymmetric septum that divides the cell laterally into two differently sized compartments (stage II) (Hitchins and Slepecky, 1969), the larger being the mother cell compartment and the smaller the developing forespore. Each compartment contains an intact chromosome. When the septum is complete, each chromosome is used for compartment-specific gene expression (reviewed in Margolis *et al.*, 1991; Errington *et al.*, 1993).
Figure 1. The stages of sporulation in *Bacillus subtilis*. This picture depicts the morphologies of the seven stages of endospore formation in *B. subtilis*. The vegetative state of the cell cycle is defined as stage 0 (top). Sporulation can be initiated only after the completion of DNA replication. The first distinct microscopically visible changes appear as the formation of a polar septum (stage II). Following engulfment of the spore (stage III), the spore cortex (stage IV) and coat (stage V) are synthesized. Once the spore has fully matured (stage VI) it is released through the proteolysis of the mother cell (stage VII).

Adapted from Losick, *et al.*, 1986.
Spore formation continues with the movement of the membrane surrounding the cytoplasm of the mother cell towards the pole of the forespore. Double membranes with opposite polarities eventually surround the forespore (stage III). Once the forespore is fully engulfed, the process is committed to spore formation (Errington, 1993). The space between the two membranes is the site of cortex formation (stage IV). The cortex, made of cell wall material resembling a loosely cross-linked form of peptidoglycan (Warth and Strominger, 1972), is thought to contribute to the heat resistance of the mature endospore (Gould, 1984). The exterior of the forespore is covered with coat proteins synthesized and assembled within the mother cell (stage V) (Jenkinson et al., 1981). Stage VI is associated with the maturation of the forespore. During this stage, the forespore acquires the traits associated with an endospore, including resistance to UV radiation, dessication, heat and organic solvents (Dion and Mandelstam, 1980; Jenkinson et al., 1981; Gould, 1984). The release of the mature spore through lysis of the mother cell (stage VII) occurs roughly 8-10 hours after sporulation initiation.

The signals involved in the initiation of sporulation are not well understood. However, knowledge of signal transmission within the cell and the processes behind initiation and regulation of sporulation is rapidly increasing.

B. Regulation of Sporulation Initiation.

1. Transition state regulators.

When *B. subtilis* is in the vegetative growth phase, the expression of *spo0* genes is largely prevented at the level of transcription, by repressors such as AbrB, SinR, and Hpr (Strauch and Hoch, 1993; Fisher *et al.*, 1994; Hueck and Hillen, 1995). Upon entering the
transition state, AbrB, SinR, and Hpr are thought to act as molecular switches within the cell to effect a commitment to sporulate, or to adopt an alternate strategy in response to nutrient limitation. Strauch and Hoch (1993) have thus suggested that these proteins be called “transition state regulators.”

2. The sigma factor cascade.

Promoter-specific transcription in *B. subtilis* occurs through the association of the RNA polymerase core enzyme (α2ββ') with one of the various σ subunits, to form the holoenzyme (Losick and Pero, 1981; Helmann and Chamberlin, 1988; Stragier and Losick, 1990). The use of alternative sigma factors provides an efficient means of regulating gene expression, both temporally and spatially. Each different σ subunit directs the RNA polymerase to transcribe a specific group of genes with common promoter sequences.

During sporulation, *spo* gene expression is controlled through an ordered series of σ subunit replacements, each of which changes the promoter specificity of the RNA polymerase (Losick and Pero, 1981; Stragier and Losick, 1990). There are six known different σ subunits involved in a “cascade” that results in the timely, sequential transcription of a subset of *spo* genes. The first group of *spo* genes (*spo0* and *spoII* genes) are transcribed by σ^A^, which is the predominant sigma factor during vegetative growth (Kenney et al., 1989; Haldenwang, 1995), or σ^H^, encoded by the *spo0H* gene and expressed maximally during stationary phase (Dubnau et al., 1987; Dubnau et al., 1988; Haldenwang, 1995).

Following stage 0, σ^E^ (*spoIIGB* gene, mother cell specific) and σ^F^ (*spoIAC* gene, forespore specific) appear, and are the first truly sporulation-specific sigma factors (Stragier and Losick, 1990; Errington, 1993; Haldenwang, 1995). Appearing lastly are σ^G^ (*spoIIIG*
gene, forespore specific) and \( \sigma^K \) (spoIVCB:spoIIC gene group, mother cell specific), that are required to transcribe those spo genes necessary to complete the construction of the developing spore (Errington, 1993; Haldenwang, 1995).

The regulation imposed on sporulation by the ordered appearance of specific sigma factors is mediated in part by sigma factor activation (for reviews, see Errington, 1996; Jenal and Stephens, 1996; Helmann, 1999; Kroos et al., 1999). Each sporulation-specific sigma factor is either translated to yield an inactive precursor form (\( \sigma^E \) and \( \sigma^K \)), or is held inactive through complex formation with a second protein (\( \sigma^F \) and \( \sigma^G \)). Each of these sigma factors requires the activation of the previously produced sigma factor before it can become active itself. Sporulation sigma factor regulation is summarized in Figure 2.

There are approximately 12 other known and putative sigma factors in B. subtilis (Kunst et al., 1997). Of those, \( \sigma^B \) transcribed genes are expressed at heightened levels during environmental stress, with many of these genes having promoters recognized by other holoenzymes (Haldenwang, 1995). \( \sigma^D \) appears to transcribe genes encoding structural proteins that form the flagellar hook-basal body complex and chemotaxis regulatory proteins (Helmann et al., 1988; Mirel and Chamberlin, 1989; Helmann, 1991). \( \sigma^L \) is involved in the transcription of a subset of degradative enzymes (Debarouille et al., 1991a; 1991b). \( \sigma^X \) is thought to be involved in the regulation of peptidoglycan synthesis and turnover (Huang and Helmann, 1998), and \( \sigma^W \) is thought to be involved in stationary phase detoxification and/or synthesis of anti-microbial compounds (Huang et al., 1999). \( \text{sig}B, \text{sig}D, \text{or sig}L \) null mutations do not appear to affect growth or sporulation in normal laboratory conditions (Haldenwang, 1995).
nutritional, cell density, and cell cycle signals

transcriptional regulator for sporulation

chromosome ^ CT

partitioning

σ^A

pro - σ^E

polar septum

σ^H

σ^F*SpoIIA

MOTHER CELL

engulfment ^ cr

spo cortex and i

spore cortex and coat synthesis X

pro - a

a
cortex and coat synthesis ^)

FORESPORE

σ^E

σ^F*spoIIA

σ^G

preservation of DNA and preparation for spore germination

σ^K

Figure 2. The regulation of sigma factor synthesis and activation\textsuperscript{a}. Solid arrows indicate a dependence relationship between sigma factors and the gene products that bring about morphological changes. The two vertical lines represent the membrane partition between the mother cell and developing forespore after formation of the asymmetric septum. Dashed arrows indicate interactions between the cell types necessary for sigma activation (short dashes) or synthesis (long dashes).

\textsuperscript{a} Adapted from Kroos, \textit{et al.}, 1999
C. Sporulation Initiation

1. Conditions required for sporulation.

*B. subtilis* will sporulate when starved for carbon, nitrogen or phosphate. However, even when starved for phosphate and/or nitrogen, in the presence of an excessive amount of a phosphotransferase system (PTS) sugar, catabolite repression will prevent sporulation from proceeding (Schaeffer *et al.*, 1965; Freese, 1981; Sonenshein, 1989). It is presumed that, during starvation, one or more critical metabolites will accumulate intra- or extracellularly and act as a signal triggering the sporulation response, but the nature of that signal remains unknown. Recent publications have implicated pH (Cosby and Zuber, 1997; Matsuno and Sonenshein, 1999; Matsuno *et al.*, 1999) and Krebs cycle activity (Jin and Sonenshein, 1994; Ireton *et al.*, 1995; Matsuno and Sonenshein, 1999; Matsuno *et al.*, 1999) as important indicators of the cell’s nutrient status, but the details of these effects remain to be elucidated.

Under normal laboratory conditions, *B. subtilis* sporulation requires high cell density (Grossman and Losick, 1988). There is good evidence that extracellular oligopeptides are secreted and processed to function as chemical messengers that communicate a sporulation signal between cells (Perego *et al.*, 1994; Perego and Hoch, 1996a,b; Perego *et al.*, 1996; Perego, 1997; Perego, 1998; Jiang *et al.*, 2000). In addition, sporulation must be coordinated with respect to the cell cycle, to ensure the presence of two fully replicated chromosomes (Hitchins and Slepecky, 1969; Mandelstam and Higgs, 1974; Dunn *et al.*, 1978; Hauser and Errington, 1995; Wu *et al.*, 1995). One chromosome will be condensed and packaged in the spore, and the other will be used as a template for gene expression in the mother cell.

2. Genes required for sporulation initiation.
The sigma factor cascade mentioned above provides an elegant means of temporally and spatially regulating the process of sporulation. However, the use of successive sigma factors cannot solely control the initiation of sporulation, as the first inducible spo genes must be transcribed by an RNA polymerase holoenzyme already active in the cell. Because of this, B. subtilis must use some other means to activate the early spo genes, which include those genes encoding the first sporulation sigma factors.

There are nine known loci with clearly defined roles in sporulation initiation. These loci were originally discovered through the examination of mutants blocking the induction of sporulation, and include the genes spoOA, spoOB, spoOE, spoOF, spoOH, spoOJ, spoOK, spoOL, and spoOP (Hoch, 1976; Errington, 1993). All of these genes are expressed during logarithmic growth, or are induced at or slightly after the onset of stationary phase. Of these genes, spo0A is a key regulator of stationary phase events, and expression of spo0A is absolutely required for sporulation initiation (Hoch, 1976). Several other stage zero mutants are suppressed by spo0A mutations (Hoch et al., 1985; Spiegelman et al., 1990; Cervin and Spiegelman, 1999). These observations suggest that at least some of the other spo0 gene products function in the regulation of spo0A expression. However, no suppressors of spo0A deletion mutants have ever been isolated.

3. The phosphorelay and signal transduction.

The cloning and sequencing of spo0A revealed that its protein product was related to a class of proteins known collectively as response regulators (Ferrari et al., 1985; Kudoh et al., 1985; Burbulys et al., 1991). Many proteins in this class are transcriptional regulators that function to positively or negatively control the expression of genes within a regulon.
Spo0A, like all response regulators, is paired with one or more proteins known as sensor kinases, and together these proteins form two-component regulatory systems, which are present in many bacterial genera (for reviews, see Kofoid and Parkinson, 1988; Stock et al., 1989; Stock et al., 1990; Bourret et al., 1991). These regulatory systems work to direct the behavior of a bacterial cell in response to specific environmental stimuli. In B. subtilis, stationary phase events such as competence, motility, chemotaxis, exoenzyme production, antibiotic production and sporulation are initiated as a response to the activation of a response regulator by sensor kinases receiving distinct environmental signals (Msadek et al., 1993). Despite the diversity of the events regulated by response regulators, all of these proteins are activated in the same way: through a signal transduction mediated via protein phosphorylation by one or more activated sensor kinases.

Sensor kinases have the ability to perceive environmental stimuli, with each sensor kinase presumably tuned to respond to a specific aspect of the extracellular environment. The distribution of sensor kinases is diverse: some are intracellular, while others are membrane bound. When a sensor kinase detects an environmental change, it will autophosphorylate, resulting in the transfer of a phosphoryl group to a highly conserved histidine residue located in the C-terminal end of the protein (Kofoid and Parkinson, 1988; Stock et al., 1989; Stock et al., 1990; Bourret et al., 1991). The activated sensor kinase can then transfer that phosphoryl group to the N-terminal end of its cognate response regulator, resulting in the activation of that response regulator (Kofoid and Parkinson, 1988; Stock et al., 1989; Stock et al., 1990; Bourret et al., 1991). The activated response regulator then mediates an adaptive response appropriate to the signal originally received.

There are three known sensor kinases paired with Spo0A: KinA, KinB, and KinC.
The major kinase involved in the sporulation response in normal laboratory conditions is KinA (Perego et al., 1989; Antoniewski et al., 1990; LeDeaux et al., 1995). Deletion of *kinA* or *kinB* causes a delay in the sporulation response, or decreases the level of spore formation in a *B. subtilis* population (Perego et al., 1989; LeDeaux et al., 1995; Dartois et al., 1996). Deletion of *kinC* alone results in a negligible effect on sporulation frequency under most conditions (Kobayashi et al., 1995; LeDeaux and Grossman, 1995; LeDeaux et al., 1995). Only a double *kinA kinB* mutation reduces the sporulation frequency to near zero, and a triple *kinA kinB kinC* mutation abolishes sporulation completely (LeDeaux et al., 1995). While KinA is a cytoplasmic protein (Perego et al, 1989; Antoniewski et al., 1990), KinB is membrane bound (Trach and Hoch, 1993), suggesting that both intracellular and extracellular factors are important to the induction of a sporulation response. KinC is thought to be membrane bound (Fabret et al., 1999).

In *B. subtilis*, Spo0A phosphorylation by a kinase occurs indirectly, through a signal transduction system called the phosphorelay (Burbulys et al., 1991; Hoch, 1993). An activated kinase phosphorylates an aspartate residue in the N-terminal end of the DNA non-binding response regulator Spo0F, which then passes the phosphate group to a histidine moiety in the phosphotransfer protein Spo0B, which then phosphorylates an aspartate within Spo0A (Burbulys et al., 1991). This extension of the well-described two-component signal transduction system exists presumably to allow for extra levels of regulation of the phosphorylation state of Spo0A (for recent reviews see Grossman, 1995; Stragier and Losick, 1996; Perego, 1998).

In terms of energy and nutrients used, sporulation is an expensive process for the
bacterial cell. Accordingly, the initiation of sporulation is regulated in a number of different ways. The presence and activity of the phosphorelay is known to be controlled directly by two sigma factors, two transition-state regulators, three kinases, one kinase inhibitor, and three phosphatases. The nature of the interactions between these diverse components is complex. The schematic presented in Figure 3 summarizes both the phosphorelay and its known repressors. The phosphorelay and regulation of the initiation of sporulation have recently been reviewed (see Grossman, 1995; Stragier and Losick, 1996; Perego, 1998; Msadek, 1999).

4. The functions of Spo0A.

The study of spo0A defective B. subtilis cells has yielded the observation that, along with being asporogenous (spo⁻), these strains fail to become competent or produce exoenzymes during stationary phase (Hoch, 1976; 1993). These phenotypes can be explained in part by the failure of spo0A mutants to repress transcription of the abrB gene. Consequently, the expression of the stationary phase genes involved in these processes that are normally repressed by AbrB during logarithmic growth are not derepressed in the transition state (Zuber and Losick, 1987; Dubnau et al., 1987; Perego et al 1988; Strauch et al., 1989a). However, spo0A abrB double mutants are still spo⁻, suggesting that Spo0A⁻P has other functions as well. Further experimentation provided data that spo0A mutants do not induce a number of early spo genes, which were subsequently found to have a transcriptional requirement for Spo0⁻P. Therefore, it was concluded that Spo0A⁻P is an "ambiactive" transcriptional regulator, with negative and positive functions affecting both transition-state regulators and spo0 genes, respectively (Perego et al., 1991b; Spiegelman et
Figure 3. The regulation of the phosphorelay and phosphorylation of Spo0A. The phosphorelay is shown in the center of the figure. Solid black arrows represent the sporulation signal transduction through the phosphorelay. Dashed black arrows represent gene transcription mediated by a specific holoenzyme, and dotted black arrows indicate the gene from which a specific sigma factor is transcribed. Solid grey lines represent direct and negative regulatory protein-protein interactions. Dotted grey lines represent negative regulatory transcriptional effects on protein production. Spo0B and Spo0F are phosphorelay components. spoOH encodes the sigma factor $\sigma^H$. Spo0E, Spo0P, and Spo0L are phosphorelay phosphatases. KipI is the KinA kinase inhibitor. AbrB and SinR are transition-state regulators.
SpoOA\textsuperscript{P} is now known to bind DNA at the consensus sequence 5'-TGNCGAA-3' (Strauch \textit{et al.}, 1990; Baldus \textit{et al.}, 1995). This sequence (termed an 0A box) can be found in pairs upstream of the promoters of several genes involved in sporulation that require SpoOA\textsuperscript{P} for transcription, including \textit{spolIG}, \textit{spolla}, and the \textit{spo0A} gene itself (Spiegelman \textit{et al.}, 1995). Details of how SpoOA\textsuperscript{P} activates and represses transcription can be found elsewhere (Spiegelman \textit{et al.}, 1995).

The fact that SpoOA\textsuperscript{P} is required for the expression of both \textit{spolIG} and \textit{spolla}, encoding sporulation specific sigma factors $\sigma^F$ and $\sigma^A$, respectively, is significant in that the expression and activation of Spo0A provides a mechanism for the initiation of sporulation. The phosphorelay links the sensing of starvation signals to the induction of the sigma factor cascade through the activation of Spo0A. Therefore, phosphorylation of Spo0A is crucial to the sporulation initiation process.

D. Review of Transcription Initiation.

1. Promoter structure.

\textit{B. subtilis} promoters are characterized by conserved DNA sequences at the –10 and –35 positions relative to the transcription start site, with an intervening spacer region with an optimal length. Both promoter sequence and spacer length vary with the sigma factor specificity of the promoter (reviewed in Helmann and Chamberlin, 1988; Haldenwang, 1995). The length of the spacer region determines the linear and angular separation of the –10 and –35 sequences on the DNA axis. Structure/function investigations of a number of promoters have determined that the –10 and –35 sequences are vital for promoter recognition by RNA polymerase. Therefore, the sequences at the –10 and –35 promoter sites, as well as
the length of the spacer between them, contribute to the transcriptional activity of a given promoter (Helmann and Chamberlin, 1988; deHaseth and Helmann, 1995). For the most part, there is good correlation between adherence to the consensus promoter sequence and strong in vitro promoter activity. Promoters that rely on positive regulation for activation have weak in vitro transcriptional activity. These promoters often have either a minimal similarity with the consensus promoter sequence, or a spacer region of non-optimal length, and interact poorly or not at all with RNA polymerase in the absence of a positive regulator (for examples, see Satola et al., 1991; 1992; Bird et al., 1993; 1996).

The tight binding of RNA polymerase to a promoter sequence is the first of three steps preceding transcription initiation (for reviews see Gralla, 1990; deHaseth and Helmann, 1995; Helmann and deHaseth, 1999). This initial enzyme-promoter complex is referred to as a closed complex. The next step involves the formation of an intermediate complex, which is characterized by a change in the structure of the RNA polymerase that coincides with an initiation of DNA strand separation localized to the –10 region of the promoter. DNA strand separation is followed by an expansion of the melted region and movement of RNA polymerase to encompass the transcription start site, resulting in the formation of an open complex. Transcription initiation can begin immediately after open complex formation. Elongation of the transcript begins with the release of the sigma factor, generally after the first 10-15 bases of the transcript have been synthesized (deHaseth and Helmann, 1995, and references therein).

2. Transcription factors.

Bacterial transcription factors usually bind to discrete DNA sequences in close
proximity to the promoters they activate. In fact, positive regulators commonly bind DNA near the –40 position, and sometimes overlap the RNA polymerase –35 binding site (for examples, see Collado-Vidas et al., 1991; Satola et al., 1991; 1992; Bird et al., 1993; 1996). Once a transcription factor is bound near a promoter site, it can affect the rate of transcription from that promoter in different ways. A positive transcriptional regulator may facilitate the binding of the RNA polymerase to a promoter. Alternatively, a transcription factor may act as a catalyst in the isomerization step after RNA polymerase has bound to a promoter, resulting in DNA strand separation and open complex formation, as is seen with the response regulator Spo0A–P on the spoIIG promoter (Rowe-Magnus and Spiegelman, 1998).

3. The spo0A promoter.

The spo0A gene has two promoters that are differentially regulated. During vegetative growth, transcription from the weak σ^A promoter (P_v; located 218 bp 5' to the translation start site) results in the presence of low Spo0A levels in the cell (Chibazakura et al., 1991; 1995). As the cells enter stationary phase, the phosphorelay is activated by kinases responding to sporulation signals. The activity of the phosphorelay results in the phosphorylation of Spo0A, with subsequent repression of the abrB gene and derepression of spo0H (Perego et al., 1988; Strauch et al., 1990; Weir et al., 1991). Eσ^H, in the presence of the transcriptional activator Spo0A–P, will bind to and transcribe from the sporulation promoter (P_s; located 52 bp 5' to the translation start site) of the spo0A gene (Predich et al., 1992). This “promoter switch,” which results in amplification of Spo0A production, has been found to be required for sporulation initiation in wild type cells (Chibazakura et al., 1991; Strauch et al., 1992; Chibazakura et al., 1995). In otherwise wild type cells, B. subtilis
spo0A promoter mutants lacking the Ps promoter produce very little Spo0A and are unable to sporulate (Strauch et al., 1992; Siranosian and Grossman, 1994).

Transcription from the spo0A Pγ promoter is unaffected by the presence of glucose in the medium, with low level transcription of the spo0A gene present during vegetative growth. However, a repressive effect of glucose-containing media on stationary phase expression of spo0A has been observed, and has been ascribed to the repression of transcription from the spo0A Ps promoter (Chibazakura et al., 1991). The transition state regulator SinR has been implicated in this repression (Gaur et al., 1988; Smith et al., 1991; Strauch and Hoch, 1993). SinR has been found to bind the spo0A Ps promoter at the -10 site (Mandec-Mulec et al., 1995). Apart from Spo0A–P and SinR, no other regulators are known to affect spo0A gene transcription.

E. Carbon Source-Mediated Catabolite Repression

Catabolite repression (CR) is a regulatory mechanism by which expression of genes required for utilization of alternative sources of carbon is prevented by the presence of a preferred substrate. This regulation of metabolic activities enables bacteria to optimize growth rates in environments providing complex mixtures of nutrients. Originally termed glucose repression, the phenomenon of CR has been known for over 50 years (Monod, 1947). The presence of glucose combined with certain additional carbohydrates in the culture medium of E. coli resulted in diauxic growth, with the first cycle of growth corresponding to exclusive utilization of glucose. Utilization of the second carbohydrate was prevented by the presence of glucose. Repression was found to be a general phenomenon in which readily metabolized carbohydrates suppress utilization of less readily metabolized
sugars, by preventing the synthesis of enzymes needed to use alternative substrates. The result of this regulation establishes priorities in the use of various carbon and energy sources. CR of synthesis of a specific enzyme is not restricted to general carbohydrate catabolic enzymes. Synthesis of enzymes required for secondary metabolites, including antibiotics, in both prokaryotic and eukaryotic microorganisms is either directly or indirectly subject to glucose repression (Martin and Demain, 1980). For *B. subtilis*, spore formation and the synthesis of certain extracellular enzymes and toxins are also repressed by readily metabolized carbohydrates (Fisher and Sonenshein, 1991).

1. Catabolite repression in *Escherichia coli*.

The mechanism of regulation of CR in *E. coli* is well understood (for recent reviews see Saier, 1996; Ferenci, 1999; Stulke and Hillen, 1999). The only common feature of *E. coli* and *B. subtilis* CR is that it is mediated at the level of transcription of target genes in both organisms. In *E. coli*, CR is effected by the catabolite repressor protein (CRP or CAP) in a complex with cAMP, which binds to specific sites in the promoter region of CR-sensitive genes or operons and activates transcription (Ullmann and Danchin, 1983; Magasanik and Neihardt, 1987). This binding is dependent upon the rate of intracellular cAMP synthesis by adenylate cyclase, which is stimulated when the phosphotransferase system (PTS) for carbohydrate uptake lacks a substrate (Postma, 1987). Thus, when a PTS sugar is present (such as glucose, fructose, or mannose), cAMP levels are low, cAMP-CRP complexes cannot form and bind to CR-regulated promoters, and transcription is not induced. In the absence of PTS sugars, cAMP levels rise, cAMP-CRP complexes bind to CR-regulated promoters and transcription is induced (Ullmann and Danchin, 1983).
2. Catabolite repression in *B. subtilis*.

Studies of the regulation of α-amylase synthesis have been used as a basis for a molecular model for the mechanism of CR in *B. subtilis* (for recent reviews see Henkin, 1996; Saier, 1996; Stulke and Hillen, 1999). Two genes, *ccpA* (Henkin *et al.*, 1991: *ccpA* is allelic to *alsA* [Zahler *et al.*, 1976]) and *ptsH* (Gonzy-Treboul *et al.*, 1989), encoding the proteins CcpA and HPr, were identified as important in CR (Hueck and Hillen, 1995; Deutscher *et al.*, 1995). CcpA is a DNA-binding protein and a member of the GalR family of repressor proteins that inhibit transcription by binding to operator sequences (Weikert and Adhya, 1992). CcpA binds to cis-active operator-like sequences called catabolite responsive elements (*cre* sites) found in the vicinity of several catabolite repressed genes (Weikert and Chambliss, 1990). HPr is a protein that is involved in phosphate transfer in the phosphoenolpyruvate (PEP)-dependent sugar transport system, the PTS (Gonzy-Treboul *et al.*, 1989). Metabolite-activated phosphorylation of HPr by an ATP-dependent kinase (Deutscher and Saier, 1983) is essential for catabolite regulation of genes whose expression also depends on the presence of *cre* and a functional CcpA. Dephosphorylation of HPr occurs under starvation conditions, and HPr phosphorylation-dephosphorylation represents a switch responding to carbon source availability and to energy levels in the cell (Reizer *et al.*, 1989; Hueck and Hillen, 1995). Experiments with purified HPr and CcpA have shown that the phosphorylated form of HPr will be retarded by CcpA on an affinity column, and that this interaction is strengthened by the addition of fructose-1,6-diphosphate (FDP) (Deutscher *et al.*, 1995).

In the proposed signal transduction pathway for catabolite repression in *B. subtilis*
(Hueck and Hillen, 1995; Deutscher et al., 1995), the presence of glucose results in a high intracellular level of FDP. High FDP levels activates the ATP-dependent kinase leading to the formation of phosphorylated HPr. HPr\~P interacts with CcpA in an FDP dependent manner. The HPr\~P::CcpA complex binds to cre sites, blocking the transcription of genes under catabolite repression. The absence of a readily useable carbon source leads to Pj-stimulated phosphatase activity leading to dephosphorylation of HPr and dissociation of the complex with CcpA and relief from CR.

Since CcpA was first described, two other catabolite control proteins have been isolated, CcpB and CcpC. CcpB (Chavaux et al., 1998), also a member of the GalR family of repressor proteins, exhibits 30% amino acid similarity to CcpA and has been shown to be involved in CR of the gluconate and xylose utilization genes. The dependence on CcpB for CR of these genes was most obvious when \textit{B. subtilis} cells were grown on solid media, or when the liquid culture agitation rate was low, indicating that physical conditions affect CcpB-mediated CR. CcpA and CcpB both bind the same \textit{cre} sequence, and are thought to mediate CR in a coordinated fashion dictated by environmental conditions. CcpC shares minimal amino acid homology with either CcpA or CcpB, and instead shares sequence identity with the LysR family of transcriptional regulators. CcpC (Jourlin-Castelli et al., 2000) has been linked to CR of the \textit{citB} and \textit{citZ} genes, as well as repressing those genes during anaerobiosis. The DNA sequence to which CcpC appears to bind is different from the \textit{cre} sequence.

The link between CR and sporulation is poorly understood. The enzymes of the citric acid cycle (CAC) in \textit{B. subtilis} are under various forms of CR during vegetative growth, such that CAC is not fully functional until the onset of stationary phase (Hederstedt, 1993; Fisher
et al., 1994). Evidence has been reported that full CAC function is required for activation of Spo0A, apparently because of a failure to activate the phosphorelay in the absence of CAC (Ireton et al., 1995; Matsuno et al., 1999; Matsuno and Sonenshein, 1999). In addition, the transition state regulator AbrB has been found to modulate the CR of certain genes via binding near cre sites and competing with CcpA (Fisher et al., 1994; Strauch, 1995a, b). Finally, the crsA mutation permits sporulation in the presence of glucose and causes the glucose resistant expression of certain, but not all, catabolite repressible enzymes, suggesting the possibility of another, unknown CR mechanism (Chambliss, 1993; Wray, Jr. et al., 1994).

3. Catabolite resistant sporulation mutants.

Mutants that sporulate in the presence of a carbon source have been isolated by irradiation followed by plating on sporulation media containing different carbon sources (Takahashi, 1979). These mutants (crs mutants, for catabolite resistant sporulation) were shown to have pleiotropic effects (Takahashi and Sun, 1984; Kawamura et al., 1985; Leung et al., 1985; Boylan, et al., 1988; Lee, et al., 1992). Certain mutants were able to sporulate in the presence of all the carbon sources tested, while some of the mutants were resistant to only some of the carbon sources, suggesting that several metabolic steps may be affected in CR of sporulation (Takahashi, 1979; Sun and Takahashi, 1982).

The crsA mutation has been localized to the sigA gene of B. subtilis, which codes for the major vegetative sigma factor, σ^A (Price and Doi, 1985). The sequence of the crsA allele has been determined (Kawamura et al., 1985), and the mutation confers a proline to phenylalanine change located between conserved region 3 (proposed to form a helix-turn-helix structure, which may bind double-stranded DNA in a sequence-specific manner,
although there is no evidence that this is the case; Helmann and Chamberlin, 1988) and region 4 (that forms a helix-turn-helix structure, and directly contacts the −35 region of promoter sequences) of the sigma factor (Helmann and Chamberlin, 1988). Proline residues often have important structural roles in proteins, and it has been suggested that this mutation alters the overall structural integrity of the σ factor (Helmann and Chamberlin, 1988).

The crsA mutation has not been found to suppress mutations in spo0F, spo0B, spo0A, spo0H, spoIIG, or spoIIA (Kawamura et al., 1985; Leung et al., 1985; Boylan et al., 1988; Lee et al., 1992). These observations suggest that the effects of the crsA mutation do not bypass the phosphorelay, or the need for Spo0A−P, σE or σF in initiating sporulation. Normal transcriptional switching between σ^A and σ^H promoters of the spo0A gene is seen in strains with the crsA mutation (Chibazakura et al., 1991). Thus, transcription of spo0A in crsA mutants in the presence of glucose is not due to Eσ^A47 transcription from the σ^H promoter (Chibazakura et al., 1991). This result is supported by the inability of the crsA mutation to rescue a spo0H mutation (Boylan et al., 1988). A catabolite repressible factor was proposed to mediate posttranscriptional control of σ^H expression (Chibazakura et al., 1991).

F. Main Research Objectives.

The integration of multiple signals (including nutrient availability, DNA replication, cell density, and chromosome partitioning) into the sporulation initiation machinery ensures that sporulation is initiated only in conditions where nutrient sources are limited and the entire process can be successfully completed. The mechanisms whereby these diverse signals are interpreted are not well understood. The work in this thesis was directed to
understanding one component of this process by examining how the presence of the \textit{crsA}
mutation causes catabolite resistant sporulation.

The \textit{crsA} mutation results in the production of an altered \(\sigma^A\) component of RNA
polymerase. This renders the cell blind to certain nutritional signals (such as the presence of
glucose) that would normally result in the repression of sporulation initiation. The initial
hypothesis was that the \textit{crsA} mutation resulted in an alteration in promoter utilization by
RNA polymerase containing \(\sigma^{A47}\), resulting in the inappropriate initiation of sporulation. \textit{In}
vivo and \textit{in vitro} studies of the mutant RNA polymerase using different promoters involved
in sporulation may indicate how the mutant phenotype occurs.

\(\sigma^A\)-dependent promoters that are either repressed or activated at the onset of
sporulation include those upstream of the \textit{spo0A, spo0F, spo0L, spo0P, spoIIG, sinR} and
\textit{abrB} genes. The mutation conferred by \textit{crsA} may result in increased or decreased
transcriptional activity from some or all of these promoters, thus permitting the phosphorelay
to be either inappropriately activated, or bypassed, resulting in sporulation. Those \(\sigma^A\)-
dependent promoters with unexplained changes in transcriptional activity \textit{in vivo} were
examined using \textit{in vitro} techniques, to investigate the potential for \(E\sigma^{A47}\) to be directly
involved in the unusual expression of these genes.

\(\sigma^H\)-dependent promoters that are repressed or activated during sporulation initiation
include those upstream of the \textit{spo0A, spo0F, kinA, spoIIA, spoVG, and sinI} genes. \(\sigma^H\)-
dependent transcription may be indirectly affected by the \textit{crsA} mutation, either \textit{via} a \(\sigma^{A47}\)-
dependent activation of \(\sigma^H\) despite the presence of glucose, or as a consequence of alterations
in \(\sigma^A\)-dependent \textit{spo} gene expression. Such changes in \(\sigma^H\)-dependent expression may also
permit either the inappropriate activation, or bypassing, of the phosphorelay.
By comparison of \textit{in vivo} and \textit{in vitro} activities of mutant and wild type RNA polymerases, and working backwards to how these changes affect the sporulation initiation pathway, it may be possible to gain insight into how these differences result in the catabolite resistant sporulation phenotype.

This thesis describes the analysis of the transcription patterns of a number of genes whose expression are important in sporulation initiation. It was found that both $\sigma^A$- and $\sigma^H$-directed transcription of several promoters were altered in the presence of the \textit{crsA} mutation. The experiments described herein indicate that the alteration of the expression of these genes was the result of three separate events: the inappropriate activation of $\sigma^H$, the unusually low transcription of the gene encoding the transition state regulator SinR, and an increase in the efficiency of transcription from the \textit{spo0A} P$_v$ promoter.
Materials and Methods

A. Bacterial strains, plasmids, and primers.

Tables 1 and 2 below list and describe the origins of the bacterial strains, plasmids, and PCR primers discussed in this thesis.

Table 1. Bacterial strains and plasmids used in this study.

<table>
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<th>Strain or plasmid</th>
<th>Genotype, phenotype or description</th>
<th>Source</th>
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<td>BGSC&quot;</td>
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**Escherichia coli strains**

| DH5α   | hsdR17 (rK-, mK+) recA1 | NEM*
| GM2163 | hsdR2 (rK-, mK+) recA1 dam13::Tn9 dcm-6 | BRL*

**Plasmids**

<p>| pDH32  | Am' Cm' promoter-lacZ fusion vector | J. Hoch |
| pJM103 | Am' Cm' vector | J. Hoch |
| pJM8114 | Am' Cm' P-kinA(-970 to +891)::lacZ | M. Perego |
| pGBS783 | Am' Cm' pJM8114ΔP-kinA(-970 to +891) | this study |
| pGEM-T  | Am' commercial vector | Stratagene |
| pDG780 | Am' + Km' cassette | BGSC |
| pGBS-0H | Am' Km' pGEM-T::spo0H(+58 to +665)::Km' | this study |
| pGBS-0H2 | Am' Cm' pJM103::spo0H (+58 to +665) | this study |</p>
<table>
<thead>
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<th>Target region</th>
</tr>
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<td>kinA</td>
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<tr>
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</tr>
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<tr>
<td>0A3</td>
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<td>spo0A</td>
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</table>

Km', Cm', MLS, and Am' refer to drug resistance of the bacterial strains. Km-kanamycin; Cm-chloramphenicol; MLS-erythromycin/lincomycin; Am-ampicillin.

Transformation with chromosomal DNA from the bacterial strain listed.

Promoter sequence regions used in plasmid construction are shown in parentheses, with values listed relative to the translational start site of the gene.

Bacillus Genetic Stock Center
New England BioLabs, Inc.
Bethesda Research Laboratories
B. Molecular biology techniques.

1. Plasmid DNA restriction endonuclease digests.

Plasmid restriction endonuclease digest reaction volumes were from 10 - 30 µL, with DNA concentrations of 100 - 250 ng/µL. Restriction endonuclease enzymes were used with buffers provided by the supplier (Bethesda Research Laboratories, New England BioLabs, Pharmacia) and were added to a concentration of 0.5 units/µL total reaction mix. Restriction digests were incubated at 37°C (unless otherwise suggested by the supplier), for a minimum of one hour. Samples were analyzed following electrophoresis through agarose gels (Materials and Methods, B.6).

2. Ligation reactions.

Insert and vector DNA fragments for use in ligation reactions were purified by agarose gel electrophoresis, followed by either electroelution of gel slices into dialysis tubing, or spin column purification of gel slices (Qiagen gel purification kit). Ligation reaction volumes were generally 10 – 35 µL, with DNA concentrations of 2-10 ng/µL. T4 DNA ligase (Bethesda Research Laboratories) was used at a concentration of 1 unit/µL total reaction mix. Cohesive end and blunt end ligations were incubated at 16°C overnight.

3. Transformation of competent cells.

3a. E. coli transformation.

DH5α cells were made competent using a modification of the protocol published by Hanahan (1983). Firstly, a single colony of DH5α was resuspended in 10 ml of prewarmed ψB (per litre: 20 g tryptone, 5 g yeast extract, 10.22 g magnesium sulfate heptahydrate; pH
adjusted to 7.6 with 1 M potassium hydroxide), and incubated at 37°C, shaking at 200 rpm, until cell growth was visible (1 to 2 hours). 100 ml of prewarmed \( \psi \)B in a 1 L flask were then inoculated with the 10 ml culture and incubation continued at 37°C until cell growth was at a spectrophotometric density of 0.45 - 0.55 with an absorbance at 550 nm. The culture was then swirled constantly on an ice bath for 5 minutes. The cells were then centrifuged at 4000 x g for 5 minutes, 4°C. The supernatant liquid was removed and the pellet resuspended, very gently, in 20 ml of ice cold TfbI (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, pH to 5.8 with acetic acid, filter sterilized). 3 ml of sterile glycerol was added, and the cells were mixed and incubated on ice for 5 minutes. The cells were centrifuged at 4000 x g for 5 minutes, 4°C. The supernatant fluid was removed and the cell pellet was resuspended in 4 ml of TfbII (10 mM MOPS, 25 mM calcium chloride, 10 mM rubidium chloride, 15% v/v glycerol, pH to 6.5 with 1 M potassium hydroxide). After a 15 minute incubation on ice, cells were then aliquoted into 0.65 mL Eppendorf tubes and frozen in dry ice and ethanol. Competent cells were stored at -70°C until use.

Ligation reactions were diluted 1:5 with distilled water prior to transformation. Competent cells and DNA were incubated on ice for 45 minutes prior to a 1 minute heat shock at 42°C. Cells were then allowed to recover for 1 - 2 hours at 37°C in L-broth (Sambrook et al., 1989) prior to plating on selective media.

3b. \( B. \) subtilis transformation.

\( B. \) subtilis cells were prepared for transformation using the method of Hoch (1991). Cells were transformed with 10 - 100 ng chromosomal DNA or 0.5 - 1.0 µg plasmid DNA,
and were allowed to outgrow in second period growth medium (Hoch, 1991), for 2 hours prior to plating on selective media.

4. Preparation of plasmid and chromosomal DNA.

4a. Plasmid DNA

Plasmid preparations were obtained from cells of overnight *E. coli* cultures (strain DH5α (New England BioLabs, Inc.) or GM2163 (Bethesda Research Laboratories) grown in L-Broth (Sambrook *et al.*, 1989) supplemented with the appropriate antibiotic. The alkaline lysis procedure was used for small-scale preparations of plasmid DNA (Sambrook *et al.*, 1989). Either the alkaline lysis or cleared lysis procedure was used for large-scale preparations of plasmid DNA, and were carried out as described by Sambrook *et al.* (1989). A CsCl density gradient procedure was used to purify large scale plasmid preparations (Sambrook *et al.*, 1989) and was followed by several butanol extractions to remove ethidium bromide. Purified DNA was then dialyzed at 4°C versus 3 exchanges of 2 L of TE buffer (Sambrook *et al.*, 1989). DNA concentration was determined by absorbance readings at 260 nm (an A_{260} of 1.0 corresponds to 50 μg/ml DNA; Sambrook *et al.*, 1989). Plasmid DNA was stored in TE buffer at 4°C.

4b. Chromosomal DNA

*B. subtilis* chromosomal DNA was prepared from 25 mL of 18 hour cultures grown at 37°C in L-broth (Sambrook *et al.*, 1989) supplemented with the appropriate antibiotic. Cells were harvested by centrifugation at 4,000 x g for 5 minutes, 4°C. The cell pellet was resuspended in 1 mL TE buffer, and 1 mL of 5 mg/mL lysozyme in TE was added. Cells
were then incubated at 37°C for 30 minutes, without agitation. 100 μL of 10 μg/mL proteinase K in TE was added, and incubation continued at 37°C for 15 minutes, without agitation. Cells were lysed by the addition of 500 μL of 10% sodium lauryl sulfate (with gentle shaking until clearing occurred). 200 μL of 3.0 M sodium acetate (pH 5.4) was added, and the mixture shaken gently until fully dispersed. Two phenol:chloroform (50:50) extractions were performed, followed by one chloroform extraction to remove residual phenol. The aqueous layer was then removed to a clean test tube, two volumes of ice cold 100% ethanol were added, and the DNA spooled out at 4°C using a glass rod. Following a wash in ice cold 70% ethanol, DNA was dissolved in 200 - 400 μL of TE and stored at 4°C.

5. Determination of sporulation frequency.

*B. subtilis* cultures used to determine sporulation frequency were grown in Schaeffer’s spore broth (SSM; Schaeffer *et al.*, 1965) pH 7.5, supplemented with tryptophan and phenylalanine at a concentration of 10 μg/mL, and when appropriate, 1% glucose. Cells were grown for 22 – 24 hours at 37°C prior to sampling.

To determine total cell and spore counts, cultures were serially diluted in fresh SSM. Aliquots of the diluted cultures were spread on SSM agar prior to (for total cell count) and after (for spore count) extraction of the diluted culture with 1/10 volume of chloroform. Agar plates with between 30-300 colonies were counted following a 20 – 24 hour incubation at 37°C. Sporulation frequencies shown are an average of results obtained from a minimum of 3 separate determinations, and were calculated as a ratio of the spore count/total cell count.
6. Agarose and polyacrylamide gel electrophoresis.

Electrophoresis of DNA, RNA, or protein was carried out in agarose or polyacrylamide gels as described by Sambrook et al. (1989). Agarose gels (0.7 – 1.2 %) for analysis of DNA were poured on 5 x 8 cm or 6.5 x 10 cm glass slides and contained 0.5 μg/mL ethidium bromide. DNA was electrophoresed in ½ X TBE (5 mM Trizma base, 5 mM boric acid and 0.5 mM EDTA) for 45 – 60 minutes at 8 – 10 volts/cm. DNA was detected by placing the gels on a UV transilluminator (Ultra-Violet Products, Inc.).

RNA polymerase extracts were examined by electrophoresis of protein samples through 12% SDS-polyacrylamide gels (Sambrook et al., 1989) at 10 – 15 volts/cm, using a mini-protean gel apparatus (BioRad, Inc.). Proteins within the gel were stained with Coomassie Brilliant Blue R (Sigma Chemical Co.).

\(^{32}\)P-labelled RNA from transcription assays was separated by electrophoresis through 7.0 M urea, 8% polyacrylamide gels. These gels were prepared and electrophoresed in ½ X TBE at 40 – 50 volts/cm. RNA bands were detected by autoradiography following an 18-24 hour exposure to x-ray film at -70°C, or by using a Molecular Dynamics PhosphorImager SI.

7. Polymerase chain reaction.

PCR reactions used either Taq (Bethesda Research Laboratories) or Vent polymerase (New England BioLabs, Inc.), and the buffer recommended by the supplier. Magnesium concentrations for reactions with Taq polymerase were held constant at 2 mM, and with Vent polymerase varied between 1-4 mM. Nucleotide triphosphates were added to a final concentration of 250 μM, and primers were added to a final concentration of 1 pmol/μL.
ng of chromosomal or plasmid template DNA were usually added per 50 μL reaction volume, and polymerase was added to a final concentration of 0.5 units/10μL.

C. Plasmid constructs.

Because of the large number of constructs involved, the details of plasmid construction are found in the Results section, immediately prior to presentation of the results obtained using each construct.

pGBS73 was created by the recircularization of the large BamHI fragment of pJM8114 (see Figure 5). pGBS783-based plasmid constructs were transformed intact into B. subtilis JH642 or GBS10 strains. Plasmid integration occurred through a single crossover event via homologous recombination between cloned B. subtilis sequence and chromosomal DNA, with selection for both the antibiotic resistance conferred by the plasmid, and the hydrolysis of X-gal present in agar plates by β-galactosidase, which resulted in the B. subtilis colonies turning blue. pDH32-based plasmid constructs were linearized with PstI (Bethesda Research Laboratories) prior to transformation, and plasmid integration occurred through a double crossover event via homologous recombination between amyE sequences bracketing the vector cloning sites and the amyE gene in the chromosome, with selection for both antibiotic resistance (conferred by the plasmid) and the hydrolysis of X-gal present in agar plates by β-galactosidase, which resulted in the B. subtilis colonies turning blue. Transformants were confirmed to be amyE' by the inability to hydrolyze 0.1% starch in L-agar (Sambrook et al., 1989) after a 24 hour incubation at 37°C. Starch remaining in solid media after 24 hours was visualized using Wescodyne disinfectant (a source of iodine) applied to the surface of the agar, which reacts with starch to form a dark blue/brown color.
D. β-galactosidase assay of reporter gene constructs.

1. Bacterial growth and sampling.

*B. subtilis* strains used for analysis of β-galactosidase activity were inoculated into 10 mL of L-broth (Sambrook *et al.*, 1989) containing appropriate antibiotic (5 µg/mL of chloramphenicol or kanamycin), and left standing overnight at 37°C. Following overnight incubation, cells were diluted 1:25 into 50 mL of Schaeffer’s spore broth, pH 7.5, containing an appropriate antibiotic and supplemented with 10 µg/mL of both tryptophan and phenylalanine. Cultures were incubated at 37°C, on a rotary shaker set at 300 rpm. Culture density was measured hourly at 525 nm, and 1 mL aliquots were taken, centrifuged at 14 000 x g for 5 minutes, and cell pellets were stored at −70°C until analyzed.

2. ONPG assay of promoter-*lacZ* activity.

β-galactosidase production in *B. subtilis* strains was assayed as previously described (Ferrari *et al.*, 1988). Enzyme specific activity (expressed in Miller units; Miller, 1972) was determined in duplicate for each data point in each experiment, and each data point shown is an average of the two determined values. Values obtained were considered reliable if the higher determined value fell within 10% of the lower determined value. Each promoter-*lacZ* expression pattern shown is a representative result chosen from a minimum of 3 separately performed β-galactosidase assay experiments with comparable patterns of expression.

E. Isolation and purification of RNA polymerase.

RNA polymerases EσA and EσA47 used in transcription assays were isolated from *B. subtilis* 168S and GBS10 strains, respectively, as described by Dobinson and Spiegelman
(1985), except that this procedure did not include the heparin-sepharose column purification step; glycerol gradient fractions with high transcriptional activity were adjusted to 50% glycerol and used directly. Enzymes were stored at −20°C.

F. In vitro transcription assay procedure.

1. $P_{A2}$ and $P_{04}$ template preparation.

The plasmid pUCA2trp was created by subcloning the fragment containing the A2 promoter from pKKA2 (Bird et al., 1993) into the HindIII/BamHI sites of the plasmid pUCIIGtrpA (Satola et al., 1991), replacing the spoIIG promoter in that plasmid (Cervin et al., 1998). When digested with PvuII, pUCA2trpA produced a 550 bp DNA fragment, containing the A2 promoter. Digested DNA was extracted two times with phenol:chloroform (1:1), once with chloroform, and was precipitated in 0.3 M sodium acetate and 2 volumes of ethanol. DNA was then resuspended in 10 mM Tris-HCl, pH 8.0, and its concentration was determined by absorption readings at 260 nm. Transcription assays performed using this DNA produced a runoff transcript 130 bp in length.

The spo0A promoter region was generated in a PCR reaction using Vent polymerase (New England BioLabs, Inc.) and primer pair 0A5/0A3 (Results, Figure 24). The DNA fragment generated was approximately 950 bp in length, and was purified by agarose gel electrophoresis followed by a gel extraction spin column kit (Qiagen, Inc.). DNA was eluted using 10 mM Tris-HCl, pH 8.0, and its concentration was determined on an agarose gel by comparison to the mass of a φ29 HindIII DNA ladder. Transcription assays performed using this DNA produced a runoff transcript 291 bp in length.
2. *In vitro* transcription assays performed on templates containing \( P_{A2} \) or \( P_{0A} \).

The volume of transcription assays was 20 \( \mu \)L with a DNA concentration varying from 1.0–9.2 nM. The assays were carried out in 0.65 mL Eppendorf tubes by mixing template DNA with 1X transcription buffer (40 mM Hepes-NaOH (pH 8.0), 5 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol and 0.1 mg/mL bovine serum albumin) to a total volume of 16 \( \mu \)L. The mixture also contained (unless otherwise stated) 0.4 mM ATP, 5 \( \mu \)M GTP, and 0.5 \( \mu \)Ci of \( [\alpha^{32}\text{P}] \)-GTP (800 Ci/mmol; NEN). Tubes containing this mixture were warmed to the appropriate temperature (usually 37\(^\circ\)C) for 2 minutes prior to initiating the transcription reaction. Transcription was initiated by the addition of 2 \( \mu \)L of RNA polymerase diluted in 1X dilution buffer (10 mM Hepes, pH8.0, 10 mM magnesium acetate, 80 mM potassium acetate, 10% v/v glycerol, 0.1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin). After a 1 minute incubation, a 2 \( \mu \)L mixture containing 0.1 mg/mL heparin, 4.0 mM CTP and 4.0 mM UTP was added, promoting transcript elongation without allowing a second round of transcription initiation to occur. After 5 minutes, 9 \( \mu \)L of transcription stop buffer was added (2X TBE, 10 M urea, 1% bromphenol blue, 1% xylene cyanol FF), and the reactions were placed on ice until electrophoresis. All transcription assays were performed a minimum of three times, and representative results are shown.

3. Transcript quantitation.

Following the separation of transcripts from free nucleotides *via* electrophoresis (Materials and Methods, B.6), the gel containing the transcripts was exposed to a phosphorimager screen, typically for 2 to 3 hours. Following exposure, the phosphorimager
screen was scanned using a Molecular Dynamics Phosphorimager SI scanner, and the data accumulated from the scan (representative of the degree of radioactive exposure from the gel used to separate the transcripts) were projected onto a computer screen using ImageQuant 1.0 software.

Using the computer software, the amount of incorporation of radioactivity in the transcripts present in each reaction, represented by the number of pixels present on the exposed screen within a selected area of the gel, corrected for background activity, was determined. A single transcript was observed from the spo0A template in the products of the \textit{in vitro} reaction. The 291 nt \textit{P}\textsubscript{0A} transcript was located relative to the 130 nt \textit{P}\textsubscript{A2} control transcript (Bird \textit{et al.}, 1993; Cervin \textit{et al.}, 1998).
Results

A. Examination of the effect of the crsA mutation on sporulation frequency.

The *B. subtilis* strains created in this thesis are all derivatives of the lab strain JH642, which is auxotrophic for both tryptophan and phenylalanine. GBS10 contains the crsA mutation in the sigA gene, but is otherwise isogenic to JH642.

The presence of the crsA mutation has been reported to cause sporulation in the presence of excess glucose (Takahashi, 1979). To confirm this report in my hands, the abilities of both JH642 and GBS10 strains to sporulate in the presence of excess glucose were examined. Table 3 shows the sporulation frequency of the two strains grown in SSM containing varying concentrations of added glucose. JH642 exhibited a glucose sensitive sporulation phenotype at all glucose concentrations tested, with a 5000-fold drop in sporulation efficiency seen with the addition of as little as 0.1% glucose. Conversely, GBS10 sporulation was clearly glucose resistant at all glucose concentrations tested, with sporulation efficiency dropping slightly only at very high glucose concentrations. This decrease in sporulation frequency observed in GBS10 in media containing 2% glucose occurred in spite of the crsA mutation. This may reflect additional controls on sporulation, but this effect was not studied further in this thesis.

The viable cell count of JH642 increasing glucose concentrations decreased with time. This decrease indicates that cells in stationary phase that cannot sporulate lose viability. The cell viability observed in GBS10 did not decrease with increasing glucose supplementation, except at very high glucose concentrations. The reasons for the loss of cell viability observed in both JH642 and GBS10 are not known.
Table 3. The effect of the *crsA* mutation on *Bacillus subtilis* sporulation in the presence of excess glucose.

<table>
<thead>
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<th>Strain / % glucose*</th>
<th>total cell count/ml</th>
<th>spore count**/ml</th>
<th>sporulation frequency (spores/total cells)</th>
</tr>
</thead>
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<tr>
<td>JH642</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0%</td>
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<td>$4.35 \times 10^8$</td>
<td>$6.50 \times 10^{-1}$</td>
</tr>
<tr>
<td>0.1%</td>
<td>$2.82 \times 10^8$</td>
<td>$3.20 \times 10^4$</td>
<td>$1.13 \times 10^{-4}$</td>
</tr>
<tr>
<td>0.5%</td>
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<td>$1.30 \times 10^4$</td>
<td>$9.71 \times 10^{-5}$</td>
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<tr>
<td>1.0%</td>
<td>$7.25 \times 10^7$</td>
<td>$5.62 \times 10^3$</td>
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</tr>
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<tr>
<td>GBS10</td>
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</tr>
<tr>
<td>0.0%</td>
<td>$3.12 \times 10^9$</td>
<td>$4.26 \times 10^9$</td>
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<tr>
<td>0.1%</td>
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<td>$2.09 \times 10^9$</td>
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<tr>
<td>0.5%</td>
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<td>$4.02 \times 10^8$</td>
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<td>2.0%</td>
<td>$9.28 \times 10^7$</td>
<td>$8.70 \times 10^6$</td>
<td>$9.38 \times 10^{-2}$</td>
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</tbody>
</table>

* Strains were grown in Schaeffer's spore medium, pH 7.5, for 22-24 hours prior to sampling.

** Spore counts were generated by treating total cell samples with 1/10 volume of chloroform prior to sampling.
In all subsequent experiments, between 0.2% and 1% glucose was added to Schaeffer's spore media. These glucose levels allowed maximal sporulation in \textit{crsA} mutant strains while clearly inhibiting sporulation in JH642.

B. Investigation of the effects of the \textit{crsA} mutation on the expression patterns of promoters involved in sporulation initiation.

Initially, the target gene or genes whose activity was affected by the \textit{crsA} mutation were completely unknown. Therefore, a survey of genes important in the initiation of sporulation (see Figure 3) was undertaken in an effort to identify promoters whose activities were altered. Promoters were cloned directly upstream of the reporter gene \textit{lacZ}, and \(\beta\)-galactosidase activity throughout the growth period used as an indicator of promoter activity. Each promoter-	extit{lacZ} expression pattern shown is a representative result chosen from a minimum of 3 separately performed \(\beta\)-galactosidase assay experiments with comparable patterns of expression.

Figure 4 shows the structure of pDH32, the plasmid used in most of the promoter-	extit{lacZ} fusions. Prior to transformation into \textit{Bacillus} strains, pDH32-based constructs containing a promoter insert were linearized with \textit{PstI}. Selection for chloramphenicol resistance would ensure that the promoter-	extit{lacZ} fusions were recombined into the nonessential \(\alpha\)-amylase gene (\textit{amyE}) by a double crossover event. All transformants with pDH32 based promoter-	extit{lacZ} fusions were confirmed to be \textit{amyE}\textsuperscript{+} as was described in Materials and Methods. One promoter-	extit{lacZ} fusion, \textit{kinA-lacZ}, was created elsewhere, using pJM783 (M. Perego, Scripps Institute), and the details are shown in Figure 5.
Figure 4. Structure of the *B. subtilis* promoter expression vector pDH32. The vector contains an *E. coli* origin of replication (ori) and an ampicillin resistance gene (amp) for growth and selection in *E. coli*. The chloramphenicol acetyltransferase gene (cat) allows for selection of the integrated plasmid in *B. subtilis*, which recombines in a double crossover event into the α-amylase gene (amyE) using the front and back portions of the gene present in the vector. Transcription from the inserted promoter sequence and subsequent translation of the reporter gene (lacZ) mRNA begins at the ribosome binding site (rbs, taken from the *B. subtilis spoVG* gene), and results in expression of the enzyme encoded by *lacZ*, β-galactosidase.
Figure 5. Creation of the 1.7 kb $kinA$ promoter-$lacZ$ reporter gene construct. The 1.7 kb chromosomal $Cla$ I fragment containing the $kinA$ promoter (represented in B) was obtained from a chromosomal DNA digest and cloned into the $Acc$ I site of pJM103 (A). The 1.7 kb $EcoR$ I / $EcoR$ V fragment of the resulting plasmid (pJM8110, not shown) was then subcloned into the $EcoR$ I / $Sma$ I sites of pJM783 (C), giving rise to the plasmid pJM8114 (not shown).

MCS – multiple cloning site
1. Genes required for the phosphorelay.

The activity of the phosphorelay has been shown to be crucial in sporulation initiation, via the generation and phosphorylation of the transcriptional regulator encoded by the *spo0A* gene (Burbulys *et al.*, 1991). Accordingly, transcription initiation at the promoters of the phosphorelay protein-encoding genes *kinA* (σ^H^ promoter), *spo0F* and *spo0A* (each with dual σ^A^/σ^H^ promoters) were examined. The growth patterns of JH642 and GBS10 strains containing *kinA-lacZ* fusions are shown in Figures 6A and 6B, respectively. The time at which the onset of stationary phase occurred (T₀) was determined by the intersection of the slopes of plots of cell number versus time during logarithmic and stationary phase growth. β-galactosidase assay times were then labeled to reflect the time of sampling relative to T₀.

Figure 6C depicts the activity of the σ^H^-dependent *kinA* promoter fused to *lacZ* in JH642. The construction of this *kinA-lacZ* fusion strain (JH12664) is described elsewhere (Dartois *et al.*, 1996), and the *kinA-lacZ* fusion was introduced into GBS10 by transformation using chromosomal DNA from JH12664. In the absence of glucose (open squares), the *kinA* promoter had a peak activity occurring just after the onset of stationary phase, and declining after T₁. This is an expected pattern of transcription and agrees with previously published results (Antoniewski *et al.*, 1990; Dartois *et al.*, 1996). In the presence of glucose (closed diamonds), promoter activity peaked earlier and at only slightly lower levels, at T₀. Expression after T₀ was depressed. Figure 6D shows the activity of the *kinA* promoter-*lacZ* fusion in GBS10. Expression of the promoter in the absence of glucose (open squares) was twice that seen in the wild type strain, with expression beginning earlier and peaking at T₀. In the presence of glucose (closed diamonds), *kinA* promoter activity in the mutant strain increased at the same time as in the wild type, but increased rapidly to peak at T₁.5 to T₂ at
Figure 6. Growth of *B. subtilis* strains containing the *kinA* promoter-*lacZ* reporter gene fusion constructed in pJM783 and inserted in the *kinA* gene, and expression of the *kinA-lacZ* fusion. Strains are: (A and C) JH642; (B and D) GBS10. Y-axis values shown are the same for both (A) and (B), and are the same for both (C) and (D), and therefore are presented only on Figures 6A and 6C. Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) and without (open squares) 0.2% added glucose.
levels 5- to 6-times that seen in wild type strains in the absence of glucose. The activity of the \textit{kinA} promoter in GBS10 was unusual, as this promoter is known to be transcribed by Erj$_{H}$. Assuming that Erj$^A_{A47}$ cannot transcribe \textit{kinA} itself, these results suggested the possibility of either unusual $\sigma^H$ activity, or that the activity of a regulator of the \textit{kinA} promoter was altered by the presence of the \textit{crsA} mutation.

Figure 7 depicts the activity of the $\sigma^A/\sigma^H$ dual \textit{spo0F} promoter-lacZ fusion in JH642 and GBS10 strains. The \textit{spo0F-lacZ} fusion was created using pDH32, inserted into the \textit{amyE} gene of JH642 (to generate strain JH12862), and was generously provided by M. Perego (Scripps Institute). This \textit{spo0F} promoter-lacZ fusion was introduced into GBS10 by transformation using chromosomal DNA from JH12862. In Figure 7A, the \textit{spo0F-lacZ} expression pattern in JH642 in the absence of glucose is shown by the open squares, and the pattern of expression agreed with previously published results (Smith \textit{et al.}, 1992; Chibazakura \textit{et al.}, 1995) Peak activity was observed around T$_1$, and declined sharply thereafter. In the presence of glucose (closed diamonds), promoter activity was roughly 60\% less, reaching a peak at roughly T$_1$ and dropping afterwards. In Figure 7B, \textit{spo0F-lacZ} expression in GBS10 in the absence of glucose began earlier, but peaked at levels similar to that seen in the wild type strain, and at roughly the same time. In the presence of glucose, however, although \textit{spo0F-lacZ} activity began at a similar time as was seen in JH642 in the presence of glucose, transcription increased rapidly to peak at T$_1$ to T$_2$ at levels roughly 4-times that seen in JH642 in the absence of glucose.

Expression of the dual $\sigma^A/\sigma^H$ \textit{spo0A} promoter-lacZ fusion is shown in Figure 8. The \textit{spo0A-lacZ} fusion was created using a pDH32-type vector, inserted into the \textit{amyE} gene of JH642 (to generate strain JH12751), and was generously provided by M. Perego (Scripps
Figure 7. Expression of the spo0F promoter-lacZ reporter gene fusion. The promoter construct was inserted in the amyE gene in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer’s spore medium, pH 7.5, with (closed diamonds) and without (open squares) 0.2% added glucose.
Institute). This *spo0A* promoter-*lacZ* fusion was introduced into GBS10 by transformation using chromosomal DNA from JH12751. In Figure 8A, promoter expression in JH642 in the absence of glucose (open squares) began at roughly T_{-1}, rose to a peak at T_{0.5}, and fell gradually thereafter. This transcription pattern agreed with previously published results (Strauch *et al.*, 1992; Mandic-Mulec *et al*., 1995). Upon the addition of glucose, *spo0A* promoter activity began at a similar time, peaked earlier (T_{0}) at lower levels, and decreased at a faster rate than was seen without glucose. In Figure 8B, *spo0A* promoter activity in GBS10 both with and without glucose began earlier (T_{-2}), and peaked higher and later than was seen in the wild type. As with the *kinA* and *spo0F* promoters, activity of *spo0A-lacZ* rose sharply in the presence of glucose, peaking at 3.5- to 4-times that seen in the wild type in the absence of glucose.

With each of the promoters mentioned above, there are two observations concerning promoter activity common to all three. Firstly, the activity of each promoter in the presence of glucose was depressed in JH642; and secondly, the activity of each promoter was increased in GBS10 in the presence of glucose, with transcription levels markedly higher and persisting for a longer duration. For the *kinA* and *spo0F* promoters, transcriptional activity in the absence of glucose in GBS10 was only marginally affected. However, *spo0A* transcription levels in GBS10 prior to the onset of stationary phase T_{0} increased early and were abnormally high, both in the presence and absence of glucose. These results show that the expression of phosphorelay genes is increased in GBS10 cells grown in the presence of glucose; this may result in higher phosphorelay activity and a greater accumulation of Spo0A in these cells.
Figure 7. Expression of the spo0F promoter-lacZ reporter gene fusion. The promoter construct was inserted in the amyE gene in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer’s spore medium, pH 7.5, with (closed diamonds) and without (open squares) 0.2% added glucose.
2. Stage II sporulation genes.

Sporulation initiation is regulated not only through the expression and activity of the phosphorelay. Additional regulatory loops exist that modulate the expression of the stage II sporulation operons *spoIIG* and *spoIIA* (see Figure 3), which encode the sporulation-specific sigma factors $\sigma^E$ (mother cell specific, encoded by *spoIIGB*) and $\sigma^F$ (forespore specific, encoded by *spoIIAC*) (Stragier and Losick, 1990; Errington, 1993; Haldenwang, 1995). Because of the possibility of altered regulation in the expression of these operons, promoter-$lacZ$ fusions were placed in GBS10 in order to examine the effects of the *crsA* mutation on transcriptional activity.

Figure 9 depicts the expression of the $\sigma^A$-dependent *spoIIG* promoter in wild type and *crsA* mutant strains. The *spoIIG-lacZ* fusion was created using a pDH32-type vector, inserted into the *amyE* gene of JH642 (to generate strain JH16304), and was generously provided by M. Perego (Scripps Institute). This *spoIIG* promoter-$lacZ$ fusion was introduced into GBS10 by transformation using chromosomal DNA from JH16304. In Figure 9A, *spoIIG* promoter activity in JH642 in the absence of glucose (open squares) began at $T_0$, rose to a low peak by $T_{1.5}$ to $T_2$, and dropped thereafter. This pattern of transcription agrees with previously published observations (Mandec-Mulec *et al.*, 1992; Baldus *et al.*, 1995; Schyns *et al.*, 1997). When glucose was present (closed diamonds), *spoIIG* promoter activity was completely repressed at $T_0$, and by $T_4$ had not been relieved of that repression. In Figure 9B, *spoIIG* expression in GBS10 in the absence of glucose was quite similar to that of JH642, beginning an hour earlier, but peaking at a similar time to levels only slightly higher than were seen in JH642. However, in GBS10 in the presence of glucose, *spoIIG* promoter activity was increased, with transcription rising sharply near $T_0$, and peaking at $T_2$ at levels
Figure 9. Expression of the \textit{spoIIG} promoter-\textit{lacZ} reporter gene fusion. The promoter construct was inserted in the \textit{amyE} gene in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) or without (open squares) 0.2% added glucose.
roughly 5-times that seen in JH642 in the absence of glucose. The \textit{spoIIG-lacZ} activity in GBS10 is interesting: in section III.B.1, transcriptional activity observed in GBS10 strains containing \textit{kinA-}, \textit{spoOF-}, and \textit{spo0A-lacZ} fusions in the presence of glucose was also increased during late stationary phase. However, the \textit{kinA}, \textit{spoOF}, and \textit{spo0A} promoters each have $\sigma^H$-dependent transcriptional activity, whereas the \textit{spoIIG} promoter activity seen in GBS10 in Figure 9 is due strictly to $\sigma^A$-dependent transcription. A similar change in \textit{spoIIG} promoter activity has been previously reported in cells containing a \textit{sinR} null mutation, with peak \textit{spoIIG} transcription levels 3.5- to 4-times higher than that observed for wild type cells (Mandic-Mulec \textit{et al.}, 1992).

The activity of the $\sigma^H$-dependent \textit{spoIIA} promoter-\textit{lacZ} fusion is shown in Figure 10. The \textit{spoIIA-lacZ} fusion was created using pDH32, inserted into the \textit{amyE} gene of JH642 (to generate strain JH16124), and was generously provided by M. Perego (Scripps Institute). This \textit{spoIIA} promoter-\textit{lacZ} fusion was introduced into GBS10 by transformation using chromosomal DNA from JH16124. In Figure 10A, transcription in the absence of glucose (open squares) followed a pattern similar to that of the \textit{spoIIG} promoter, with activity beginning roughly at $T_0$ and peaking at $T_{1.5}$. The pattern of transcription shown here is representative of previously published results (Mandec-Mulec \textit{et al.}, 1992; Baldus \textit{et al.}, 1995). In the presence of glucose (closed diamonds), the \textit{spoIIA} promoter was completely repressed. In GBS10 without glucose (Figure 10B, open squares) a low level of \textit{spoIIA} promoter activity was detected earlier, but did not rise substantially until shortly before the onset of stationary phase, and peaked at roughly the same time and to the same levels as was seen in JH642. In the presence of glucose, transcription began to increase at $T_0$, rose sharply, and peaked at $T_2$ to $T_3$ a level 3-times that seen in the wild type in the absence of glucose.
Figure 10. Expression of the spoIIA promoter-lacZ reporter gene fusion. The promoter construct was inserted in the amyE gene in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) or without (open squares) 0.2% added glucose.
The above results clearly indicate that the activity of both the *spoIIA* and *spoIIG* operons was altered in the presence of the *crsA* mutation. Transcriptional restraints in common to both promoters include negative regulation by the transition state regulator SinR (Smith *et al.*, 1991; Mandec-Mulec *et al.*, 1992; Strauch and Hoch, 1993), and a requirement for the transcriptional activator Spo0A–P (Bird *et al.*, 1993; 1996; Baldus *et al.*, 1994). The results shown in section III.B.1 suggest that, in contrast to the wild type, there may be a substantial level of Spo0A–P present in GBS10 in the presence of glucose, which could explain the increased level of promoter activity seen with both *spoIIA* and *spoIIG*.

3. Later stage sporulation genes.

*spoVG* was originally defined as a gene whose knockout was manifested as a late stage sporulation defect (Rosenbluh *et al.*, 1981). More recent examinations suggest that SpoVG plays a role in the early stages of sporulation, causing a change in the timing of initial events, which are not visibly manifested until a much later stage in sporulation (Matsuno and Sonenshein, 1999; Matsuno *et al.*, 1999). When the regulation of the *spoVG* promoter was examined, it was found to be a $\sigma^H$-dependent promoter under the negative control of the transition state regulator AbrB (Zuber and Losick, 1987; Healy *et al.*, 1991). The *spoVG* promoter has been viewed as useful as a means of gauging $\sigma^H$ activity, as it is the only known $\sigma^H$ promoter with a relatively simple and defined regulation. Because of its apparent simplicity, the *spoVG* promoter was used here in an attempt to define the activity of $\sigma^H$, which, from the results in Figures 6 and 7 appeared to be altered in the presence of the *crsA* mutation.

Figure 11 depicts the expression patterns of the *spoVG-lacZ* fusion in wild type and
mutant strains. The construction of the spoVG-lacZ fusion strain (IS688) is described elsewhere (Smith et al., 1992), and the spoVG-lacZ fusion was introduced into both JH642 and GBS10 by transformation using chromosomal DNA from IS688. In Figure 11A, transcription from the spoVG promoter in JH642 in the absence of glucose (open squares) was shown to begin shortly before the onset of stationary phase, peaking at T1, and gradually decreasing thereafter, an expression pattern similar to previously published results (Healy et al., 1991; Smith et al., 1992; Matsuno and Sonenshein, 1999). When glucose was added (closed diamonds), transcription from spoVG began at a similar time, but peaked earlier and to a level 1/3 of that seen without glucose. In the presence of the crsA mutation (Figure 11B), transcription in the absence of glucose began earlier than that seen in wild type cells, peaked at T1 to a level somewhat less than that seen in JH642 (roughly 2/3), and subsequently decreased. In the presence of glucose, however, the regulation of spoVG promoter activity was again changed, with transcription beginning to increase at the same time, but peaking at T1 to levels 5-times that seen in JH642 in the absence of glucose.

The results in Figure 11, combined with previous results (Figures 6, 7, 8 and 10), suggested that σH activity was not repressed in GBS10 grown in the presence of glucose, and in fact was stimulated. Collectively then, the effects of the crsA mutation on sporulation initiation seemed to center on the inappropriate presence of both Spo0A and active σH, both of which were normally repressed in sigA+ cells grown in the presence of glucose. The effects of the crsA mutation on the negative regulators of sporulation are shown below.

4. Phosphorelay phosphatases.

The data in Results, section B.1 indicated that transcription of the genes encoding
Figure 11. Expression of the spoVG promoter-lacZ reporter gene fusion. The promoter construct was inserted upstream of spoVG, in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) and without (open squares) 0.2% added glucose.
phosphorelay proteins was altered in GBS10 in the presence of glucose. One possible reason for the unusual increase in expression would be a change in the activity of the SpoOF phosphatases, which serve to negatively regulate the phosphorelay (and therefore Spo0A-P accumulation) via the removal of phosphate, in response to nutritional signals (in the case of Spo0P, also known as RapB), or in response to competence development (as seen with Spo0L, or RapA) (Perego et al., 1994; Perego and Hoch, 1996a, b; Perego et al., 1996; Perego, 1997; 1998; Jiang et al., 2000). Consequently, the transcription patterns of the promoters of the genes encoding these two phosphatases were examined.

Figure 12 shows the results of the analysis of the expression of $\sigma^A$-dependent spo0P promoter-lacZ fusion in JH642 and GBS10 strains. The spo0P-lacZ fusion was created using a pDH32-type vector, inserted into the amyE gene of JH642 (to generate strain JH12866), and was generously provided by M. Perego (Scripps Institute). This spo0P promoter-lacZ fusion was introduced into GBS10 by transformation using chromosomal DNA from JH12866. In Figure 12A, promoter expression in the absence of glucose (open squares) was low but steady through late logarithmic growth, rising to a small peak at roughly $T_{-0.5}$, and subsequently decreasing. This transcription pattern is similar to that seen previously (Perego, personal communication; Perego et al., 1994). In JH642 in the presence of glucose (closed diamonds), spo0P-lacZ activity rose sharply at $T_0$, peaking much later and higher at $T_2$. In Figure 12B, spo0P activity in GBS10 in the absence of glucose showed a very similar pattern to that in the wild type strain, peaking at $T_{-0.5}$ to a slightly higher level. In GBS10, initial transcriptional activity in the absence of glucose was similar to that of wild type cells, but dropped off more slowly after $T_0$ than in JH642. In GBS10 in the presence of glucose, transcriptional activity of the fusion began to increase at roughly $T_{-0.5}$ to a peak at $T_{1.5}$ at a
Figure 12. Expression of the spoOP promoter-lacZ reporter gene fusion. The promoter construct was inserted in the amyE gene in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) or without (open squares) 0.2% added glucose.
level slightly less than that seen in the wild type strain in the presence of glucose.

The results of the analysis of the $\sigma^A$-dependent spo0L promoter-lacZ activities in JH642 and GBS10 strains are shown in Figure 13. The spo0L-lacZ fusion was created using a pDH32-type vector, inserted into the amyE gene of JH642 (to generate strain JH12981), and was generously provided by M. Perego (Scripps Institute). This spo0L promoter-lacZ fusion was introduced into GBS10 by transformation using chromosomal DNA from JH12981. In Figure 13A, transcription patterns in JH642 showed a sharp induction of the spo0L promoter, in the presence and absence of glucose, which began at $T_{-2}$ to $T_{-1.5}$ and peaked at $T_0$. This transcription pattern was similar to that seen previously (Perego, personal communication; Perego et al., 1994). In Figure 13B, transcriptional activity of the fusion in GBS10 in the absence of glucose (open squares) began to increase at the same time as was seen in the wild type, but peaked at levels roughly 30% of that of wild type, and subsequently dropped off gradually, suggesting the possibility that the competence signals that induce spo0L transcription were at lower than normal levels. In the presence of glucose (closed diamonds) in GBS10, the initial transcription pattern mimicked that seen in JH642, but transcription after $T_0$ was maintained at high levels for roughly 2.5 hours, suggesting the possibility that the competence signals that induce spo0L transcription persist well into stationary phase in GBS10 strains grown in the presence of glucose.

The above results suggest that the expression of the Spo0F phosphatases in GBS10, when glucose was present, was very nearly equal to that seen in JH642. Therefore, assuming that the activity of the spo0L and spo0P promoters seen in GBS10 in the presence of glucose reflect the levels of Spo0L and Spo0P protein, the high level of activity of phosphorelay gene promoters (which are stimulated by phosphorylated Spo0A) cannot be attributed to a
Figure 13. Expression of the *spo0L* promoter-*lacZ* reporter gene construct. The promoter construct was inserted in the *amyE* gene in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer’s spore medium, pH 7.5, with (closed diamonds) or without (open squares) 0.2% added glucose.
decrease in Spo0F phosphatase activity. These results suggest that the levels of Spo0A–P and of σ^H activity in GBS10 are sufficient to activate spo gene transcription, despite the inhibitory effects of phosphorelay phosphatases.

5. Transition state regulators.

There are two transition state regulators directly involved in the regulation of sporulation initiation; AbrB (that negatively controls expression of spo0H and spoVG genes) and SinR (that negatively control expression of spoOA, spoIIG, and spoIIA genes; see also Figure 3) (Smith et al., 1991; Strauch and Hoch, 1993; Errington, 1993; Grossman, 1995; Stragier and Losick, 1996). As the levels of both of these regulators control the activity of several of the promoters discussed above, the expression of these genes were examined in the presence of crsA.

Figure 14 shows the results obtained from the σ^A-dependent abrB promoter-lacZ fusion expression in JH642 and GBS10 strains. The construction of this abrB-lacZ fusion strain (JH12604) is described elsewhere (Strauch et al., 1989b), and the abrB-lacZ fusion was introduced into GBS10 by transformation using chromosomal DNA from JH12604. The expression of the abrB promoter in JH642 in the absence of glucose (open squares) was roughly constant in logarithmic growth and decreased throughout stationary phase (Figure 14A). The expression pattern of the abrB promoter shown here is similar to previously published results (Perego et al., 1989; Strauch et al., 1992). When glucose was present in the medium (closed diamonds), the reduction of abrB-lacZ transcription was delayed (by approximately an hour) past the onset of stationary phase, and the peak level of transcription was not substantially different from the culture grown without glucose. The significance of
Figure 14. Expression of the \textit{abrB} promoter-\textit{lacZ} reporter gene fusion. The promoter construct was inserted in the \textit{amyE} gene in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) or without (open squares) 0.2% added glucose.
this delay is questionable, as it was based on a single data point not seen in other experiments with this promoter construct (data not shown). The *abrB* promoter activity in GBS10 in the absence of glucose was slightly higher than in wild type cells, but peak activity occurred at roughly the same time (T.i), and decreased similarly throughout stationary phase (Figure 14B). In the presence of glucose, the pattern of transcription was not substantially different from that seen without glucose. The *abrB-lacZ* transcriptional activity in GBS10 was not maintained into stationary phase in the presence of glucose, as was seen in Figure 14A.

The *sin* genes are transcribed from a dicistronic operon regulated in a complex manner from three promoters. The first gene in the operon, *sinI*, is preceded by putative σ^H and σ^E promoters, and the transcriptional regulators, SpoOA, Hpr and AbrB have been shown to bind in the vicinity of these promoters (Kallio *et al.*, 1991; Strauch and Hoch, 1993). Transcription of *sinI* and *sinR* genes from these promoters is minimal during vegetative growth, is induced at the onset of stationary phase in a Spo0A–P dependent manner, and is subject to catabolite repression by glucose (Gaur *et al.*, 1988; Strauch and Hoch, 1993). The second gene in the operon, *sinR*, is preceded by a putative σ^A promoter, located between the *sinI* and *sinR* genes. *sinR* is transcribed from this promoter at low constitutive levels during vegetative growth, with increasing transcription seen at the onset of stationary phase (Gaur *et al.*, 1988). This transcript is thought to be poorly translated throughout the *B. subtilis* growth cycle (Gaur *et al.*, 1988; Mandic-Mulec *et al.*, 1992).

SinR forms a tetramer of identical subunits (Lewis *et al.*, 1998) that negatively regulates the transcription of several genes, including *spo0A* (Mandic-Mulec *et al.*, 1995), *spoIIG*, and *spoIIA* (Mandic-Mulec *et al.*, 1992). For sporulation to proceed, SinR repression must be overcome, and this has been shown to occur in part through the interaction of SinR
with SinI, which is synthesized under conditions that favor sporulation (Mandic-Mulec et al., 1992; Bai et al., 1993). The relative levels of each of these two proteins within the cell affect the degree of SinR repression of transcription (Gaur et al., 1991; Smith et al., 1991). SinI binds tightly to SinR, disrupting the tetramer structure to form SinI-SinR heterodimers, thereby preventing binding of the SinR tetramer to DNA and alleviating its transcriptional repression of sporulation (Bai et al., 1993; Lewis et al., 1998).

Construction of the sinI-lacZ and sinR-lacZ fusion strains (IS423 and IS424, respectively) are described elsewhere (Gaur et al., 1988), and these fusions were introduced into JH642 and GBS10 strains by transformation using chromosomal DNA from either IS423 or IS424. The data shown in Figure 15 indicate that in wild type cells in the absence of glucose (Figure 15A, open squares), sinI transcription levels increased throughout the late logarithmic growth, and peaked at T₀. sinR transcriptional activity increased throughout late logarithmic growth and into stationary phase (Figure 15C, open squares), presumably in part due to readthrough from the sinI promoters (Gaur et al., 1988; Strauch and Hoch, 1993), as well as from the sinR promoter. The expression patterns shown here were similar to those observed by others (Gaur et al., 1988; Mandic-Mulec et al., 1992). When glucose was added, sinI transcription (Figure 15A, closed diamonds) remained relatively low during stationary phase, whereas sinR transcription (Figure 15C, closed diamonds) remained roughly the same as in the absence of glucose.

If transcriptional activity is representative of protein levels, in the absence of glucose in JH642, a roughly 20-fold excess of SinI over SinR would represent the level of SinI needed to complex and sequester SinR between T₀ and T₁.5. In the presence of glucose, the level of expression after induction of the sinI promoters in JH642 was reduced. Thus, the
lower level of SinI may not block SinR repression of sporulation.

*sinl* promoter expression in GBS10 is shown in Figure 15B. In these cells, the transcriptional activity from the *sinl* promoter in the absence of glucose was lower than that seen in the wild type. *sinl-lacZ* activity was low throughout late logarithmic growth, rising only slowly to peak at T0.5, at levels 25 to 30 percent of wild type. *sinl-lacZ* activity was also altered in GBS10 grown in the presence of glucose, with transcription rising from T1.5 to peak sometime after T2 at levels 5-times higher than was seen in wild type cells in the presence of glucose. Figure 15D shows the *sinR-lacZ* activity in GBS10. In the absence of glucose, transcription through *sinl* and from the *sinR* promoter was greatly reduced from that seen in wild type cells, peaking at very low levels at roughly T0 and decreasing after that. In the presence of glucose, transcription rose sharply from T2 to peak at T0 at levels similar to those achieved in JH642 in the presence or absence of glucose.

Again, if transcriptional activity of the promoter fusions is representative of protein levels, then in the absence of glucose in GBS10, even though SinR and SinI levels were reduced from that seen in wild type cells, the ratio would be similar to that seen in JH642, and thus it seems that SinR would be blocked by SinI during early stationary phase. When glucose was added, a large induction of the *sinl* promoter was observed. Thus, unlike in JH642 where the addition of glucose decreased the ratio of expression of *sinl:sinR*, in GBS10 the ratio of *sinl:sinR* was the same, with and without added glucose. This apparent alteration in transcriptional activity of the *sin* operon in GBS10 could explain the higher levels of transcription seen in the *spo0A*, *spoIIG* and *spoIIA* promoters in late stationary phase in the presence of glucose, but it does not explain the similar result seen with the *kinA-lacZ* fusion, as the *kinA* promoter has been shown to not be affected by SinR (Mandic-Mulec et al., 1992).
Figure 15. Expression of the *sinl* and *sinR* promoter-*lacZ* reporter gene fusions. The promoter constructs were inserted in the *sinl* gene (A and B), or *sinR* gene (C and D), in strains JH642 (A and C) and GBS10 (B and D). Strains were grown in Schaeffer’s spore medium, pH 7.5, with (closed diamonds) or without (open squares) 0.2% added glucose.
Therefore, there are at least two distinct effects on spo gene transcription in the presence of the crsA mutation: one effect resulting in the increased transcription of SinR-regulated promoters (such as spoIIG and spoIIA), and a second effect resulting in increased transcription of SinR-independent promoters (such as kinA).

C. Investigation of the activity of the kinA promoter.

The kinA-lacZ results shown in Figure 6 were notable for two reasons: firstly, expression in wild type cells was repressed by the presence of glucose, and this repression was absent in crsA mutant cells; secondly, activity of the kinA-lacZ fusion in GBS10 reached levels both in the presence and absence of glucose that were substantially higher than were observed in JH642. These results, plus published observations of differential kinA promoter activity observed when the amount of glucose added to the growth medium was varied (Asai et al., 1995), suggested that an unknown mechanism regulates kinA transcription in response to glucose availability, and that this mechanism was altered in GBS10. The observed increase in kinA promoter transcription may be important in increased expression of phosphorelay genes and stage II genes (via the phosphorylation of Spo0A, and subsequent transcriptional activation of spo0F, spo0A, spoIIG, and spoIIA promoters), which may be important in the glucose resistant sporulation phenotype of GBS10. Accordingly, the kinA promoter region was subjected to analysis in an effort to uncover a DNA sequence at which a regulator may act.

1. Construction of the kinA promoter fragments.

Fusions between fragments of the kinA promoter region and lacZ were generated in
both pDH32 and pGBS783. As described in Materials and Methods, pGBS783 integrates at the \textit{kinA} promoter \textit{via} a single recombination event. As a result of the integration, the \textit{kinA} promoter-\textit{lacZ} clone is inserted into the \textit{kinA} locus so that the 3' end of the promoter driving \textit{lacZ} expression is determined by the fusion construct, but the 5' end is the intact chromosomal structure. Thus, only the effect of changes in the 3' end of the promoter can be measured with these constructs. In contrast, pDH32 integrates \textit{via} a double recombination event at the \textit{amyE} locus, and the sequence of the promoter driving \textit{lacZ} expression can be varied at both the 5' and 3' ends.

A schematic diagram of the \textit{kinA} promoter is shown in Figure 16A, along with primer binding sites used to generate, \textit{via} PCR, a 125 bp \textit{kinA} promoter, a 350 bp promoter, and a 350 bp promoter variant. Within the 350 bp promoter, a small palindrome was detected 100 bp upstream of the \textit{kinA} transcription start site. This palindrome was potentially significant for two reasons: firstly, DNA binding proteins often bind to palendromic sequences; secondly, this palindrome overlapped a sequence similar to the catabolite control protein (CcpA) binding site, \textit{cre}. Therefore, this sequence was altered by the PCR introduction of a \textit{XbaI} site, to investigate its importance to \textit{kinA} promoter expression. However, the mutation of the small palindrome did not affect \textit{kinA}-\textit{lacZ} activity (data not shown).

Figures 16A and 16B also detail the origins of the \textit{kinA} promoters that were used to analyze the effect of sequences outside of the 125 bp promoter on transcriptional activity. The 2.8 kb promoter clone (Figure 16B) was created to evaluate the idea that readthrough from \textit{orfX} in GBS10 may influence \textit{kinA} promoter activity. The sequence of \textit{orfX} encodes a putative penicillin binding-like protein preceded by a \(\sigma^A\)-like promoter sequence (SubtiList
Figure 16. Creation of the 125 bp (pGS125), 350 bp (pGS350), 780 bp (pGS780), 1.7 kb (pGS17), and 2.8 kb (pGS28) kinA promoter-lacZ constructs in pDH32 (Figure 4). PCR with Vent polymerase (New England BioLabs) of pJM814 (Figure 5) using primers listed below yielded two promoter fragments (A). To create the 350 bp promoter-lacZ fusion, primer pair 1A/2B was ligated directionally into EcoRI/BamHI digested pDH32 using T4 DNA ligase. For the 125 bp promoter-lacZ fusion, primer pairs 1A/1B (225 bp) and 2A/2B (125 bp) were used. The two fragments generated were digested with XbaI, purified using a Qiagen spin column, then ligated together at the XbaI ends, and amplified using primer pair 1A/2B. This variant 350 bp promoter was ligated directionally into EcoRI/BamHI digested pDH32. The 125 bp promoter clone was generated from the variant 350 bp promoter clone via digestion with EcoRI and XbaI, filling in the cohesive ends of the large DNA fragment with the Klenow fragment of E. coli DNA polymerase (Gibco BRL), and religation. The 1.7 kb BamHI fragment of pJM8114 (A) was removed and either cloned directly into BamHI digested pDH32 to generate the 1.7 kb promoter-lacZ fusion (A), or was digested with BclI to generate the 780 bp kinA promoter clone (A), which was ligated into the BamHI site of pDH32. The 2.8 kb of upstream sequence was created, via PCR, from chromosomal B. subtilis DNA using the OX5/OX3 primer pair (B), and was ligated into the EcoRI site of pDH32. The 350 bp kinA promoter was subcloned from pGS350 into the EcoRI/BamHI sites of pGBS783 (not shown; created from recircularization of the 5.7 kb BamHI fragment of pJM8114, see Figure 5), creating pGBS350. pDH32-derived plasmids were linearized with PstI prior to transformation into JH642 and GBS10 strains. pGBS350 was transformed into GBS10 and JH642 intact, for integration into kinA.

1A: 5' CGGAATTTCATCATACACAATCTGACTT 3' (EcoRI)
1B: 5' TGTCTAGACATTTTTGAATAAAAG 3' (XbaI)
2A: 5' TTTCATGATACCATAAGAATAGAAGGA 3' (XbaI)
2B: 5' TCGGATCCACAGAATCCCTCCTTT 3' (BamHI)
OX5: 5' GGGAATTTCCTTTCGCTGATGCTTGC 3' (EcoRI)
OX3: 5' TCGGAATTACAGATCCCTCCTTT 3' (EcoRI)

Note: Restriction sites present in primers are shown in bold type.
database), which was included in the 5’end of the DNA fragment present in the 2.8 kb promoter clone.

2. Analysis of the activity of kinA promoter fragments.

Figure 17 shows the results of β-galactosidase assays of the expression of the 350 bp wild type kinA promoter, cloned into either pGBS783 (pGBS350; Figures 17A and 17B) or pDH32 (pGS350; Figures 17C and 17D). The expression patterns from pGBS350 in both JH642 and GBS10 strains (Figures 17A and 17B) were identical to the expression patterns of the 1.7 kb kinA promoter clone originally assayed (inserted in kinA, shown in Figure 6). Because of the nature of the promoter fusion created, the 5’ end of the kinA promoter fused to lacZ was the same as the wild type chromosomal sequence. Using a single crossover integration, no conclusions could be made concerning the potential for sequences upstream of the 5’ end of the cloned DNA being associated with kinA promoter regulation. However, single crossover integrations do affect the 3’ end of the promoter associated with the lacZ gene. Since the expression pGBS350 was identical to that of 1.7 kb kinA promoter (see Figure 6), it was concluded that the sequence downstream of the translational start site of the kinA gene, present in the 1.7 kb kinA promoter clone, but removed from pGBS350, did not contain the binding site of a protein that affected the expression of kinA.

Figures 17C and 17D show the results of the assay for the expression of pGS350 (in amyE). The first observation made was that the overall activity of pGS350 was dramatically lower than that seen with pGBS350 (Figures 17A and 17B). The reasons for the dramatic drop in transcriptional activities shown in Figures 17C and 17D from those of Figures 17A and 17B were not apparent. The activity difference could arise from translational effects, or
Figure 17. Expression of the 350 bp wild type kinA promoter-lacZ reporter gene fusion inserted in the *kinA* gene (A and B) and in *amyE* gene (C and D), in strains JH642 (A and C) and GBS10 (B and D). Strains were grown in Schaeffer’s spore medium, pH 7.5, with (closed diamonds) or without (open squares) 0.2% added glucose.
from differences in the 5' region of the promoters.

The second observation made from Figures 17C and 17D concerned the increase in pGS350 activity observed in JH642 in the presence of glucose (Figure 17C, closed diamonds) over that seen in the absence of glucose (open squares). The stimulation in the presence of glucose was characteristic of the *kinA* transcriptional patterns seen in *crsA* mutants (Figures 6D, 17B, and 17D). The pattern of expression from pGS350 in JH642 and GBS10 in the absence of glucose (Figures 17C and 17D, open squares) were typical of previous results (Antoniewski *et al.*, 1990; Dartois *et al.*, 1996). Promoter expression in JH642 rose from T.1.5 to a low peak at T0.5, and declined thereafter; whereas promoter expression in GBS10 strains began earlier, peaked at a higher level than was seen in JH642, and declined thereafter. When glucose was added, the GBS10 pGS350 strain (Figure 17D, closed diamonds) showed a dramatic increase in *kinA* expression, peaking later in stationary phase (T1) at levels 6-times that seen for GBS10 cells grown in the absence of glucose, and maintained substantial activity beyond T3. The level of peak expression from pGS350 in GBS10 cells grown in excess glucose was 3-times that seen for GBS10 cells grown in the absence of glucose.

The assumption made by comparing the levels of transcription in Figure 17 was that in the presence of glucose, a negative regulator of *kinA* transcription was not able to bind and repress transcription from the smaller promoter. The activity of the proposed regulator would be altered in the presence of the *crsA* mutation, possibly contributing to the glucose resistant sporulation phenotype. The existence of a nutritional regulator of *kinA* expression has been suggested previously (Asai *et al.*, 1995). The difference in the levels of peak expression shown in Figures 17C and 17D (closed diamonds) could be explained by the
observed increase in $\sigma^H$ activity in GBS10 strains (described in Results, section B), which did not seem to occur in JH642.

The cloning and analysis of the remaining kinA promoter-lacZ fusion constructs in pDH32 was done in an attempt to roughly define the minimum sequence upstream of the kinA promoter that was required to restore a "normal" glucose response to both JH642 and GBS10 strains. Five of the promoter clones were assayed (125 bp, 350 bp, 780 bp, 1.7 kb, and 2.8 kb DNA segments, see Figure 16), each with different 5' ends. The expression patterns for all 5 clones were identical to those shown in Figures 17C and 17D.

The data obtained from the various kinA-lacZ fusions introduced into cells using pDH32 showed higher levels of transcription from the kinA promoter in the presence of glucose than in its absence. This finding was counter to what is known about sporulation initiation, and directly contradicted previously published results showing a decrease in kinA promoter activity in the presence of glucose (Asai et al., 1995). It is interesting to note that the results published by Asai et al. (1995) were generated using a kinA-lacZ fusion inserted in the kinA gene. Therefore, it was concluded that the pattern of kinA transcriptional activity shown in Figure 6 and in Figures 17A and 17B reflects the in vivo activity. The reasons for the differences in the kinA transcriptional activity seen in JH642 and GBS10 strains shown in Figures 6, 17A and 17B can be attributed to $\sigma^H$ activity. The causes of the loss of transcription when the kinA promoter fragments are inserted into the amyE site were unknown.

3. Gene knockout effects on sporulation frequency.

As further tests of the alteration of kinA expression seen in GBS10, several gene
knockouts were introduced into JH642 and GBS10. These disruptions were designed to examine three separate questions: firstly, what effect a *kinA crsA* double mutant would have on glucose resistant sporulation; secondly, what effect a loss of potential readthrough from the *orfX* promoter may have on glucose resistant sporulation; and lastly, was $\sigma^H$ required for sporulation of GBS10.

Figures 18 and 19 are diagrams of the creation of the *spoOH* and *orfX* knockout constructs. The *spoOH* knockout was created by inserting a kanamycin resistance cassette into the *spoOH* gene using a double recombination event (Figure 18). The *orfX* knockout was created by inserting a kanamycin resistance cassette into the 3' end of *orfX* via a single crossover event, such that the direction of transcription of the kanamycin resistance gene was opposite to the direction of *orfX* transcription (Figure 19). M. Perego (Scripps Institute) graciously provided the *kinA* construct (JH12638), which was created by insertion of a transposon (Perego *et al.*, 1989).

Table 4 gives the sporulation frequencies of JH642 and GBS10 strains containing various gene knockouts. In JH642, assuming that the kanamycin resistance cassette did prevent transcription of *orfX*, the interruption of the *orfX* gene did not affect sporulation in the presence or absence of glucose, suggesting that transcription readthrough from this gene into *kinA* either did not occur, or was very minor. This conclusion was supported by the sporulation frequencies of the *crsA orfX* double mutant, which were unaffected.

The *kinA* gene knockout resulted in a 77% reduction in the sporulation frequency in the absence of glucose in otherwise wild type cells. This result is similar to published data, in which the minor phosphorelay kinases KinB and KinC were shown to be responsible for the low level of sporulation seen in the absence of KinA (Perego *et al.*, 1989; LeDeaux *et al.*, 1989).
Figure 18. Creation of the clone used to assay sporulation in spo0H\textsuperscript{−} strains. The primers listed below were used to generate the 557 bp PCR product of the region internal to spo0H (A). This PCR product was ligated into pGEM-T (Stratagene, not shown), utilizing the A overhangs left by Taq polymerase (New England BioLabs), creating pGEM-0H (not shown). pGEM-0H was digested with Hind\textsc{III}, removing a 16 bp internal fragment of spo0H, and the 1.4 kb kanamycin resistance cassette from pDG780 (not shown; Guerout-Fleury \textit{et al.}, 1995) was inserted, creating pGBS-0H (not shown). pGBS-0H was linearized with Spe\textsc{I} at a unique restriction enzyme site in pGEM-T prior to transformation into JH642 and GBS10 strains (B, at top). The double crossover is shown in B, at the bottom. For the assay of spo0A promoter activity in the absence of spo0H, pGEM-0H was digested with Sac\textsc{I} and Sp\textsc{Hl} (engineered into primer sequences), and the 557 bp spo0H fragment was ligated into Sac\textsc{I}/Sp\textsc{Hl} digested pJM103 (see Figure 5), creating pGBS-0H2 (not shown), which contains the chloramphenicol resistance marker needed for selection. pGBS-0H2 was transformed intact into spo0A-lacZ containing JH642 and GBS10 strains, with plasmid insertion occurring \textit{via} a single crossover event.

\textbf{OH UP:} 5’ CT\textbf{GAGCTC}ACGAGCAGGCCATGGA\textbf{A} 3’ (Sac\textsc{I})
\textbf{OH DO:} 5’ TA\textbf{GATGCGTTCGTTTCAACGCTGA} 3’ (Sp\textsc{Hl})

Note: Restriction sites engineered into primer sequences are shown in bold type.
Figure 19. Creation of the clone used to assay sporulation in orfX− strains. The primers listed below were used to generate the 650 bp PCR product of the region upstream of kinA (A). The PCR product was digested with EcoRI and SaeI, and the 578 bp fragment internal to orfX was ligated into EcoRV/EcoRV the multiple cloning site of pBSK(-) (Stratagene, not shown). The 1.4 kb HindIII fragment of pDG780 (Guerout-Fleury, 1995) containing the kanamycin resistance cassette (not shown) was ligated into the pBSK (-) multiple cloning site already containing the orfX fragment. The resultant clone, pGBS5 (B, at top), was inserted into orfX of Bacillus subtilis strains via single integration (B, at the bottom), using selection for kanamycin resistance.

UK5: 5’ ATGAATTCCATAATACGCGAGTTG 3’ (EcoRI)
UK3: 5’ ACGGATCTTGTTTATGACCCACCGG 3’ (BamHI)

Note: Restriction sites engineered into primer sequences are shown in bold type.
In the presence of glucose, the sporulation frequency in \textit{kinA}^{-} cells mimicked that of \textit{kinA}^{+} cells, resulting in a $10^{5}$-fold repression of sporulation, suggesting that glucose inhibition of sporulation is not achieved through a reduction in \textit{kinA} expression. In the \textit{crsA kinA} double mutant in the absence of glucose, the sporulation frequency was identical to that of the \textit{crsA} mutant alone. This was an unexpected result, and suggested the possibility of increased expression of one or the other of the minor phosphorelay kinases. Since \textit{kinB} is known to be repressed by SinR (Dartois \textit{et al.}, 1996), and the \textit{sinl:sinR} ratio was increased in GBS10 in the presence of glucose (see Figure 13), this hypothesis is not unreasonable.

Table 4 also shows the effect of a \textit{spoOH} knockout on sporulation in both JH642 and GBS10 strains. As was expected, the loss of the sporulation sigma factor $\sigma^H$ resulted in a drastic drop in sporulation efficiency, in the presence and absence of glucose. No bacterial growth was observed in chloroform-treated cultures (not shown). The \textit{spoOH} disruption was previously reported to be not suppressed by the presence of the \textit{crsA} mutation (Boylan \textit{et al.}, 1988).

Collectively, these results suggest that the presence of KinA contributes to glucose resistant sporulation in GBS10, but is not the sole cause of glucose resistant sporulation. Furthermore, \textit{kinA} transcription levels were not affected by readthrough from the \textit{orfX} promoter, even in the presence of the \textit{crsA} mutation, and \textit{kinA} expression was regulated by a means as yet undetermined. Lastly, the presence of $\sigma^H$ was necessary for sporulation in both JH642 and GBS10.

D. Investigation of $\sigma^H$ activity.
Table 4. The sporulation efficiencies of JH642 and GBS10 strains containing \(\Delta kinA, \Delta orfX,\) and \(\Delta spo0H\) mutations.

<table>
<thead>
<tr>
<th>genotype</th>
<th>sporation efficiency*</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JH642</td>
<td>GBS10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SSM**</td>
<td>SSMG***</td>
<td>SSM</td>
</tr>
<tr>
<td>strain only</td>
<td>6.5 x 10^-1</td>
<td>1.0 x 10^-5</td>
<td>9.5 x 10^-1</td>
</tr>
<tr>
<td>(kinA^-)</td>
<td>1.5 x 10^-1</td>
<td>2.0 x 10^-5</td>
<td>9.3 x 10^-1</td>
</tr>
<tr>
<td>(orfX^-)</td>
<td>7.1 x 10^-1</td>
<td>8.5 x 10^-5</td>
<td>9.1 x 10^-1</td>
</tr>
<tr>
<td>(spo0H^-)</td>
<td>&lt;7 x 10^-6</td>
<td>&lt;4 x 10^-7</td>
<td>&lt;9 x 10^-6</td>
</tr>
</tbody>
</table>

* sporulation efficiency calculated as # spores/total cell count
** Schaeffer's spore medium, pH 7.5
*** Schaeffer's spore medium + 1.0 % added glucose
The observations described in Results, sections B.1 and B.2 showed a dramatic increase in the activity of the $\sigma^H$-dependent promoters in GBS10 in the presence of glucose (Figures 6, 7, 8, 10, 11 and 15), at a time in stationary phase when $\sigma^H$ activity decreased in wild type *B. subtilis*. It was previously suggested that changes in the *sinL:sinR* ratio might result in lower levels of free SinR, which may result in the increased activity of some $\sigma^H$-dependent promoters, as SinR is known to negatively regulate the $\sigma^H$-dependent promoters of the *spo0A* and *spoIIA* genes (Mandic-Mulec *et al.*, 1992; Mandic-Mulec *et al.*, 1995). However, published results indicate that SinR does not regulate either the *spoOF* or *kinA* genes (Mandic-Mulec *et al.*, 1992), which also showed increased transcriptional activity in GBS10. Recently published data suggests that other factors, such as expression of the Clp and Lon proteases (Nanamiya *et al.*, 1998; Liu *et al.*, 1999) and pH (Cosby and Zuber, 1997; Matsuno *et al.*, 1999; Matsuno and Sonenshein, 1999) may also affect the observed $\sigma^H$ activity. Consequently, a more in depth examination of $\sigma^H$ activity was undertaken.

1. AbrB effect on *spoVG* promoter activity.

The *spoVG* promoter was discussed earlier (pp. 53-55) with respect to its use in examining $\sigma^H$ activity. As shown in Figure 11, *spoVG-lacZ* activity was found to be altered in GBS10 in the presence of glucose. However, *spoVG* promoter activity is modulated by the transition state regulator AbrB (Zuber *et al.*, 1988), which means that *spoVG-lacZ* activity alone cannot be used directly as a measure of $\sigma^H$ activity. A *spoVG* promoter mutant (*spoVG42*) has been described whose transcriptional activity is independent of AbrB regulation (Youngman *et al.*, 1984). The *spoVG42-lacZ* fusion strain (ZB456, generously provided by P. Zuber, Oregon Graduate Institute of Science and Technology) was created
using Tn917, and was inserted in SPβ2Δ2 (Youngman et al., 1984). This spoVG42 promoter-lacZ fusion was introduced into both JH642 and GBS10 by transformation using chromosomal DNA from ZB456, for comparison of spoVG42 expression in the presence and absence of glucose in these strains. Similar experiments were attempted using spoVG-lacZ in strains lacking the abrB gene. While the spoVG-lacZ, abrB genetic background had a negligible impact on sporulation in cells with wild type σ^A, the crsA abrB double mutant barely grew, having a doubling time in excess of three hours, and then only when high levels of yeast extract and casamino acids were added. Sporulation in the double mutant was also severely impaired (data not shown). This unusual growth remains unexplained, but because of the difference in doubling times of the abrB mutant and the crsA abrB double mutant, meaningful comparisons between the two strains were not possible.

Figures 20C and 20D show spoVG-lacZ activities in wild type and crsA strains, taken from Figure 11, to be used as a comparison to spoVG42-lacZ activities shown in Figures 20A and 20B. In Figure 20A, the spoVG42 promoter activity in JH642 in the absence of glucose (open squares) began to increase slightly earlier and rose to a level 2.5-times higher than was seen with the wild type spoVG promoter counterpart in Figure 20C. The maximum activity of both spoVG42-lacZ and spoVG-lacZ occurred at roughly the same time (T_t). When glucose was present (closed diamonds), both spoVG42 and spoVG promoter activities (in A and C, respectively) were reduced, with a low peak at similar levels around T_0 followed by decreasing activity. The decrease in transcription from spoVG42 when glucose was present was presumably because of repressive effect of glucose on the activation of σ^H.

Figure 20B depicts the spoVG42-lacZ activity in GBS10 in the presence (closed diamonds) and absence (open squares) of glucose. When glucose was absent from the
medium, *spoVG42* promoter activity peaked at the same time (T₁) at levels roughly 4.5-times greater than the wild type promoter in GBS10 (Figure 20D). However, when glucose was present in the medium, similar transcriptional activity was observed at T₀ for both promoters, with activity continuing to increase beyond the onset of stationary phase. In the absence of AbrB regulation, *spoVG42* promoter activity continued to increase into the stationary phase; this was not seen with the *spoVG-lacZ* promoter fusion.

The results seen for the *spoVG42* promoter suggest that σ^H^-activity in GBS10 in the presence of glucose continued beyond the initial stages of sporulation. Recent publications have shown that in the absence of the ClpC ATPase, expressed as part of the stress response and during sporulation, σ^H^-activity in late stationary phase is not eliminated, suggesting that ClpC functions in part in the degradation of σ^H^ (Nanamiya et al., 1998). However, mutants lacking ClpC sporulate roughly 500-times less well than wild type strains (Nanamiya et al., 1998), suggesting that accumulation of σ^H^-activity alone into late stationary phase does not increase sporulation frequency.

2. The effect of pH on *kinA* σ^H^-dependent promoter activity.

Another possible effector of σ^H^-activity is pH. During logarithmic growth, *B. subtilis* do not express a complete Krebs cycle (Hederstedt, 1993; Fisher et al., 1994). Consequently, acidic glycolytic by-products are excreted and accumulate extracellularly as cells consume glucose, and as the cell number increases, the pH of the medium begins to drop. *B. subtilis* cells respond to this pH change as a growth phase signal, which may aid in triggering the activation of genes required for stationary phase (Ireton et al., 1995; Cosby and Zuber, 1997; Matsuno and Sonenshein, 1999; Matsuno et al., 1999). When the Krebs cycle is fully
Figure 20. Expression of the spoVG42 promoter-lacZ reporter gene fusion inserted in the amyE gene (A and B), and of the spoVG promoter-lacZ reporter gene fusion inserted upstream of the spoVG (C and D), in strains JH642 (A and C) and GBS10 (B and D). Strains were grown in Schaeffer’s spore medium, pH 7.5, with (closed diamonds) and without (open squares) 0.2% added glucose.
induced, the glycolytic by-products are eventually taken up and used as a source of energy for the initiation of stationary phase events, and as a consequence, the pH of the external medium increases (Cosby and Zuber, 1997; Matsuno et al., 1999).

Recent publications have provided evidence that pH affects $\sigma^H$ activity. A culture medium containing high levels of glucose, but buffered to a neutral pH, yielded cells with higher $\sigma^H$-dependent transcription than cells grown in an unbuffered glucose rich medium. The conclusions made from these observations were: 1) the increase in pH that occurs after the induction of the Krebs cycle was linked with the activation of $\sigma^H$; 2) this activation is affected by cellular levels of both Spo0A-P and AbrB (Ireton et al., 1995; Cosby and Zuber, 1997; Matsuno and Sonenshein, 1999).

Because the work in this thesis is focused on the activity of $\sigma^H$ in JH642 and GBS10 cells, the effect of pH under conditions used here was examined. Figure 21 depicts the pH change that occurred in wild type and crsA mutant strains grown in SSM medium unbuffered but adjusted to pH 7.5 prior to inoculation, with and without added glucose. In both strains in the absence of glucose (Figures 21C and 21D, open squares), there are only minor changes in medium pH during exponential and post-exponential phase growth. When glucose was added (closed diamonds), medium pH dropped sharply as cellular density increased, but began to rise again at roughly $T_0$. The growth conditions I used in the present study resulted in only a transient drop in pH in both JH642 and GBS10 strains (Figures 21C and 21D). Therefore, the repression of JH642 sporulation seen in the presence of glucose was not due to a pH effect that is absent from GBS10. These results do not address the effect of pH on $\sigma^H$ in GBS10, so the effects of external pH on the activity of the kinA promoter were examined.

Previously published experiments examining the pH/glucose effect used rich media
Figure 21. Growth pattern and pH profile of *B. subtilis* strains JH642 (A and C) and GBS10 (B and D). Strains were grown in unbuffered Schaeffer's spore medium, pH 7.5, with (closed diamonds) or without (open squares) 0.2% added glucose.
buffered at pH 7.5 and σ^H-dependent promoter-lacZ fusions (Cosby and Zuber, 1997). In the experiment described below, a low pH buffer (70 mM MES buffer at pH 5.2) was used to examine the potential for a low pH environment to inhibit σ^H activation. Figure 22 depicts the effects of low pH on the expression of the 1.7 kb \textit{kinA-lacZ} fusion (inserted in \textit{kinA}) introduced into JH642 and GBS10. The pattern of transcription in JH642 in the unbuffered medium (Figure 22A, open squares) was typical of the results described earlier (Figure 6), with a peak occurring shortly after T_0 and subsiding thereafter. When the medium was buffered at pH 5.2 (Figure 22A, closed diamonds), \textit{kinA} promoter expression changed only slightly and the peak activity occurred marginally later, at levels identical to that seen in unbuffered media. When glucose was added, transcriptional activity in JH642 in the unbuffered medium (Figure 22B, open squares) peaked slightly earlier (roughly T_{0.5}) and at slightly lower levels than in the absence of glucose. When the medium was buffered at pH 5.2 (Figure 22B, closed diamonds), no obvious change in \textit{kinA} promoter activity was observed from that of the unbuffered medium conditions. These results suggest that pH played little to no role in altering σ^H activity under the conditions used in this study.

Figures 22C and 22D show \textit{kinA-lacZ} activity in GBS10 in neutral (open squares) and low (closed diamonds) pH environments, in the presence (Figure 22D) and absence (Figure 22C) of added glucose. As was seen in JH642 cells, lowering the pH of the medium caused minimal changes in σ^H-dependent transcriptional activity. In Figure 22C, buffered media allowed for a marginally higher level of \textit{kinA} promoter expression during the early phase of sporulation initiation than was seen in unbuffered medium, with peak activity unchanged. When glucose was added, \textit{kinA-lacZ} activity increased sharply, regardless of pH, and the timing of expression was only marginally affected, with peak levels in unbuffered medium.
Figure 22. Expression of the *kinA* promoter-*lacZ* reporter gene fusion in the *kinA* gene in strains grown in media at different pH. Strains are JH642 (A and B) and GBS10 (C and D). Strains were grown in Schaeffer's spore medium at pH 5.2 (closed diamonds; used 70 mM MES buffer) or at pH 7.5 (open squares; unbuffered), with (B and D) or without (A and C) 0.2% added glucose.

Note: in buffered media (closed diamonds) pH throughout the growth cycles of JH642 and GBS10 strains did not exceed pH 5.5 (data not shown). Unbuffered media exhibited a pH profile identical to that shown in Figures 21C and 21D.
occurring roughly an hour earlier (T_{1.5} to T_2) than was seen in buffered media (T_{2.5} to T_3).

The results obtained with GBS10 media buffered at low pH suggest that in conditions used in this thesis, pH played a negligible role in the activity of \( \sigma^H \), either in the presence or absence of glucose. Therefore, the increase in \( \sigma^H \)-dependent transcription observed in GBS10 promoter fusion strains in the presence of glucose was not due to altered regulation in response to pH.

**E. In vivo investigation of spo0A promoter activity.**

The third observation made from the promoter-\( \textit{lac} \)Z fusion analyses in Results, section B concerned the unusual early expression from the \( \textit{spo0A} \) dual \( \sigma^A/\sigma^H \) promoters in GBS10. This early expression was also seen in previously published data, in which the early derepression of the \( \textit{spo0A} \) \( \sigma^A \) promoter was noted in a \( \textit{crsA} \) mutant strain using an S1 nuclease protection assay (Chibazakura \textit{et al.}, 1991). While that report stated that \( \textit{spo0A} \) \( \sigma^H \)-dependent promoter activity was increased early in the presence of the \( \textit{crsA} \) mutation, early \( \textit{P_v} \) promoter activity and the high \( \textit{P_S} \) promoter activity were not discussed (Chibazakura \textit{et al.}, 1991).

Results presented in sections C and D examined in some detail observations made about the unusual expression of the \( \textit{kinA} \) gene and the extended activity of \( \sigma^H \) in GBS10 in the presence of glucose. Below, the unusual activity of the \( \textit{spo0A} \) promoter in the \( \textit{crsA} \) mutant was also examined in greater detail.

1. Effect of a \( \textit{spo0H} \) knockout on \( \textit{spo0A} \) promoter activity.

The details of the creation of the \( \textit{spo0H} \) knockout construct are given in Figure 18.
pGBS-0H2 was transformed into strains containing the spo0A-lacZ fusion originally examined in Figure 8, recombining into the spo0H gene in the chromosome with a single crossover event. In the constructs, transcription of the chloramphenicol resistance gene was in the direction opposite from that of the spo0H gene.

Figure 23 depicts the expression of spo0A-lacZ in JH642 and GBS10 strains. In Figure 23A, the activity of the promoter in spo0H+ (open squares) and spo0H (closed diamonds) JH642 cells in the absence of glucose is shown. Transcription levels in cells with the intact spo0H gene were typical for this strain (see Figure 8). In the spo0H strain, only a very low level of spo0A transcription was observed, and the expected increase in promoter activity at the onset of stationary phase was not observed. These results agree with previously published data (Chibazakura et al., 1995). When glucose was added (Figure 23B), transcription from the spo0A promoter in the presence and absence of σ^H appeared virtually identical. No induction of the promoter was seen at T₀.

In Figure 23C, spo0A promoter activity +/− σ^H in GBS10 in the absence of glucose is detailed. In crsA spo0H+ cells (open squares), transcription increased earlier than was seen in JH642 (compare to Figure 23A), and peaked at higher levels. In the crsA spo0H strain (closed diamonds), spo0A-lacZ activity appeared to increase slightly from basal transcription levels, with a maximum activity at T₀ roughly 2.5-times that seen in JH642 spo0H+ cells. When glucose was added (23D, open squares), transcription in crsA spo0H+ cells was also typical of other promoters studied in this background, with increased transcription earlier than was seen in JH642 (compare to Figure 23B) and peaking at a maximum of 4- to 5-times higher than in JH642 cells. In the crsA spo0H− strain in the presence of glucose (Figure 23D,
Figure 23. Expression of the spo0A promoter-lacZ reporter gene fusion in spo0H* and Δspo0H B. subtilis strains. The promoter construct was inserted in the amyE gene in strains JH642 (A and B) and GBS10 (C and D) with (closed diamonds) and without (open squares) a kanamycin-linked spo0H insertional knockout. Strains were grown in Schaeffer’s spore medium, pH 7.5, with (B and D) or without (A and C) 0.2% added glucose.
closed diamonds), transcription appeared to increase early, similar to the pattern observed in the GBS10 spoOH+ strain, peaking at roughly 3-times the level seen in JH642 spoOH in the presence of glucose. Transcription did not increase sharply at T₀, presumably due to the lack of σH. It is interesting to note that the level of transcription seen in the GBS10 spoOH strain in the presence of glucose was roughly 80% of that seen in the JH642 spoOH+ cells in the absence of glucose, and the timing of transcriptional activity was very similar.

The results seen in Figure 23 suggest that spoOA promoter activity in GBS10 spoOH cells in the presence of glucose was roughly equivalent to the spoOA promoter activity in JH642 spoOH+ cells in the absence of glucose. Clearly, the transcriptional regulation of this promoter was altered in GBS10; both σA- and σH-dependent expression of spoOA was much higher than normal. It was not clear why the presence of glucose resulted in an increase in transcription in GBS10 (Figure 23D).

2. Construction of the spoOA Ps promoter deletion.

Because the loss of σH due to the spoOH gene disruption could have other effects which may impact on the regulation of stationary phase genes (such as sinI, spoOF and kinA), which in turn may affect the apparent activity of the spoOA-lacZ fusion, a second approach was used in examining the σA-dependent activity of the spoOA promoter. Figure 24 details the creation of the spoOAΔPs-lacZ construct for use in JH642 and GBS10 strains. This construct was created using the restriction enzymes SspI and Hpal located 5’ and 3’ of the σH promoter (respectively, from bases -59 to +18 relative to the Ps transcription start site). This deletion simultaneously deleted both the σH promoter, and the SinR regulator binding site (from bases -23 to -3, relative to the Ps transcription start site) that overlaps the Ps promoter
Figure 24. The plasmid pJH1408 and spoOA promoter-lacZ cloning strategy. To remove the sporulation promoter from the spoOA promoter fragment on pJH1408, the plasmid was first linearized by a 2 minute SspI digest, and then fully digested with HpaI. The DNA was then recircularized in a dilute ligation reaction (400 ng/ml). The resultant clone, pJH14-M, did not contain the 77 bp fragment containing the whole of the sporulation promoter. pJH14-M was then subjected to PCR using the primer pair shown below, to amplify the 950 bp fragment of the mutant spoOA promoter with engineered EcoRI/BamHI ends, which was then ligated directionally into EcoRI/BamHI digested pDH32 (see Figure 1).

0A5: 5’ CGTGAATTCCGATATGGACACAAAG 3’ (EcoRI)
0A3: 5’ TCGGATCCATGTCTTCCTGTCCTT 3’ (BamHI)

Note: Engineered restriction sites in the primers are shown in bold type.
Note: Regions of DNA at which Spo0A-P binds are denoted by a grey bar. (==)
Because SinR is thought to repress transcription from the spo0A promoter by interfering with Eσ^H binding to the Ps promoter (Mandic-Mulec et al., 1995), the deletion of the SinR binding site in the spo0AΔPs mutant was expected to lack the SinR-dependent negative effect on the spo0A P_V promoter transcriptional activity.

3. Effect of the spo0AΔPs deletion on spo0A promoter activity.

Figure 25 depicts the spo0AΔPs-lacZ expression patterns in JH642 (A and B) and GBS10 (C and D) strains in the absence (A and C) and presence (B and D) of glucose. In all cases, the activity of the wild type spo0A promoter is portrayed by open squares, and the activities shown were typical for the strains and conditions used. In Figures 25A and 25C, the activity of the spo0AΔPs promoter (closed diamonds) in the absence of glucose in the two strains was virtually identical. A high basal level of σ^A-dependent transcription was observed during logarithmic growth, which gradually decreased prior to and after the onset of stationary phase. In Figures 25B and 25D, the activity of the spo0AΔPs promoter in the presence of glucose in both strains were, again, quite similar. In these cases, the spo0AΔPs promoter showed a large induction in media containing glucose prior to and at the onset of stationary phase. After T_o, promoter activity began to decline. The activity of the spo0AΔPs promoter in JH642 in the absence of glucose shown in Figure 25A agrees with previously published observations (Strauch et al., 1992).

Two issues arose from this experiment: firstly, why did transcription from the spo0AΔPs promoter in both JH642 and GBS10 strains drop after T_1 to T_o, in the absence of glucose; secondly, what caused the induction from the spo0AΔPs promoter in both strains between T_2 and T_o seen in the presence of glucose? Observations from a previously
Figure 25. Expression of the spo0A promoter- and the spo0AΔPs promoter-lacZ reporter gene fusions (open squares and closed diamonds, respectively) in strains JH642 (A and B) and GBS10 (C and D). Strains were grown in Schaeffer’s spore medium, pH 7.5, with (B and D) and without (A and C) 0.2% added glucose.
published paper may shed light on the first issue. Strauch, et al. (1992) created both the spo0AΔP\text{S} (identical to the mutant created in this thesis) and the spo0AΔP\text{V} promoter-lacZ mutants, and assayed the activity of these promoter constructs in the presence and absence of an intact spo0A gene. It was found that the patterns of activity seen in these different strains supported the idea that Spo0A\text{P} inhibited transcription from the spo0A P\text{V} promoter after T\text{0} and that Spo0A\text{P} activated the spo0A P\text{S} promoter after T\text{0}. If this were the case, it would explain the decrease in transcription noted in Figures 25A-D in the spo0AΔP\text{S}-lacZ constructs after the onset of stationary phase. However, the in vivo data provided by Strauch, et al. (1992) have not been corroborated by any in vitro experiments. With respect to the induction observed prior to T\text{0} in both Figures 25B and 25D, there is no obvious explanation at this time. No glucose-specific regulators of the spo0A promoter have ever been identified.

4. Gene knockout effects on sporulation frequency.

Because of the increase in spo0A expression in GBS10 cells shown in Figures 9 and 23, the sporulation ability of the spo0AΔP\text{S} knockout and the sinR knockout became of interest. Two hypotheses to explain the high levels of spo0A transcription seen in GBS10 prior to stationary phase can be made based on the experimental evidence. Firstly, that Eσ\text{A47} had a higher affinity for the spo0A P\text{V} promoter than Eσ\text{A}, and therefore there was a higher level of spo0A transcription in GBS10 prior to the onset of stationary phase. Secondly, that a change in SinR negative regulation in GBS10 resulted in a lack of transcriptional repression from the spo0A P\text{S} promoter at the onset of stationary phase. Inherent in these ideas is the notion that a higher level of Spo0A at the onset of stationary phase, coupled with both the deregulation of the phosphorelay caused by inappropriate activation of σ\text{H} and a lack of SinR
negative regulation, is sufficient to allow sporulation in the presence of glucose.

To construct the spo0AΔPs knockout in JH642 and GBS10 cells, the EcoRI-BamHI PCR fragment described in Figure 24 was subcloned into pJM103 (see Figure 5) and transformed into JH642 and GBS10 so that chromosomal integration occurred via a single crossover event. I. Smith (New York Institutes of Health) graciously provided the sinR null strain (IS875), created by a plasmid insertion event. The ΔsinR mutation was introduced into JH642 by transformation using chromosomal DNA from IS875. Table 5 shows the results of sporulation assays using these gene knockouts in the presence and absence of glucose.

The JH642 and GBS10 strains assayed with and without added glucose had sporulation frequencies typical of previous experiments (see Table 3). When the JH642, spo0AΔPs knockout strain was assayed, in the absence of glucose a severe inhibition of sporulation was observed, roughly equivalent to that seen in JH642 spo0A+ cells grown in the presence of glucose. This result agrees with previously published observations (Siranosian and Grossman, 1994) and implies two things: firstly, that the spo0A promoter switch and consequent upregulation of transcription is critical for sporulation initiation; secondly, that the spo0A promoter switch is indeed negatively regulated by the presence of glucose. However, when the crsA spo0AΔPs double mutant was assayed, sporulation in the absence of glucose occurred at a frequency roughly equal to that seen in JH642 spo0A+ cells grown in the absence of glucose. Furthermore, when glucose was present, the frequency of sporulation in the crsA spo0AΔPs strain was only slightly affected (29% of that seen in GBS10 in SSMG, as opposed to 0.022% of that seen in JH642 in SSMG). These results suggest that the observed altered regulation of the spo0A promoter in GBS10 is in large part
Table 5. The sporulation efficiencies of JH642 and GBS10 strains containing $spo0A\Delta P_s$ and $\Delta sinR$ mutations.

<table>
<thead>
<tr>
<th>genotype</th>
<th>sporulation efficiency*</th>
<th></th>
<th>GBS10</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>JH642</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SSM**</td>
<td>SSM***</td>
<td></td>
</tr>
<tr>
<td>$spo0A^+/sinR^+$</td>
<td>6.5 x 10^{-1}</td>
<td>1.0 x 10^{-5}</td>
<td></td>
</tr>
<tr>
<td>$spo0A \Delta P_s$</td>
<td>2.5 x 10^{-5}</td>
<td>2.2 x 10^{-7}</td>
<td></td>
</tr>
<tr>
<td>$sinR^-$</td>
<td>7.4 x 10^{-1}</td>
<td>2.5 x 10^{-1}</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td>GBS10</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>SSM</td>
<td>SSMG</td>
<td></td>
</tr>
<tr>
<td>$spo0A^+/sinR^+$</td>
<td>9.5 x 10^{-1}</td>
<td>1.0 x 10^{0}</td>
<td></td>
</tr>
<tr>
<td>$spo0A \Delta P_s$</td>
<td>6.0 x 10^{-1}</td>
<td>2.9 x 10^{1}</td>
<td></td>
</tr>
<tr>
<td>$sinR^-$</td>
<td>ND^A</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* sporulation efficiency calculated as # spores/total cell count
** Schaeffer’s spore medium, pH 7.5
*** Schaeffer’s spore medium + 1.0 % added glucose
^A not determined
responsible for the glucose insensitive phenotype caused by the presence of the crsA mutation.

Table 5 also shows the effects of a sinR knockout on sporulation in JH642. In the absence of glucose, the sporulation frequency of the sinR knockout strain was equivalent to that of the wild type strain. However, when glucose was present, the sporulation frequency was only minimally affected, dropping by 67%. These data agree with the observations of others (I. Smith, personal communication), and suggest that the repression of the spo0A promoter by SinR was involved in the inhibition of sporulation by glucose.

The above sets of results indicate that increased transcription from the spo0A Pv promoter and an alteration in SinR regulation at spo0A may be sufficient to overcome glucose inhibition of sporulation. It was previously proposed that Spo0A~P is antagonistic to SinR negative regulation by activating transcription from the σH-dependent sinI promoter to increase the sinI:sinR transcript ratio, resulting in a decrease in free SinR and an increase in SinI:SinR heterodimer formation. Therefore, it is possible that the alteration in the pattern of sin operon transcription observed in GBS10 in the presence of glucose (which is implicated in a glucose resistant phenotype; compare Figure 17 and Table 5) was caused by an early increase in the amount of Spo0A, because of the increased transcription seen from the spo0A Pv promoter (which is implicated in a glucose resistant phenotype, compare Figure 23 and Table 6). It is assumed that Spo0A is phosphorylated by the active phosphorelay, which is not suppressed in GBS10 cells in the presence of glucose (see Figures 6 and 7).

F. In vitro spo0A promoter analysis.
The data in Figures 23B and 23D suggested the possibility that EσA47 may have a higher affinity for the spo0A σA promoter than EσA. As a direct test of this hypothesis, EσA47 was purified from GBS10 for examination in in vitro transcription assays.

1. Isolation of EσA47.

Figure 26A shows an SDS-PAGE of purified EσA47 fractions obtained from the glycerol gradient step of the purification process, along with less purified samples of the enzyme, and a sample of purified wild type RNA polymerase. Glycerol gradient fraction 8 was used in all transcription assays described below.

Figure 26B shows a sample of the 950 bp spo0A promoter fragment used in transcription assays. The fragment used was generated by PCR with Vent polymerase (New England BioLabs, Inc.), using the primer pair 0A5/0A3 shown in Figure 24. Assuming the start site reported by Chibazakura, et al. (1991), a runoff transcript from this DNA fragment would generate an RNA of 291 bases in length.

2. Characterization of initiation conditions using EσA47.

Figures 27 through 29 show preliminary characterizations of the activity of EσA47 on the spo0A σA promoter. Figure 27 depicts the results from an initiation assay to determine requirements for heparin resistance. Heparin is a non-competitive inhibitor of RNA polymerase used in vitro to limit transcription in a reaction to a single round (Walter et al., 1967). Some B. subtilis RNA polymerase-promoter interactions are stable enough that a simultaneous addition of all four NTPs and heparin allows the enzyme to initiate and
Figure 26. Purification of protein and DNA components of the transcription reaction. (A) Coomassie blue stain of a 12% SDS-PAGE gel of purified EoA47, with the RNA polymerase core enzyme components (αββ′) and σA highlighted on the right. (B) Ethidium bromide stain of a 0.7% agarose gel of purified spoOA promoter fragment generated by PCR using the primer sequences shown below. 1-Partially purified cellular fraction applied to a DNA cellulose column. 2-The fraction of the column load that did not bind to the DNA cellulose. 3-A previously purified EoA shown for comparison. 4-Fraction 3 of a glycerol gradient fractionation of the eluate of the DNA cellulose column. 5 to 9-Fractions 4 through 8 of the glycerol gradient. 10- Purified spoOA promoter fragment (60 ng; see Figure 24 for PCR details). 11-Purified spoOA promoter fragment (120 ng). 12-φ29 DNA digested with HindIII. Fragment sizes are listed to the right.

0A5: 5' CGTGAATTCCGATATGGACACAAAG 3' (EcoRI)
0A3: 5' TCGGATCCATGTCTTCCTTCTCCTCCTT 3' (BamHI)

Note: Engineered restriction sites in the primers are shown in bold type.
elongate. However, for many enzyme-promoter complexes, the simultaneous addition of heparin with nucleotides prevents transcription initiation and elongation. In these cases, one or more nucleotides must be added prior to the addition of the remaining nucleotides plus heparin to allow elongation in the presence of heparin. In transcription assays shown below, RNA synthesis was followed by the incorporation of $\alpha^{32}$P-GTP into the transcript. Once the reaction was completed, the synthesized transcripts were separated from free nucleotides by electrophoresis through an 8% PAGE gel containing 7M urea. Transcripts were then detected by exposure of the radioactive gel to either X-ray film, or a phosphorImager screen (Molecular Dynamics PhosphorImager SI). Quantitations of transcript levels were carried out using ImageQuant 1.0 software.

Figure 27A shows the autoradiograph of the polyacrylamide gel used to separate the transcripts formed in the transcription assay carried out to determine requirements for heparin resistance. The nucleotides included in the initiation mix are shown at the top. RNA polymerase and DNA were mixed with the nucleotides and incubated for 2 minutes, and then elongation was permitted by the addition of the remaining nucleotides plus heparin, followed by incubation for 5 minutes. A single transcript was observed on the gel. Figure 27B is a graphical representation of the data in Figure 27A. Values shown are relative intensities of the radioactivity incorporated into the transcripts, generated using a phosphoimager. The results in Figure 27A indicated that the $Eo^{A47}$-spo0A promoter complex alone was not stable enough to initiate in the presence of heparin. The level of transcripts generated was extremely low. An initiated complex using ATP only (which permits the synthesis of an AA dimer) was also not stable in the presence of heparin. However, when ATP and GTP were used to form the initiated complex (which permits the synthesis of an AAGA tetramer), the
Figure 27. Nucleotide requirements for heparin resistance at the $\sigma^A$ dependent spo0A promoter. (A) Autoradiograph of the gel used to separate the transcription products. (B) A graphical representation of the results shown in (A), using relative intensity calculated from a phosphorimager scan. Various combinations of nucleotides were preincubated with EoA$^{47}$ and template for 2 minutes prior to addition of heparin and the remaining nucleotides necessary for elongation. The concentration of spo0A promoter template used was 5 nM. NTP-nucleotide triphosphate; A-ATP; G-GTP; U-UTP; C-CTP.
amount of transcript generated increased dramatically. The addition of UTP or UTP and CTP to the initiation mix resulted in only minor increases in the amount of transcripts generated over that seen with ATP and GTP. These results were similar to those obtained with wild type RNA polymerase and the spo0A promoter (data not shown). All transcription assays described below were performed using an ATP + GTP initiation, followed by the addition of a UTP, CTP, and heparin mix.

Figure 28 shows the effects of temperature on the initiation reaction using Eσ^{A47} at the spo0A promoter. Figure 28A shows the autoradiograph of the gel used to separate transcripts formed in the transcription reaction, and Figure 28B is a graphical representation of the data in Figure 28A. As was expected, transcription was sensitive to temperature change. The temperature at which transcript production was maximal was 37°C. When the temperature of the reaction was shifted 5°C in either direction, transcript production was halved, and was halved again when the temperature was dropped to 28°C.

In vitro transcription is normally very sensitive to salt concentration (Shaner et al., 1983; Roe, et al., 1984; Leirmo and Record, 1990). This sensitivity is thought to be due to the accumulation of cations next to the DNA phosphate backbone, which forms a steep ion concentration gradient when compared to the ion concentration in the reaction mix as a whole. When RNA polymerase binds to a promoter site and melts the DNA helix, counterions are displaced into the solution, which provides a large entropic contribution to the initiation of transcription (Lohman et al., 1978; Shaner et al., 1983; Lohman, 1985). Thus, transcription reactions are more active in conditions of lower ionic strength, as a steeper ion gradient provides a larger increase in entropy.
Figure 28. The effect of temperature on EσA47 transcription of the spo0A σA dependent promoter. All transcriptions included a 2 minute A+G pre-initiation. Pre-initiation, initiation and elongation were carried out at the indicated temperature, and the transcripts produced were separated by electrophoresis (A) Autoradiograph of the polyacrylamide gel used to separate the transcribed mRNA. (B) A graphical representation of the results shown in (A), using relative intensities calculated from a phosphorimager scan.
Figure 29 shows the effects of varying potassium acetate concentration on transcript production, using both EsA47 and EsA. Salt concentrations in the reaction mix varied from 50 mM to 125 mM. Figure 29A shows an autoradiograph of a portion of the gel used to separate transcripts produced in this assay. Figure 29B is a graphical representation of the data shown in Figure 29A. EsA transcription increased slightly between 50 and 95 mM salt, and began to drop at 110 mM potassium acetate. EsA47 transcription was roughly constant between 50 and 80 mM salt, dropped slightly at 95 mM salt, and decreased sharply at 110 and 125 mM salt. These results were reproducible (data not shown) and suggested that EsA47 was more salt-sensitive at the spo0A promoter at higher salt concentrations than was EsA. This experiment demonstrated that the potassium acetate concentration used in the remainder of the in vitro experiments, 80 mM, was within the range of salt concentrations at which both polymerases transcribed maximally; thus, differences in the transcriptional activity of the two RNA polymerases shown in the following experiments are not due to differences caused by salt sensitivity.

3. The effect of DNA concentration on transcription from the spo0A promoter.

The results in Figure 25 showed that the spo0AΔP5 promoter was transcribed in vivo at higher levels in GBS10 than in JH642. This suggested the possibility that EsA47 had a higher activity on the spo0A σA promoter than did EsA. In order to compare EsA47 and EsA transcription from the spo0A promoter, an experiment was devised in which the activity of each enzyme on a standard template, the φ29 phage A2 promoter, was compared to the activity on the spo0A promoter. RNA polymerase transcribing from the φ29 A2 promoter
Figure 29. The effect of potassium acetate concentration on transcription from the *spo0A* promoter. (A) Autoradiograph of the gel used to separate the transcription products produced by $E\sigma^{A47}$ (left) and $E\sigma^A$ (right). Potassium acetate concentrations, from left to right, are 50 mM, 65 mM, 80 mM, 95 mM, 110 mM, and 125 mM. (B) A graphical representation of the results shown in (A), using relative intensities calculated from a phosphorimager scan.
also requires ATP + GTP preinitiation to become heparin resistant (Dobinson and Spiegelman, 1987), but it is not regulated by any known effectors, and has been extensively characterized (Dobinson and Spiegelman, 1985; 1987). Figure 30 shows the results of a transcription assay containing a constant amount of each of the two enzymes, ΕσΑ, and ΕσΑ47, and varying amounts of the A2 promoter. Figure 30A shows the autoradiograph of the gel used to separate the transcripts, with DNA concentrations used in the assay decreasing from left to right. Figures 30B and 30C are graphical representations of the results shown in Figure 30A, generated by a phosphorImager scan of an exposed screen.

To analyze the data given in Figures 30B and 30C, the initial slopes of the DNA input curves were estimated by drawing lines from 0 through the initial points in each graph, where the intensities were nearly linear with DNA input. The slopes of the lines were 137700 pixels/nM DNA for ΕσΑ in Figure 30B, whereas Figure 30C had a slope of 65334 pixels/nM DNA for ΕσΑ47. Given these numbers, it can be stated that the wild type RNA polymerase appeared to transcribe the A2 template 2.1-times more efficiently than the crsA mutant enzyme (slope of graph B/slope of graph C). This comparison was used to calculate the differences in the combination of the specific activities and absolute amounts between the ΕσΑ and ΕσΑ47 enzyme preparations.

Figure 31 depicts the results of a transcription assay done concurrently with, and in an identical manner to, the assay described in Figure 30, except the spo0A Pv promoter was used as the DNA template. In this assay, the slopes of the lines in Figures 31B and 31C were calculated at 15000 pixels/nM DNA and 15563 pixels/nM DNA, respectively. Given these numbers, it appears that the crsA mutant RNA polymerase transcribed the spo0A template
Figure 30. DNA input assay using the φ29 phage A2 promoter DNA. (A) Transcription from the A2 promoter using RNA polymerase isolated from JH642 (left) and GBS10 (right), and with increasing DNA concentrations. DNA levels from left to right are 1.0 nM, 2.2 nM, 3.4 nM, 4.6 nM, 5.8 nM, 7.0 nM, and 8.2 nM. (B and C) Intensities of the transcription bands were graphed vs. template concentration. (B) wild type RNA polymerase. (C) crsA mutant RNA polymerase.
Figure 31. DNA imput assay using spo0A promoter DNA. (A) Transcription from the spo0A $\sigma^A$ promoter using RNA polymerase isolated from JH642 (left) and GBS10 (right), and with increasing DNA concentrations. DNA levels from left to right are (for $E\sigma^A_{47}$ only) 2.0 nM, 3.2 nM, 4.4 nM, 5.6 nM, 6.8 nM, 8.0 nM, and 9.2 nM (for both RNA polymerases). (B and C) Intensities of the transcription bands were graphed vs. template concentration. (B) wild type RNA polymerase. (C) crsA mutant RNA polymerase.
with approximately equal efficiency (1.04-times) to the wild type enzyme (slope of graph C/slope of graph B). Using the difference in transcriptional activity between Eσ^A and Eσ^A47, based on the φ29 promoter as calculated in Figure 30, Eσ^A47 appeared to have a two-fold higher activity on the spo0A promoter than Eσ^A. In short, these data suggest that the crsA mutant polymerase transcribes the spo0A promoter template twice as efficiently as the wild type enzyme.

4. The effect of RNA polymerase concentration on transcription from the spo0A promoter.

A second comparison of the activities of Eσ^A47 and Eσ^A was performed by keeping the DNA concentration constant and varying the RNA polymerase concentration. Figure 32 depicts the results from transcription assays with 5.5 nM φ29 A2 promoter DNA as a template. Figure 32A shows an autoradiograph of the gel used to separate the transcripts generated, with increasing enzyme concentration from left to right. Figures 32B and 32C are graphical representations of the data shown in Figure 32A.

The initial slope of the amounts of the 130 nt transcript versus polymerase input, estimated from the initial points in the graphs shown in Figures 32B and 32C, were calculated as 743750 pixels/enzyme dilution and 3422500 pixels/enzyme dilution, respectively. The ratio of the slopes of the RNA polymerase transcriptional activities for the two enzymes on the A2 promoter was 4.60 (slope of graph C/slope of graph B).

Figure 33 depicts a transcription assay done concurrently with, and in an identical manner to, the assay described in Figure 32, except that 5.5 nM spo0A promoter fragment
Figure 32. RNA polymerase input assay using 5.5 nM φ29 phage A2 promoter DNA. (A) Transcription from the A2 promoter using increasing concentrations of RNA polymerase isolated from GBS10 (left) and JH642 (right). (B and C) Intensities of the transcription bands were graphed vs. template concentration. (B) crsA mutant RNA polymerase. (C) wild type RNA polymerase.
Figure 33. RNA polymerase input assay using 5.5 nM spo0A promoter DNA. (A) Transcription from the spo0A promoter using increasing concentrations of RNA polymerase isolated from GBS10 (left) and JH642 (right). (B and C) Intensities of the transcription bands were graphed vs. template concentration. (B) crsA mutant RNA polymerase. (C) Wild type RNA polymerase.
was used as the DNA template. In this assay, the initial slopes of the lines in Figures 33B and 33C were calculated at 172500 pixels/enzyme dilution and 398334 pixels/enzyme dilution, respectively, and the ratio of the slopes was calculated as 2.31 (slope of graph C/slope of graph B). Normalizing the activity of the two enzymes using the data from Figure 32, the apparent difference in transcriptional activity between Eσ^A47 and Eσ^A at the spo0A promoter was 1.99. Again, the results suggested that the crsA mutant polymerase transcribed the spo0A promoter template roughly twice as well as did the wild type enzyme.

The results in Figures 30 through 33 support the hypothesis that RNA polymerase containing σ^A47 transcribed the spo0A σ^A promoter twice as efficiently than the wild type RNA polymerase. This increased enzyme activity would explain the transcription patterns shown in Figure 23, in which the GBS10 strain had a higher spo0A-lacZ activity in the presence and absence of σ^H than JH642. The sporulation frequencies in Table 5 support the idea that the higher level of spo0A transcription seen in GBS10 is sufficient to allow sporulation.
Discussion

The crsA mutation is a 2-base change that results in a single amino acid change from proline to phenylalanine in the B. subtilis major vegetative sigma factor, $\sigma^A$ (Kawamura et al., 1985). This mutation enables wild type B. subtilis cells to sporulate in what is a prohibitive environment: media containing glucose (concentrations studied range from 0.1% to 1.0%: Schaeffer et al., 1965; Dawes and Mandelstam, 1970; Coote, 1974; Cooney et al., 1977; Takahashi, 1979). The objective of this work was to investigate the mechanism that allows B. subtilis cells containing the crsA mutation to sporulate in the presence of glucose.

In Results, section B, the in vivo effects of the RNA polymerase containing $\sigma^{A47}$ on the expression of genes crucial to sporulation initiation were described. There were three discernible effects on the sporulation initiation pathway in the crsA mutant: 1) changes in the activity of the $\sigma^H$-dependent promoters; 2) changes in the activity of the promoters of the $\sin$ operon (and resultant regulatory effects on spo gene transcription), and; 3) changes in the activity of the dual $\sigma^A/\sigma^H$ spo0A promoter. Each of these effects was examined in some detail and is discussed below.

A. $\sigma^H$ and sporulation initiation.

Sporulation in B. subtilis is controlled, in part, by a cascade of RNA polymerase sigma factors whose appearance is both temporally and spatially regulated (recently reviewed in Kroos et al., 1999). The earliest-acting sigma factors in the cascade are $\sigma^H$, the protein product of the stage 0 gene spo0H (Carter and Moran, 1986; Dubnau et al., 1988; Zuber et al., 1989), and $\sigma^A$ (Errington, 1993). $\sigma^H$ is dispensable for vegetative growth, but is required
for the initiation of both competence and sporulation (reviewed in Grossman, 1995). In
addition, some genes involved in the general stress regulon (Hecker et al., 1996; Varon et al.,
1996; Gaidenko and Price, 1998; Hecker and Volker, 1998), as well as some genes involved
in the Krebs cycle (Price et al., 1989; Tatti et al., 1989; Jin and Sonenshein, 1994), the sigA
gene coding for the vegetative sigma factor, \( \sigma^A \) (Carter et al., 1988) and other genes (Jaacks
et al., 1989), require \( \sigma^H \) for normal transcriptional activity during the \( B. subtilis \) growth
cycle.

There is a poor correlation between the pattern of spoOH transcription, \( \sigma^H \) protein
levels, and the expression of \( \sigma^H \)-dependent genes in stationary phase cells grown in a
nutritive medium (Healy et al., 1991; Weir et al., 1991), suggesting that ancillary factors, i.e.,
repressors or activators, may play an important role in the activity of \( \sigma^H \) and/or the
transcriptional control of genes in the \( E\sigma^H \) regulon, in response to environmental cues
(discussed in more detail below). These factors may act on \( \sigma^H \) itself, via post-translational
mechanisms; alternately, the transcription of genes in the \( E\sigma^H \) regulon may be controlled by a
common regulator. Because of the requirement for \( \sigma^H \) in the transcription of several genes
involved in sporulation initiation, it was hypothesized that either \( \sigma^H \) activity, or the activity
of a regulator of \( \sigma^H \)-dependent promoters may be altered in the presence of the \( crsA \)
mutation. Therefore, \( \sigma^H \)-dependent transcription was examined in some detail, to gain
insight into whether \( \sigma^H \) activity or the regulation of \( \sigma^H \)-dependent promoters was altered in
GBS10 in the presence of glucose, contributing to the glucose resistant sporulation
phenotype of the mutant.
1. *spoOH* transcription and σ^H^-directed transcription vary differently in response to nutrient availability.

Many of the early observations detailing σ^H activity used the *spoVG* promoter (Healy et al., 1991; Weir et al., 1991), a sporulation gene whose expression depends on σ^H* (Carter and Moran, 1986; Zuber et al., 1989) and whose transcription is rapidly stimulated at the onset of stationary phase (Zuber and Losick, 1983; Zuber, 1985; Zuber et al., 1988). The *spoOH* and *spoVG* genes are connected via a negative regulatory loop involving the transcriptional repression of each gene by the transition state regulator AbrB, and a positive regulatory loop involving σ^H*, the product of the *spoOH* gene itself (reviewed in Grossman, 1995; Stragier and Losick, 1996).

The *spoOH* gene is transcribed from a σ^A*-specific promoter that is negatively regulated by AbrB (Weir et al., 1991). Because of the steady state levels of AbrB in vegetative cells (Perego et al., 1988), *spoOH* is transcribed at low levels until the late exponential phase of growth (Weir et al., 1991). At this time, AbrB levels begin to drop, due to inhibition of *abrB* transcription by Spo0A~P, which is phosphorylated in the phosphorelay by activated KinB and/or KinC, (Perego et al., 1988; Strauch et al., 1990; Siranosian and Grossman, 1994; LeDeaux and Grossman, 1995). Repression of *abrB* leads to derepression of *spoOH* (Weir et al., 1991; Strauch, 1995a), and ultimately to σ^H* protein accumulation.

The results shown in Figure 14 depict the expression of *abrB* in JH642 and GBS10 cells. The primary reason for determining the expression of *abrB* in GBS10 cells was that it was conceivable that the *abrB* promoter was poorly transcribed in GBS10 cells, thus resulting in the altered regulation of *spo* gene expression. As can be seen in Figure 14, *abrB* expression was not substantially different in JH642 and GBS10 and was unaffected by the
presence of glucose. Given the \( abrB-lacZ \) expression observed, there was no obvious negative impact of \( E\sigma^{A47} \) on \( abrB \) transcription as compared to that seen with \( E\sigma^A \). If it is assumed that transcriptional activity was roughly representative of protein levels, then AbrB levels in GBS10 were not substantially different from those in JH642, and therefore the negative regulation of \( spo0H \) transcription by AbrB should not be altered in GBS10.

Previous studies have demonstrated a lack of correlation between the pattern of \( spo0H \) expression and \( spoVG \) induction (Weir et al., 1991). This observation, plus those of others (Zuber et al., 1988; Price et al., 1989; Healy et al., 1991), suggest the existence of post-transcriptional mechanisms governing the activation of \( \sigma^H \). The mechanism of \( \sigma^H \) activation is discussed below, in section A.2.

Published observations show that \( \sigma^H \) is active in stationary phase cells both in the absence and (to a lesser extent) in the presence of glucose (Siranosian and Grossman, 1994; Asai et al., 1995). Given that sporulation is inhibited by the presence of glucose, this observation may seem paradoxical; however, both \( \sigma^H \) activity and the presence of glucose are required for the development of competence in stationary phase cells. The need for \( \sigma^H \) activity in competence development has been established, but its role has not yet been defined (Sadaie and Kada, 1983; Albano et al., 1987; Siranosian and Grossman, 1994).

In JH642 (\( \text{sig}A^+ \) background) grown without glucose, the \( \sigma^H \)-dependent promoter fusions [Figures 6 \( (\text{kinA}-lacZ) \), 7 \( (\text{spo0F}-lacZ) \), and 11 \( (\text{spoVG}-lacZ) \)] exhibited a typical, transient \( \sigma^H \)-dependent induction, with transcription increasing at or slightly before \( T_0 \), peaking at \( T_1 \) to \( T_{1.5} \), and decreasing thereafter. When each of these constructs was assayed in GBS10 (\( \text{crs}A \) background), the pattern of induction was slightly different, with transcription beginning to rise as much as an hour earlier, and peaking at levels similar to or
slightly higher than seen in JH642 cells. These results suggested that $\sigma^H$ was activated earlier in the GBS10 transition phase than in JH642. However, these results did not suggest that $\sigma^H$ activity was unusually increased during the early stationary phase of GBS10 cells grown without excess glucose.

The low level $\sigma^H$-dependent promoter activities in JH642 strains in glucose containing media shown in Figures 6, 7 and 11 supported the observation of others that there is some active $\sigma^H$ present in stationary phase cells in the presence of glucose (Siranosian and Grossman, 1994; Asai et al., 1995). However, in crsA mutant strains grown in the presence of glucose, the $kinA$, $spo0F$, and $spoVG$ promoter activities were greatly increased relative to in JH642, indicating that the level of $\sigma^H$-dependent promoter expression was elevated in GBS10 cells grown in the presence of glucose.

2. Possible mechanisms for $\sigma^H$ activation.

A number of observations have been made in recent years concerning the regulation of the activities of some sigma subunits in *B. subtilis*. Control of sigma factor activity has been demonstrated at many levels of expression, from transcription initiation of the sigma-encoding gene to the turnover of the sigma protein (reviewed in Haldenwang, 1995; Stragier and Losick, 1996; Helmann, 1999; Kroos et al., 1999). The complex regulation patterns remain the subject of intense investigation. Some of the mechanisms through which *B. subtilis* controls sigma factor activation are briefly outlined below.

2a. Release from anti-sigma factor complexes.

The activities of the *B. subtilis* $\sigma^F$ and $\sigma^G$ sporulation sigma factors are each governed
by partner-switching mechanisms involving the binding of an anti-sigma factor to either the corresponding sigma factor, or to an anti-anti-sigma factor (for reviews, see Haldenwang, 1995; Stragier and Losick, 1996; Helmann, 1999; Kroos et al., 1999). Prior to asymmetric septation of cells, $\sigma^F$ is held in an inactive complex, which is regulated as shown in the schematic below (adapted from Kroos et al., 1999).

$$\text{ADP} \quad \text{SpoIIAA} + \text{SpoIIAB} \cdot \sigma^F \xrightleftharpoons{\text{ATP}} \text{SpoIIAA} \cdot \text{SpoIIAB} + \sigma^F$$

$\text{SpoIIIE phosphatase}$

$$(\sigma^F \text{ inhibition}) \text{ SpoIIAA} \cdot \text{P} \quad \text{SpoIIA} \cdot \sigma^F$$

$\text{SpoIIAB kinase}$

The anti-anti-sigma factor SpoIIAA can complex with either $\sigma^F$ or the anti-sigma factor and kinase SpoIIAB, depending on the phosphorylation state of SpoIIAA (Min et al., 1993), which is affected by the presence of the phosphatase SpoIIE (Duncan et al., 1995; Feucht et al., 1996; Lewis et al., 1996). $\sigma^G$ has been shown to be held inactive as a result of binding to SpoIIAB (Kellner et al., 1996); in this instance, $\sigma^G$ activity correlates with the degradation of SpoIIAB (Lewis et al., 1996). In each case, the timing of sigma factor release from sequestration in the developing forespore is coupled to events occurring within the mother cell (reviewed in Haldenwang, 1995; Stragier and Losick, 1996; Helmann, 1999; Kroos et al., 1999). There is no evidence suggesting that $\sigma^H$ activity in $B.\ subtilis$ is, or is not modulated via sigma factor binding to an anti-sigma factor. However, $\sigma^H$ binding to an anti-sigma factor has not been demonstrated, and mutants that would indicate anti-sigma factor
activity (Schmidt et al., 1990; Margolis et al., 1993) have not been reported. As well, the anti-sigma factor SpoIIAB involved in sporulation in *B. subtilis* is encoded by a gene found in the same operon spoIIAC encoding $\sigma^E$ (Schmidt et al., 1990;; Margolis et al., 1991). spo0H is transcribed as a single gene (Weir et al., 1984; Dubnau et al., 1988).

2b. Sigma factor cleavage.

The *B. subtilis* sporulation sigma factors $\sigma^E$ and $\sigma^K$ are initially made as inactive precursors that must undergo proteolytic processing prior to becoming active; pro-$\sigma^E$ cleavage involves the removal of 27 N-terminal amino acids (LaBell et al., 1987; Miyao et al., 1993), and pro-$\sigma^K$ cleavage involves the removal of 20 N-terminal amino acids (Stragier et al., 1989; Cutting et al., 1990; Lu et al., 1990). In each case, the timing of proteolytic cleavage in the mother cell is coupled to events occurring within the developing forespore (reviewed in Haldenwang, 1995; Stragier and Losick, 1996; Kroos et al., 1999). There is no evidence that $\sigma^H$ activity in *B. subtilis* is controlled by a pro-sigma factor cleavage event, as the apparent molecular weight of $\sigma^H$ does not change in exponential and post-exponential phase cells, as demonstrated by immunoblot and immunoprecipitation experiments (Healy et al., 1991).

2c. Protein stabilization.

The products of three genes have been implicated in the post-translational control of $\sigma^H$ protein levels and therefore $\sigma^H$ activity. Cells defective in *lonA* and *lonB*, both encoding proteases induced under conditions of temperature, osmotic stress, and oxidative stress (Hecker et al., 1996) lack the ability to degrade $\sigma^H$, via proteolysis, in response to acid stress.
(Liu et al., 1999). The ClpX ATPase has been found to be required for activation of $\sigma^H$ in media with neutral pH, in response to nutritional stress (Liu et al., 1999). However, a mechanism for ClpX activation of $\sigma^H$ has not been demonstrated.

Levels of $\sigma^H$-dependent promoter expression seen in GBS10 cells in the presence and absence of glucose are not likely to arise from increased transcription from the spo0H promoter over that in JH642 cells, because the major regulator of spo0H expression, AbrB, was not affected by the presence of the crsA mutation. If transcriptional activity is used as a rough indicator of protein levels, the abrB-lacZ assay results in GBS10 (Figure 14) suggested that AbrB levels were not lower than was seen in JH642 in exponential phase growth, and therefore the spo0H promoter should have been repressed appropriately. Furthermore, even if there had been an increase in spo0H transcription during the exponential phase in GBS10, it would not be predicted to result in higher early $\sigma^H$ activity (Healy et al., 1991). This conclusion is based on an analysis of spo0H expression at $T_2$ under the control of the IPTG-inducible $P_{SPAC}$ promoter. Induction of spo0H resulted in low spoVG promoter activity during the exponential phase, but spoVG induction occurred at the onset of stationary phase, even though IPTG was added 2 hours earlier (Healy et al., 1991). Thus, the unusual $\sigma^H$ activity observed in GBS10 cells seen in this thesis was not likely to be due to transcriptional regulation of spo0H. Differences in $\sigma^H$ protein stability during exponential and post-exponential growth stages (Weir et al., 1991; Nanamiya et al., 1998), increased stability or translation of spo0H mRNA (Weir et al., 1991), and stimulation of $\sigma^H$ activity (Liu et al., 1999) have been observed to affect both the level and the timing of $\sigma^H$-dependent promoter expression, and so are good candidates for regulating $\sigma^H$ activity.
3. $\sigma^H$-dependent transcription in the crsA mutant was deregulated.

The increase in $\sigma^H$-dependent promoter activity in GBS10 cells grown in the absence of glucose began earlier in the *B. subtilis* growth cycle, but both the peak promoter activity and the time at which promoter activity decreased were similar to the timing and levels of promoter activity in JH642. There are two possible explanations for the altered pattern of expression of $\sigma^H$-dependent genes in GBS10. It is possible that the *spoOH* gene was more actively transcribed in GBS10 cells than in JH642 cells, resulting in higher $\sigma^H$ protein levels. These higher levels cannot be due to decreased AbrB repression of *spoOH*, since *abrB-lacZ* assay results (Figure 14) suggest that AbrB levels were not unusually low in the late exponential phase of growth.

The second possibility was that the $\sigma^H$ protein was more stable in GBS10 cells than in JH642 cells in late logarithmic and early stationary phases of growth. It has been demonstrated that the $\Delta clpP$ mutant (ClpP is implicated in $\sigma^H$ proteolytic degradation; Liu *et al.*, 1999) exhibits earlier expression of the *spoVG42-lacZ* fusion than *clpP*+ cells, although *spoVG42-lacZ* expression in the $\Delta clpP$ mutant was not increased beyond that seen in *clpP*+ cells (Liu *et al.*, 1999). The transcription pattern of *spoVG42-lacZ* observed in $\Delta clpP$ cells during the late exponential phase of growth was similar to the pattern of $\sigma^H$-dependent promoter-*lacZ* expression seen in GBS10 in this thesis, suggesting that a premature increase in $\sigma^H$ protein stability may be involved in the early $\sigma^H$-dependent transcription seen in GBS10 cells grown in the absence of glucose.

$\sigma^H$-dependent promoter activity in GBS10 cells grown in the presence of glucose began earlier in the growth cycle, and peak expression from these promoters in GBS10 was dramatically higher than was seen in JH642 grown in the presence or absence of glucose.
The early $\sigma^H$ activity in GBS10 grown in the presence of glucose may be a consequence of increased protein stability in exponential phase growth, as discussed above for cells grown without glucose. However, the reason for the increase in peak expression from the $\sigma^H$-dependent promoters observed in GBS10 cells grown in the presence of glucose is unclear. ClpX is implicated in $\sigma^H$ activation at neutral pH; however, although $\Delta$clpX mutants exhibit higher $\sigma^H$ protein levels in the presence of excess glucose, $\sigma^H$-dependent promoter activity was not increased (Liu et al., 1999). Lon proteases are implicated in $\sigma^H$ degradation during acid stress; however, $\Delta$lon mutants also do not exhibit dramatically high $\sigma^H$-dependent expression in the presence of excess glucose (Liu et al., 1999). There are no currently published observations of abnormal $\sigma^H$ activity with a pattern of $\sigma^H$-dependent transcription in the presence of glucose similar to that observed in this thesis. Therefore, no conclusions can be made concerning the mechanism through which the presence of $\sigma^{A47}$ resulted in increased $\sigma^H$ activity in strains grown in the presence of glucose.

4. $\sigma^H$ activation in the crsA mutant was not affected by reduction of pH.

Although $\sigma^H$ activation is reported to be controlled in the post-exponential phase in response to acid stress, the mechanism for this control is poorly understood. A recent study reported that a decrease in culture pH, because of the accumulation of acidic glycolytic by-products in a glucose-rich medium, resulted in the repression of $\sigma^H$-dependent spoVG expression in stationary phase cells (Cosby and Zuber, 1997). The reduction in $\sigma^H$-dependent gene expression was explained by the reduced intracellular concentration of $\sigma^H$, which was associated with the continued presence of AbrB well past the onset of stationary phase. Stimulation of $\sigma^H$-dependent promoter activity was observed after adjusting the
medium pH with a neutral buffer. The answer to the question of how the low pH decreased $\sigma^H$ activity was not apparent: $spo0H$ transcription patterns were only minimally affected by the presence of glucose and an extracellular low pH. It is possible that the rate of $\sigma^H$ turnover was affected, because an earlier study showed different half-lives for $\sigma^H$ of 20-30 minutes in vegetative cells and 90-130 minutes in cells after a drug-induced sporulation initiation using decoynine (Healy et al., 1991).

The pH effect on $\sigma^H$-directed transcription under the conditions used in this thesis was shown in Figure 22, using the 1.7 kb $kinA$ promoter-$lacZ$ fusion inserted in $kinA$. $kinA$ expression in the presence and absence of glucose in unbuffered media (open squares) was typical for both JH642 and GBS10 strains, exhibiting $\sigma^H$-dependent $kinA$ promoter activity despite the pH drop incurred through the metabolism of glucose (see Figures 21C and 21D). The persistence of $\sigma^H$ activity observed here despite the low pH was supported by the work of others, who have shown a negligible affect of adding low levels of glucose to culture media on the transcription of a $kinA$-$lacZ$ fusion (Asai et al., 1995). However, addition of MES buffer, pH 5.2 (closed diamonds), which prevented media pH from rising above pH 5.5, did not appear to inhibit $\sigma^H$ activity at the $kinA$ promoter in either strain, either with or without glucose. This result contradicts previously published results that showed a similar decrease in culture pH resulted in a dramatically lowered $\sigma^H$-directed transcription from the $spoVG$, $spoIIA$ and $spo0A$ P$_S$ promoters (Cosby and Zuber, 1997; Liu et al., 1999).

The buffered medium used in the experiment whose results are shown in Figure 22 maintained a pH at or less than pH 5.5 (results not shown). The amount of glucose used to supplement the medium was 0.2%. In the published experiments mentioned above, the medium pH was decreased due to the metabolism of 1% glucose and 0.1% glutamine, and
restoration of $\sigma^H$-dependent promoter activity occurred after pH adjustment of the medium with a neutral pH buffer (Cosby and Zuber, 1997; Liu et al., 1999). The difference in the setup between these experiments could have been critical with respect to the effect on apparent $\sigma^H$ activity: in a low pH, low glucose-containing medium, the effect on $\sigma^H$ activity was negligible; in a low pH, high glucose and glutamine containing medium (glucose and glutamine together have been shown to further reduce Krebs cycle activity beyond that seen in the presence of glucose alone; Cosby and Zuber, 1997), $\sigma^H$ activity was repressed (Cosby and Zuber, 1997; Liu et al., 1999).

The above observations suggest that the published pH effect on $\sigma^H$ activity may be caused by the extent of the acidic metabolite buildup in the culture medium, which would increase, to a certain extent, with the amount of glucose added. If this is true, then there is unlikely to be a “pH effect” on the activities of any of the $\sigma^H$-dependent promoters examined in the presence of glucose in this thesis, simply because the small amount of glucose added would result in less acidic by-products produced through glycolysis than was seen elsewhere (Cosby and Zuber, 1997; Liu et al., 1999). If the repressive, low pH signal was dependent on bacterial sensing of acidic glycolytic by-products, then the artificially low pH caused by the addition of a buffer may not have affected $\sigma^H$ activation. If this were true, however, then the mechanism behind the activation of $\sigma^H$ in media containing high levels of acidic glycolytic by-products via adjustment of the pH with a neutral buffer must involve bacterial sensing of a different signal then just pH.

Because of the lack of a substantial repressive effect on kinA-lacZ expression in JH642 caused by either the metabolism of 0.2% glucose (and the resultant pH drop) or by the addition of a low pH buffer, it was concluded that the activity of $\sigma^H$ in both JH642 and
GBS10 under the conditions used in this thesis was not unduly affected by a mild acid stress. Therefore, it is unlikely that the increased $\sigma^H$-dependent promoter activity observed with the $\text{kinA-lacZ}$ fusion in GBS10 in the presence of 0.2% glucose involved a $\sigma^{A47}$-mediated immunity to the effects of low pH.

5. The activity of the $\text{kinA}$ promoter.

The high level of $\text{kinA}$ expression observed in GBS10 grown in the presence of glucose (Figure 6) provided information to propose a potential mechanism for the catabolite-resistant sporulation phenotype of the $\text{crsA}$ mutant. The observed increase in the expression from both $\text{spo0F}$ and $\text{spo0A}$ promoters in GBS10 could be due to kinase activation of the phosphorelay and the buildup of Spo0A~P, and the subsequent positive feedback loop would lead to the induction of the $\text{spo0F}$ and $\text{spo0A}$ $\sigma^H$-dependent promoters via increased Spo0A~P production (Chibazakura et al., 1991; Strauch et al., 1992; Ireton et al., 1993; Hoch, 1993). This positive feedback loop would require a sufficient input of phosphate to overcome the induction of phosphorelay phosphatase genes $\text{spo0P}$ and $\text{spo0L}$ during the transition state (see Figures 12 and 13). The increase in $\text{kinA}$ expression may result in sufficient KinA levels to overcome the activity of the phosphorelay phosphatases. Because the increase in $\text{kinA}$ expression in GBS10 could not be explained by an increase in phosphorelay activity, the $\text{kinA}$ promoter was examined in more detail to reveal potential regulatory sequences involved in $\text{kinA}$ expression.

5.a. $\text{kinA}$ transcription is independent of the phosphorelay.
The *kinA* gene is transcribed from a $\sigma^H$-dependent promoter that is upregulated as cells enter the post-exponential growth phase (Antoniewski *et al.*, 1990; Predich *et al.*, 1992). Two mechanisms contribute to the increase in $\sigma^H$ activity at this time. Firstly, transcription of the *spoOH* gene (encoding $\sigma^H$) is negatively regulated by AbrB during exponential growth (Weir *et al.*, 1991). As the cell enters stationary phase, *abrB* gene transcription is repressed by increasing levels of Spo0A-P (Strauch *et al.*, 1989b; 1990; Weir *et al.*, 1991; Strauch and Hoch, 1993; Asai *et al.*, 1995). Secondly, $\sigma^H$ protein is stabilized by post-transcriptional mechanisms at the onset of stationary phase (Healy *et al.*, 1991; Nanamiya *et al.*, 1998; Liu *et al.*, 1999). There is a sufficient amount of $\sigma^H$ accumulated intracellularly immediately prior to $T_0$ to activate *kinA* transcription, even in the absence of the phosphorelay, since *B. subtilis* strains containing mutations in genes encoding phosphorelay proteins were shown to still upregulate stationary phase expression of a *kinA* promoter-*lacZ* fusion (Asai *et al.*, 1995).

5.b. *kinA* transcription varies in response to nutrient availability.

There are no known positive or negative regulatory effectors of *kinA* transcription. However, the pattern of transcription from the *kinA* promoter has been shown to vary in stationary phase cells with the medium composition. In the study published by Asai *et al.* (1995), increasing the glucose concentration from 0.1% to 1.0% resulted in a drop in *kinA* expression. When glutamine combined with high levels of glucose were added to the medium, *kinA* transcription was fully repressed.

In contrast to the nutritional repression of the *kinA* promoter, the addition of glucose and glutamine to the growth medium resulted in only a minor change in transcription from
the $\sigma^A$-dependent spo0H promoter (Frisby and Zuber, 1994; Asai et al., 1995). Based on the available data, Asai et al. (1995) proposed that since the reduction in kinA expression seen under conditions of excess glucose and glutamine was due neither to the repression of spo0H transcription, nor to a decrease in $\sigma^H$, additional factors were involved that regulate kinA expression in response to nutritional conditions. The nature of the "additional factors" were not discovered.

5.c. kinA expression was increased in the crsA mutant.

The assay of kinA-lacZ fusion activity (Figure 6) in JH642 and GBS10 cells indicated an unusually high $\sigma^H$-dependent expression in GBS10 cells in the presence of glucose. It was possible that the extent of kinA activity observed in GBS10 was, in part, due to changes in the pattern of $\sigma^H$ activity seen in GBS10 grown in the presence of glucose. However, the possibility exits that the crsA mutation altered the activity of a hypothetical regulator of kinA expression, contributing to the kinA-lacZ activity observed in Figure 6D. In Figure 6C, kinA-lacZ expression in JH642 was clearly negatively affected by the presence of glucose. Given the extent of kinA activation observed in GBS10 cells, this system was considered useful to create mutations in the kinA promoter to search for a DNA sequence required for glucose regulation.

5.d. kinA promoter analysis failed to reveal regulatory DNA sequences.

Figures 17A and 17B show the results of the analysis of JH642 and GBS10 strains containing pGBS350, a 350 bp fragment of the kinA promoter fused to lacZ and inserted in the kinA gene. The expression of this construct in GBS10 and JH642 cells was identical to
those shown in Figure 6, with a kinA-lacZ fusion containing an additional 887 bp of sequence downstream of the kinA translational start site. The results seen in Figures 17A and 17B suggested that the 887 bp of kinA gene sequence was not involved in the glucose regulation of the kinA promoter activity as observed in Figure 6.

Figures 17C and 17D show the results of the analysis of expression in JH642 and GBS10 strains containing pGS350, a 350 bp kinA promoter inserted in the amyE gene. As was mentioned in the Results section, amyE gene insertions were used to search for sequences upstream of the kinA promoter at which a regulator may act. Initially, the assumption made from the comparisons of Figures 17C and 17D with those of 17A and 17B was that a negative regulator of kinA transcription expressed in the presence of glucose was not able to bind and repress transcription from the 350 bp promoter, but could repress the wild type promoter. If the activity of the proposed regulator were altered in the presence of the crsA mutation, the change could contribute to the glucose resistant sporulation phenotype. However, further examination of the sequences upstream of the kinA promoter using a pDH32 based cloning vehicle (described in Figure 16) showed that each promoter construct assayed produced identical results to those shown in Figures 17C and 17D, with each promoter fusion expressed at much lower levels, and (for JH642) with a different pattern of expression than was seen in Figure 6 and Figures 17A and 17B (inserted in kinA).

The reason for the low expression of the kinA-lacZ fusion inserted in amyE compared to that seen when the fusion was inserted in kinA is unknown, as is the reason for the change in the pattern of expression of the kinA fusions in JH642. pDH32 was previously shown to contain a 2-bp deletion which interfered with one of three stop codons present (one in each reading frame) between the promoter cloning sites and the lacZ gene, raising the possibility
that some of the promoter-*lacZ* fusions created in this study are translational rather than transcriptional (Kraus et al., 1994). Translational effects arising from the loss of this stop codon could cause unexpected or abnormal results in affected promoter fusions. However, examination of the cloning strategies used to create the various fusions (in both pDH32 and pGBS783) indicated that only one fusion, the 2.8 kb *kinA-lacZ* promoter clone in pDH32, would be affected by the loss of the stop codon. The pattern of expression of this fusion did not differ from the 125 bp, the 350 bp, the 780 bp, or the 1.7 kb *kinA-lacZ* fusions, also created in pDH32, that were examined in this thesis, suggesting that translational effects arising from the 2-bp deletion in the 2.8 kb *kinA-lacZ* promoter clone were minor or absent. Furthermore, the loss the stop codon due to the 2-bp deletion is abrogated by the presence of another stop codon, located 14-bp downstream of the *lacZ* ribosome binding site. This downstream stop codon is located such that all three reading frames contain a stop codon, and thus all promoter fusions are transcriptional rather than translational.

If the results obtained with *lacZ* fusions inserted at *amyE* are reliable, then there are other possible explanations that may account for the discrepancies in the *kinA-lacZ* expression between the two chromosomal insertion sites. There may actually be a regulator of the *kinA* promoter that responds to nutritional signals, which interacts with DNA farther upstream than the largest promoter clone examined at *amyE* (2.8 kb). Alternatively, the *kinA* and *amyE* genes are located in quite different regions of the chromosome (*kinA* is located at 125.5° and *amyE* at 27.9° on a 360° map of the *B. subtilis* chromosome; SubtiList database), and the chromosome structure or activity in these areas may be different, as has been found in other cases (Ogasawara et al., 1983; Vold, 1985; Jarvis et al., 1988). Furthermore, expression from the two sites may be different in the presence and absence of the *crsA*
mutation. It is also possible that the differences in *kinA-lacZ* expression in GBS10 and JH642 seen in Figure 6 arose solely from differences in the activity of $\sigma^H$ in these strains, and that there is no nutritional regulator affecting *kinA* expression.

6. $\sigma^H$ activity in later stages of sporulation.

The loss of $\sigma^H$ activity in late stationary phase *B. subtilis* is controlled by the proteolytic degradation of this sigma factor, a process involving expression of the ClpC ATPase, shown to be induced as part of an operon controlled by $\sigma^A/\sigma^B$ dual promoters during heat shock and stationary phase growth (Nanamiya *et al.*, 1998). *ΔclpC* strains overproduce a repressor of *clpP* transcription (the ClpP protease is implicated in $\sigma^H$ degradation), and contain high levels of $\sigma^H$ protein as late as $T_4$ in stationary phase. Furthermore, the activity of the *spo0A* $P_5$ promoter in the *ΔclpC* mutant was found to be transcribed at levels roughly twice those seen in wild type cells after the onset of stationary phase, and continued to increase until at least $T_4$, suggesting, along with other results, that $\sigma^H$ was more stable in the mutant than in wild type cells (Nanamiya *et al.*, 1998).

The $\sigma^H$-dependent promoter activities shown in Figures 6 (*kinA-lacZ*), 7 (*spo0F-lacZ*), and 11 (*spoVG-lacZ*) in GBS10 strains grown in the presence of glucose all had a similar pattern of transcription: the levels were 2- to 4-times higher than were seen in GBS10 cells in the absence of glucose, and as much as 6-times higher than were seen in JH642 cells in the absence of glucose. Although $\sigma^H$-dependent transcription of these promoters in the presence of glucose persisted at high levels as late in stationary phase as $T_{3.5}$, the expression from these promoters began to drop from the peak activity observed by approximately $T_{1.5}$ to $T_2$, suggesting that the activity or amount of $\sigma^H$ began to decrease during this time, and that
the degradation of $\sigma^H$ was not affected in GBS10. Therefore, the $\sigma^H$-dependent gene expression observed in GBS10 cells grown in the presence of glucose was most likely not due to a loss of control over $\sigma^H$ degradation during late stationary phase.

One experiment presented in this thesis, the analysis of the $spoVG42$-lacZ fusion, contradicts the claim that $\sigma^H$ activity was not present in the late stationary phase in GBS10 cells grown in the presence of glucose (Figure 20). Unlike the pattern of expression seen in other $\sigma^H$-dependent promoters examined, when glucose was added (Figure 20B, closed diamonds) $spoVG42$ promoter expression in GBS10 mimicked that seen in JH642 during the onset of stationary phase (Figure 20A, closed diamonds) and remained relatively low until T$_{2.5}$, at which time activity rapidly increased. The expression from every other $\sigma^H$-dependent promoter examined in this thesis [including the $kinA$ promoter (lacking any known transcriptional regulator), the $spo0F$ promoter (activated by Spo0A~P), and the $spoVG$ promoter (repressed by AbrB)] decreased in GBS10 cells grown in the presence of glucose at roughly T$_{1.5}$ to T$_2$, suggesting that $\sigma^H$ activity or amount was downregulated at this time. The reason for the difference in the pattern of transcription seen with the $spoVG42$ promoter (mutated such that it is no longer negatively regulated by AbrB) in GBS10 cells grown in the presence of glucose is unknown.

B. The transition state regulator SinR and sporulation initiation.

1. SinR regulates $spo$ gene transcription.

Regulation of the $sin$ operon is described in some detail in Results, Section B.5. The $sinR$ gene is constitutively expressed from a $\sigma^A$-dependent promoter throughout exponential and post-exponential growth of $B. subtilis$ (Gaur et al., 1988). SinR inhibits the expression of
several genes that are important to the initiation of sporulation, including spo0A (Mandic-Mulec et al., 1995) and kinB (Dartois et al., 1996), as well as spoIIG and spoIIA (Mandic-Mulec et al., 1992). SinR inhibition of expression of these genes is negatively regulated by Spo0A∼P levels, which, along with active σ^H, stimulate increased transcription of the sinI gene (Strauch and Hoch, 1993). SinI antagonizes SinR activity via a protein-protein interaction that serves to sequester SinR, thus preventing SinR-mediated repression of promoter activity (Bai et al., 1993). Spo0A∼P competes with SinR binding at promoter sites (Cervin et al., 1998) and activates transcription from the spo0A, spoIIG and spoIIA genes (Satola et al., 1991; Satola et al., 1992; Bird et al., 1993, 1996; Baldus et al., 1995; Chibazakura et al., 1995; Schyns et al., 1997).

Sporulation is inhibited in the presence of glucose, in part because glucose inhibits the transcription of the sinI gene (Gaur et al., 1988). The inhibition of sinI expression in the presence of glucose is possibly due to insufficient Spo0A∼P levels to allow sinI promoter activation, but may also involve negative regulation of sinI expression by other regulators (Kallio et al., 1991). Inadequate transcription of sinI results in a sinI:sinR transcript ratio insufficient to result in full sequestration of SinR and relief from SinR transcriptional repression of sporulation genes (Bai et al., 1993).

2. sin operon expression was altered in the crsA mutant.

sinI transcription in JH642 was induced during the transition state and was inhibited by glucose (Figure 15A). sinR-lacZ expression showed no difference in the presence and absence of glucose (Figure 15C). Therefore, in JH642, in the absence of glucose, sinI transcription yielded a high sinI:sinR transcript ratio that was conducive to sporulation,
whereas in the presence of glucose, sinI transcription was lower, presumably resulting in the persistence of free SinR at the onset of stationary phase and repression of sporulation.

Expression of both sinI-lacZ and sinR-lacZ in the absence of glucose was decreased in GBS10 relative to that seen in wild type cells (Figures 15B and 15D). The pattern of expression was similar to that reported elsewhere (Louie et al., 1992). However, the ratio of the levels of transcription of sinI and sinR remained roughly equal to that observed in JH642, suggesting that the sinI:sinR transcript ratio in GBS10 cells grown in the absence of glucose was roughly equivalent to that seen in JH642. If the logic is sound, free SinR would be normally regulated during sporulation. The reason for the decrease in the transcription levels seen in Figures 15B and 15D are unknown.

Expression of the σH-dependent sinI-lacZ fusion in GBS10 in the presence of glucose (Figure 15B) paralleled that observed for the other σH-dependent promoters examined in this thesis, supporting the hypothesis presented above that σH is activated in stationary phase GBS10 despite the presence of glucose. Expression of the sinR-lacZ fusion (Figure 15D) also increased in GBS10 cells grown in the presence of glucose. What was startling about the sinI- and sinR-lacZ expression seen in GBS10 cells in the presence of glucose was that the ratio of the levels of promoter transcription of sinI:sinR at T₀ appeared to be roughly 4.5-times greater than that seen in JH642 in the presence of glucose. The increase in the ratio of sinI:sinR transcription levels observed in GBS10 cells grown in the presence of glucose presumably would lead to a level of SinI protein levels that would reduce the level of free SinR. If this were the case, it would increase the sporulation efficiency of GBS10 cells in the presence of glucose.
Table 6 summarizes the transcriptional activities of the promoters of the sin operon in JH642 and GBS10 strains at various times as measured by sinL:sinR transcriptional activity. For the following analysis of Table 6, two assumptions were made: firstly, that the ratio of sinL:sinR expression is directly related to the ratio of SinL:SinR protein levels present in the cell; secondly, that the smallest sinL:sinR expression ratio obtained in JH642 grown in the absence of glucose (20:1) represents the minimum ratio of SinL:SinR protein levels required to sequester SinR and permit sporulation initiation. Given these assumptions, three conclusions were made: 1) the SinL:SinR ratio prior to and during stationary phase in GBS10 cells grown in the absence of glucose would permit sporulation; 2) the SinL:SinR ratio prior to the onset of stationary phase in GBS10 cells grown in the presence of glucose would inhibit sporulation; and 3) the SinL:SinR ratio during stationary phase in GBS10 cells grown in the presence of glucose was sufficient to inhibit sporulation.

The proposed alteration in SinR regulation of sporulation in GBS10 cells and its affect on sporulation in excess glucose is supported by the sporulation frequency of the ΔsinR mutant (Table 5), which sporulated 25000-times better in the presence of excess glucose than the sinR^+ strain. The sporulation frequency of the ΔsinR mutant, combined with the observations made from the expression of the sin operon in GBS10, suggests that SinR regulation of sporulation is important for CR of sporulation.

3. The expression of SinR-regulated spo genes was altered in the crsA mutant.

SinR is involved in the negative regulation of three spo genes examined in this thesis: spo0A (Mandic-Mulec et al., 1995), spoIIIG and spoIIA (Mandic-Mulec et al., 1992). It has been suggested that SinR interferes with spoIIA and spoIIIG transcription by binding in the
Table 6. Relative transcriptional activities of the promoters of the *sin* operon in *B. subtilis*.

<table>
<thead>
<tr>
<th>time (h) relative to the onset of stationary phase, ( T_0 )</th>
<th>ratio(^a) of observed <em>sinI</em>:<em>sinR</em> transcription levels</th>
<th>( \text{JH642 SSM}^b )</th>
<th>( \text{SSM}^c )</th>
<th>( \text{GBS10 SSM} )</th>
<th>( \text{SSMG}^c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T_{-1} )</td>
<td>50:1</td>
<td>10:1</td>
<td>20:1</td>
<td>11:1</td>
<td></td>
</tr>
<tr>
<td>( T_0 )</td>
<td>35:1</td>
<td>5:1</td>
<td>40:1</td>
<td>22:1</td>
<td></td>
</tr>
<tr>
<td>( T_1 )</td>
<td>20:1</td>
<td>8:1</td>
<td>40:1</td>
<td>26:1</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) ratios were calculated using the data in Figure 15
\(^b\) SSM - Schaeffer's spore medium, pH 7.5
\(^c\) SSMG - Schaeffer's spore medium + 0.2% glucose
vicinity of 0A boxes upstream of the −35 sites, thus preventing Spo0A−P activation of these promoters (Mandic-Mulec et al., 1992; Cervin et al., 1998). It has been shown that SinR binds to the spoIIA promoter in the region bound by −110 to −30 (relative to the transcription start site; Mandic-Mulec et al., 1992), which contains five 0A boxes and is required for in vivo spoIIA transcriptional activity (Trach et al., 1991; Wu et al., 1991; Baldus et al., 1995). A more recent study suggested that SinR inhibits transcription from the spoIIG promoter either by competing with Spo0A−P binding to upstream 0A boxes, or by distorting promoter DNA such that bound Spo0A−P is prevented from interacting with RNA polymerase (Cervin et al., 1998). Transcriptional regulation of the spo0A gene will be discussed in more detail in section C, below.

In contrast to JH642, the expression of the spoIIG and spoIIA promoters in GBS10 cells were transcribed prior to T₀ in the absence of glucose (Figures 9 and 10), suggesting three things about the GBS10 sporulation pathway: 1) SinR negative regulation was less pronounced at these promoters prior to the onset of stationary phase; 2) there was enough Spo0A−P present to activate transcription from these promoters prior to the onset of stationary phase; and 3) there was adequate active σ^{H} present to drive spoIIA transcription prior to the onset of stationary phase. In a previously published study, the transcription patterns of the spoIIA and spoIIG promoters were found to be altered in a ΔsinR strain during post-exponential phase growth, although the patterns were different from those observed in this thesis (Mandic-Mulec et al., 1992). In the ΔsinR mutant, spoIIG transcription in the absence of glucose was found to be roughly 4-times that seen in sinR⁺ cells, whereas spoIIA transcription was only slightly increased in the absence of SinR. The timing of expression of
both promoters was moderately affected, with promoter activity increasing roughly one half hour earlier than that seen in \(\text{sinR}^+\) cells.

The patterns of the \(\text{spoIIG}\) and \(\text{spoIIA}\) promoter expression in GBS10 seen in the presence of glucose were somewhat different than that seen in the absence of glucose (Figures 9 and 10). In the presence of glucose, activation of these two promoters was repressed until the onset of stationary phase, a timing that paralleled the dramatic increase in \(\text{sinI}\) transcription observed in GBS10 cells grown in the presence of glucose (Figure 15), and with the \(\text{sinI}:\text{sinR}\) ratios shown in Table 6.

The expression of \(\text{sinI}-\text{lacZ}\) and \(\text{sinR}-\text{lacZ}\) fusions in GBS10 grown in the presence of glucose, combined with the activity of both \(\text{spoIIG}\) and \(\text{spoIIA}\) promoters, suggest that the presence of the \(\text{crsA}\) mutation resulted in the inappropriate relief from SinR transcriptional repression of these sporulation promoters. SinR regulation of \(\text{spo}\) gene transcription has been identified as a checkpoint in the control of sporulation initiation (Mandic-Mulec \(\text{et al.},\ 1992;\ 1995\) that appears to be bypassed in GBS10 cells grown with and without glucose.

C. The activity of the \(\text{spo0A}\) promoter.

1. \(\text{spo0A}\) transcription is regulated by nutrient availability.

A current model for the transcription from the dual \(\sigma^A/\sigma^H\) \(\text{spo0A}\) promoters throughout the \(\text{B. subtilis}\) growth cycle is as follows (see also Chibazakura \(\text{et al.},\ 1991;\ Strauch \(\text{et al.},\ 1992;\ Hoch, 1993;\ Ireton \(\text{et al.},\ 1993\): 1) during exponential growth, the \(\sigma^A\)-dependent \(\text{spo0A}\) promoter is transcribed at low levels, to provide a minimal level of Spo0A to be available for sporulation initiation sensing; 2) as cells begin to deplete available nutrients, metabolic and other signals trigger the activation of one or more protein kinases,
such as KinA, and inactivate phosphatases, so the phosphorelay protein Spo0F becomes phosphorylated, ultimately leading to phosphorylation of the available Spo0A; 3) Spo0A\textasciitilde P binds with high affinity to DNA target sites (0A boxes); 4) Spo0A\textasciitilde P represses transcription initiation from the *abrB* promoter by binding to downstream 0A boxes, and the resultant decrease in AbrB levels allows derepression of the *spoOH* gene, causing an increase in \( \sigma^H \) protein production; 5) Spo0A\textasciitilde P and \( \sigma^H \) together induce the activity of the *sinI* \( \sigma^H \)-dependent promoter, resulting in increased SinI production, sequestration of SinR, and derepression of the \( \sigma^H \)-dependent *spo0A* promoter located downstream of the *spo0A* \( \sigma^A \)-dependent promoter; 6) binding of Spo0A\textasciitilde P at the 0A boxes upstream of the *spo0A* \( \sigma^H \)-dependent promoter is required for the activation of the \( \varepsilon \sigma^H \) transcription of *spo0A*, and results in amplification of Spo0A production; and 7) during this time, Spo0A\textasciitilde P and \( \sigma^H \) also activates transcription from the *spo0F* promoter, increasing phosphorelay components that in conjunction with activated sporulation kinases increase the overall phosphorylation of Spo0A, creating a positive feedback loop leading to increased Spo0A production and phosphorylation.

The repressive effect of glucose on the expression from the dual \( \sigma^A/\sigma^H \) *spo0A* promoter has been associated with a lack of the \( \sigma^A \) (vegetative promoter, or \( P_v \)) to \( \sigma^H \) (sporulation promoter, or \( P_S \)) promoter switch described above (Chibazakura *et al.*, 1991). This promoter switch has been proposed to be prevented by the continued repression of the \( P_S \) promoter by SinR (Mandic-Mulec *et al.*, 1995), through glucose repression of the \( \sigma^H \)-dependent *sinI* promoter (Gaur *et al.*, 1988), which prevents stationary phase accumulation of SinI and sequestration of SinR via SinI:SinR interaction (Bai *et al.*, 1993; Lewis *et al.*, 1998).

2. The *spo0A* promoter switch was deregulated in the *crsA* mutant.
In JH642 cells grown in the presence and absence of glucose, glucose repression of spo0A P_s transcription was indicated by the failure of spo0A-lacZ expression to increase past the onset of stationary phase: instead, β-galactosidase activities gradually dropped, possibly as a consequence of Spo0A~P-mediated inhibition of P_v promoter expression (Figure 8A, Strauch et al., 1992). In the absence of glucose, spo0A-lacZ expression continued to rise after the onset of stationary phase, presumably because of derepression of the P_s promoter.

The pattern of spo0A-lacZ expression observed in GBS10 was different from that of JH642 (Figure 8). In the absence of glucose, spo0A expression in GBS10 began to rise earlier than was seen in JH642, and peaked at higher levels. It is possible that the increased expression of spo0A-lacZ seen in GBS10 cells was due to an early promoter switch, that occurred in combination with the early σ^{H} activation or stabilization (section A), and/or the absence of SinR inhibition suggested in section B, above. However, the pattern of spo0A transcription observed in GBS10 cells grown in the presence of glucose suggested that an early promoter switch was not the cause of the observed high spo0A-lacZ expression, since the transcriptional activity of the spo0A promoters prior to the onset of stationary phase was identical in the presence and absence of glucose. Analysis of the sin operon expression (above) suggested that free SinR should be present in GBS10 cells grown in the presence of glucose until T_0. If so, this free SinR would repress transcription from the P_s prior to the onset of stationary phase, so the overall activity would be independent of the P_s promoter. Therefore, activity of the spo0A promoter prior to the onset of stationary phase in the presence of glucose, and by extension without glucose, was due to Eσ^{A47}-dependent transcription from the P_v promoter. In the presence of glucose, relief from SinR repression in GBS10 cells at T_0 would allow the promoter switch to occur at this time.
The \textit{spo0A} promoter switch was analyzed in a \textit{crsA} mutant by the use of an S1 nuclease protection assay (Chibazakura \textit{et al.}, 1991). mRNA from the \textit{P_S} promoter was shown to appear in wild type cells at roughly \textit{T_1}, and was absent in cells grown in 2% glucose. In \textit{crsA} mutant cells mRNA from the \textit{P_S} promoter appeared regardless of glucose supplementation. The finding (discussed above) that SinR regulation of \textit{spo} genes was altered in GBS10 cells, leads to the possibility that the abnormal \textit{spo0A} promoter switch observed by Chibazakura \textit{et al.} (1991) in \textit{crsA} mutant cells in the presence of glucose occurred because of a decrease in or a lack of SinR repression of \textit{P_S}. Similarly, the possibility that glucose repression of the \textit{sinl} promoter resulted from low \textit{\sigma^H} activity and Spo0A-P levels indicated that the promoter switch observed in GBS10 in the presence of glucose may have been due to increased \textit{spo0A} transcription from the \textit{P_V} promoter by Ec\textsuperscript{A47} (raising the level of Spo0A), and abnormal \textit{\sigma^H} activity (raising the level of phosphorelay components).

The effect of Ec\textsuperscript{A47} on \textit{spo0A} \textit{P_V} expression was initially addressed by an analysis of \textit{spo0A-lacZ} activity in \textit{\Delta spo0H} strains (Figure 23). Deletion of the \textit{spo0H} gene has the potential to affect \textit{spo0A-lacZ} expression in three ways: the loss of \textit{\sigma^H}-dependent \textit{sinl} expression; the loss of phosphorylation of Spo0F by KinB, through the repression of \textit{kinB} transcription by SinR (Dartois \textit{et al.}, 1996); and the loss of \textit{\sigma^H}-dependent \textit{kinA} and \textit{spo0F} transcription. In JH642 \textit{\Delta spo0H}, \textit{spo0A} \textit{P_V} promoter activity was observed at low but constant levels throughout the growth cycle in the absence of glucose. Somewhat surprisingly, \textit{spo0A} \textit{P_V} promoter activity in JH642 \textit{\Delta spo0H} cells grown in the presence of glucose was slightly increased during late exponential phase growth; the reason for this is unknown, but a glucose-associated increase in the activity of the \textit{spo0A} \textit{P_V} promoter was
noted by Chibazakura et al. (1991). In the absence of glucose, GBS10 Δspo0H cells generated a higher level of spo0A P_v promoter activity prior to the onset of stationary phase than was seen in JH642 Δspo0H cells. This result also suggests that Eσ_A47 transcribed the spo0A P_v promoter better than did Eσ_A. The reason for the decrease in the expression from the spo0A P_v promoter after T_0 is unknown, but it is possible that Spo0A~P generated from phosphorylation by KinC in the phosphorelay (whose transcription should be unaffected by the Δspo0H mutation: LeDeaux and Grossman, 1995; LeDeaux et al., 1995) was sufficient to repress P_v transcription via Spo0A~P binding at downstream 0A boxes. The same repression of the spo0A P_v promoter may have occurred in JH642 Δspo0H cells, but was not obvious because of the low transcriptional activity.

In the presence of glucose, GBS10 Δspo0H cells generated a higher level of spo0A P_v promoter expression prior to the onset of stationary phase than was seen in these cells in the absence of glucose. The reason for this is unknown, but may involve the same glucose-associated increase in P_v promoter activity noted above for JH642 Δspo0H cells. The observed decrease in transcription may be due to repression of P_v transcription by Spo0A~P, as was also suggested above.

P_v promoter expression in spo0H^ strains was examined using a spo0AΔPs promoter mutant, as another approach to examine RNA polymerase transcriptional activity (Figure 25). The deletion that created the spo0AΔPs promoter mutant removed both the Ps promoter and the SinR binding site (Mandic-Mulec et al., 1995). Two of the three 0A boxes, implicated in P_v promoter repression, remained. In both JH642 and GBS10 cells grown in the absence of glucose, transcription from the spo0AΔPs promoter mutant during exponential phase growth was constant, and decreased in late-exponential and post-exponential phase growth, possibly
due to repression of the P_v promoter by the accumulation of Spo0A-P. The spo0AΔP_s-lacZ expression patterns in JH642 and GBS10 cells grown in excess glucose were similar to each other, but entirely unexpected. In both strains, the addition of glucose resulted in increased transcription from the spo0AΔP_s-lacZ promoter over that seen without glucose, with expression maintained until well into stationary phase. The molecular mechanism for the increase in spo0AΔP_s-lacZ expression is unknown. It is possible that the increased promoter activity observed with the addition of glucose was due to the expression of the spo0P and spo0L genes encoding the Spo0F phosphatases observed in the presence of glucose (Figures 12 and 13). Increased phosphatase activity would result in decreased Spo0A-P by the reversal of the phosphorelay, alleviating the repression of the spo0A P_v promoter by Spo0A-P as observed by others (Strauch et al., 1992).

3. Eσ^A_47 transcribes the spo0A σ^A-dependent promoter more efficiently than Eσ^A.

The spo0A-lacZ expression patterns in GBS10 suggested that Eσ^A_47 activity at the P_v promoter may be higher than that of Eσ^A. Therefore, the *in vitro* transcription from the spo0A P_v promoter by these RNA polymerases was examined.

Chibazakura *et al.* (1991) proposed two overlapping sets of putative promoter sequences for the spo0A P_v promoter, shown below by lines drawn above and below the promoter sequence.

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<table>
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<tr>
<th>&quot;-35&quot;</th>
<th>&quot;-10&quot;</th>
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<td>TAtaaT</td>
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\[
\text{CCCTTTCACTTTCAGAATACATACGGTAAATATACAAAAGAAGAT}
\]
Each promoter shares four of six bases in the –35 region and four or five of six bases in the –10 region with the consensus sequence (at top, with the most highly conserved bases in upper case and the more weakly conserved bases in lower case), and contains a 17 bp spacing that is optimal for σA promoters (Haldenwang, 1995). A conserved TG dinucleotide commonly found two bases upstream of the 5' end of the –10 promoter sequence in B. subtilis σA promoter sequences, known to be important for transcription from some weak promoters, is absent from both proposed spo0A σA promoter sequences (Voskull et al., 1995; Helmann, 1995). It is not known which of the putative promoter sequences functions in vivo. The transcription start site of the Pv promoter is indicated by an arrow. The distances between the putative –10 promoter sequences and the Pv promoter transcription start site is 5 bp (top) and 10 bp (bottom). The average distance between the 3' end of the –10 promoter sequence and the transcription start site is 7 bp for B. subtilis σA-dependent promoters, but distances of 5 and 10 bp have been shown to function in a small number of promoters (Helmann, 1995). The spo0A Pv promoter forms unstable complexes with RNA polymerase when challenged in vitro with heparin prior to the addition of nucleotides ATP and GTP (G. B. Spiegelman, unpublished results), and is considered to be a weakly active promoter in vivo (Chibazakura et al., 1991). Given the sequences and spacings of these putative promoter sites, which are not extremely divergent from the σA consensus promoter, the reason for the weak activity of EσA on this promoter is not obvious. It is interesting to note that the proposed promoter sequence shown at the bottom has greater homology to the highly conserved residues of the consensus promoter sequence than the one shown at the top. The distance between the 3' end of this proposed –10 promoter sequence and the transcription
The crsA mutation results in an amino acid change from proline to phenylalanine (Kawamura et al., 1985), which is located between conserved regions 3 and 4 (Helmann and Chamberlin, 1988). Because of its molecular structure, a proline residue restricts the mobility of a peptide chain, and often plays an important role in protein architecture (Stryer, 1988). Proline residues are cyclic, and because the reactive nitrogen of this amino acid is contained within the ring structure, the presence of a proline residue results in a relatively inflexible bend within the peptide chain (Stryer, 1988). The proline to phenylalanine change in $\sigma^{A47}$ is interesting, as both $\sigma^D$ and E. coli $\sigma^A$ also have proline residues at the same relative position, suggesting that this position may be important for the overall structure of some sigma factors (Helmann and Chamberlin, 1988).

The effects of the crsA mutation on the activity of Eo$\sigma^{A47}$ at different promoters have not been characterized. Because the crsA mutation may affect the structure of this sigma factor, it is possible that the orientation of or the distance between $\sigma^A$ conserved regions 2 (shown to directly contact the $-10$ promoter sequence, and be involved in promoter melting; Helmann and Chamberlin, 1988) and 4 (shown to directly contact the $-35$ promoter sequence; Helmann and Chamberlin, 1988) may be altered, and thus may affect either promoter recognition by the sigma factor, or affect the kinetics of open complex formation (reviewed in Whipple and Sonenshein, 1992; deHaseth and Helmann, 1995; Helmann and deHaseth, 1999).

Comparative analysis of the transcriptional activities of Eo$\sigma^A$ and Eo$\sigma^{A47}$ on a standard template, the $\phi29$ A2 promoter versus the spo0A promoter, by measuring the effects of both
template and enzyme inputs on transcript production, suggested that Eσ^{A47} did in fact transcribe the spo0A P_v promoter more efficiently than Eσ^A, by a factor of approximately 2. The in vitro demonstration of higher activity of Eσ^{A47} at the P_v promoter than was seen for Eσ^A supports the in vivo observations described above concerning the patterns of spo0A-lacZ expression seen in GBS10 cells +/- σ^H prior to the onset of stationary phase.

The increase in transcriptional activity of the spo0A P_v promoter caused by the crsA mutation in σ^A may be important in the ability of GBS10 to sporulate in the presence of glucose. This hypothesis was examined by the removal of the chromosomal spo0A P_s promoter in both JH642 and GBS10, and determination of the sporulation frequencies of the resultant strains in media with and without glucose (Table 5). The spo0AΔP_s promoter mutant in JH642 cells sporulated at low levels, as has been seen by others (Chibazakura et al., 1991; Siranosian and Grossman, 1994). The sporulation defect can be attributed to the prevention of full σ^H activation and persistence of SinR negative regulation of spoIIA and spoIIG promoter activity due to low levels of Spo0A production. However, the sporulation frequency of the GBS10 spo0AΔP_s promoter mutant was several thousand-fold higher than that seen in JH642 spo0AΔP_s cells, both in the presence and absence of glucose. The sporulation frequency seen in GBS10 spo0AΔP_s cells strongly suggests that Eσ^{A47} transcription of the spo0A P_v promoter in these cells, which was accompanied by σ^H activation and expression of spoIIG and spoIIA, was sufficient to allow sporulation, regardless of the presence of glucose.

D. Sporulation initiation in the crsA mutant.

The data presented in this thesis indicate that three elements in the regulatory network
that controls sporulation initiation are altered as a result of the crsA mutation, and these changes are involved with the glucose resistant sporulation phenotype associated with this mutation. These three elements are described below.

1. $\sigma^H$ activation.

The patterns of transcription of $\sigma^H$-dependent genes in cells grown in the presence and absence of glucose indicated that the regulation of $\sigma^H$ activity was altered in crsA mutant cells. In GBS10 grown in the presence of glucose, inappropriately high $\sigma^H$ activity was suggested by increased expression from $\sigma^H$-dependent promoter-$\text{lacZ}$ fusions. The level of $\text{abrB-lacZ}$ expression in GBS10 suggested that increased transcription of the primary transcriptional regulator of spoOH, AbrB, was not involved in the observed stationary phase increase in $\sigma^H$ activity. Rather, this increase in $\sigma^H$ activity is thought to be due to altered post-translational regulation of $\sigma^H$ protein, which would occur as a result of changes in $\sigma^{A47}$-dependent gene expression. Because the mechanisms involved in the activation of $\sigma^H$ are poorly understood, it is unknown whether $\sigma^{A47}$ regulation of $\sigma^H$ activity was direct, or indirect.

2. $\text{spo0A}$ transcription.

The presence of the crsA mutation had a direct effect on the expression of the $\text{spo0A}$ gene. A higher level of exponential phase transcription from the $\text{spo0A P}_V$ promoter was suggested from the analysis of $\text{spo0A-lacZ}$ expression patterns in vivo, both in the presence and absence of an intact $\text{spo0H}$ gene. The in vitro demonstration of a higher affinity of Ec$^{A47}$ for the $\text{spo0A P}_V$ promoter than that seen with the wild type enzyme
confirmed that the \textit{crsA} mutation resulted in increased expression of \textit{spo0A} prior to the onset of stationary phase. Furthermore, the \textit{spo0AΔPs} deletion in GBS10 sporulated at high levels, indicating that the increased transcription of \textit{spo0A} from the \textit{Pv} promoter in GBS10 compensated for the lack of transcription from the \textit{Ps} promoter, resulting in the attainment of threshold levels of Spo0A without the promoter switch. The observed sporulation efficiency of the \textit{crsA, spo0AΔPs} double mutant contrasted with the \textit{spo0AΔPs} deletion in \textit{sigA}+ cells, which showed a severe sporulation deficiency under all conditions tested. An increased \textit{in vivo} transcriptional efficiency of \textit{Ec}A47 would result in a higher basal level of Spo0A during the exponential phase of growth than would occur in wild type cells. An increase in Spo0A levels, combined with an inappropriate increase in \textit{σH} activity (discussed above), would trigger activation of the phosphorelay (see Figure 3). At the onset of stationary phase, inappropriate activation and/or stabilization of \textit{σH} would result in a high level of transcription of \textit{kinA} and \textit{spo0F}, and the resultant increase in phosphorelay components would increase the levels of phosphorylated Spo0A, despite \textit{spo0L} and \textit{spo0P} expression. Spo0A~P would repress \textit{abrB} transcription, alleviating the transcriptional repression of \textit{spo0H} and resulting in increased \textit{σH} protein production. These events would trigger the \textit{spo0A} promoter switch, identified as a checkpoint in the control of sporulation initiation that is sensitive to the presence of glucose in wild type cells (Chibazakura \textit{et al.}, 1991), and would result in further accumulation of Spo0A~P in GBS10, despite the presence of glucose.

Because of the accumulation of Spo0A~P and active $\sigma^H$, the activity of $\varepsilon \sigma^{47}$ produced a third, indirect effect on the regulation of sporulation. The alteration in the patterns of transcription of the sin operon described for GBS10 (resulting in part from stimulation of the sinI $\sigma^H$-dependent promoter by Spo0A~P) suggested that SinR repression of spo gene expression was reduced or absent in GBS10 at $T_0$ in the presence of glucose. The ratio of sinI:sinR suggested that the crsA mutation resulted in a lowering of the level of SinR to that seen in stationary phase JH642 grown without glucose. The high frequency of sporulation of the JH642 ΔsinR mutant in the presence of glucose supported the idea that SinR regulation was involved in the repression of sporulation by glucose. Given that $\sigma^H$ has been shown to be somewhat active in stationary phase cells grown in the presence of glucose, SinR repression of the $\sigma^H$-dependent promoters of both spo0A and spoIIA would constitute a critical point in preventing sporulation in the presence of glucose. The proposed loss of SinR repression of these promoters in the crsA mutant, coupled with inappropriately high $\sigma^H$ activity and increased levels of Spo0A~P, would result in the activation of the spo0A promoter switch and the expression of stage II genes spoIIA and spoIIG, ultimately resulting in spore formation, despite the presence of glucose.

The scenario presented in 1-3 above is a reasonable model for how the crsA mutation leads to glucose resistant sporulation. This scenario is also depicted in the schematic shown in Figure 34. There are, however, two unresolved major regulatory changes highlighted by my examination of the effects of the crsA mutation on sporulation. Firstly, the mechanism of glucose repression of sinI induction is thought to involve inhibition of both $\sigma^H$ activation and Spo0A~P accumulation, but this has not been addressed directly, here or elsewhere. Other
Figure 34. The effects of the crsA mutation on the sporulation initiation pathway. The accumulation and phosphorylation of Spo0A–P in the presence of glucose is shown at center. At the top (grey), EσA in wild type cells interacts with promoters to result in low levels of phosphorelay proteins (Spo0F and Spo0B, not shown), including Spo0A. Minimal Spo0A–P accumulation, coupled with both persistence of SinR negative regulation, and no σH activation prevents sporulation from initiating. At the bottom (black), EσA47 in crsA mutant cells interacts with the spo0A Pν promoter more efficiently than is seen in wild type cells, resulting in increased Spo0A production. EσA47 also results in increased σH activity, through an unknown mechanism. High σH activity results in increased kinase production (not shown), resulting in increased Spo0A phosphorylation. High σH activity and increased Spo0A–P activates the transcription of promoters of phosphorelay genes, resulting in high levels of Spo0A phosphorylation. High Spo0A–P levels and high σH activity result in increased SinI production and removal of SinR negative regulation of spoIIG, spoIIA, and spo0A promoters. The removal of SinR negative regulation, accompanied by active σH and high levels of Spo0A–P result in the activation of transcription from the spo0A P5 promoter, and spoIIG and spoIIA promoters, ultimately resulting in spore formation. Arrows represent either protein production arising from transcriptional activity of the RNA polymerase, protein activation, or protein phosphorylation. Solid arrows represent normal activity, and bold arrows represent a level of activity greater than what is seen in wild type cells. Solid lines represent negative regulatory effects. Circled question marks indicate unknown regulatory effects.
regulatory mechanisms are thought to affect sin operon expression (Kallio et al., 1991; Strauch and Hoch, 1993), and it is unknown how these mechanisms affect sin expression, or whether they could be affected by the activity of EaA47. Secondly, the regulatory events surrounding the activation and stabilization of $\sigma^H$ during stationary phase remain poorly understood. It is possible that changes in Spo0A and/or SinR levels, caused by the presence of the crsA mutation, resulted in the alteration of $\sigma^H$ activity seen in GBS10 cells. It is also possible that the presence of the crsA mutation affected $\sigma^H$ protein activity more directly, via altered transcription of genes involved in the post-translational control of $\sigma^H$.

There are a variety of experiments remaining to be done to further elucidate the effects of the crsA mutation on sporulation initiation. These experiments can be grouped into three categories, reflecting the three known effects of the crsA mutation discussed above. Experiments to be done include:

1. $\sigma^H$ protein levels and $\sigma^H$ activity. What is the mechanism through which EaA47 results in increased $\sigma^H$-dependent transcription despite the presence of glucose?
   - the in vivo examination of increased $\sigma^H$ protein levels using multicopy spo0H, and the potential of higher $\sigma^H$ protein levels to effect the sporulation efficiency of wild type cells in the presence of glucose.
   - the in vivo examination of the regulatory impact of EaA47 on $\sigma^H$ activation, by the analysis of suppressor mutations that result in a decrease in $\sigma^H$-dependent promoter-lacZ expression in complex media, and a loss of the crs phenotype.

2. spo0A promoter transcription. How is the activity of RNA polymerase altered by the proline to phenylalanine mutation?
- the *in vitro* examination of the interaction of $\sigma^A_{47}$ versus $\sigma^A$ on the spo0A $P_{\gamma}$ promoter, including transcription rate assays, electrophoretic mobility shift assays, and DNA footprint analysis.

- the *in vivo* and *in vitro* examination of the effects of targeted mutagenesis of the spo0A $P_{\gamma}$ promoter on transcriptional efficiency of the wild type and mutant enzymes, and sporulation efficiency of JH642 and GBS10.

3. *sin* operon regulation, SinR regulation of transcription, and SinR regulation of $\sigma^H$ activity. What is the mechanism of glucose repression of *sinI*?

- the *in vivo* examination of the *sinI* promoters in JH642 and GBS10 to determine the effects of known regulators (such as AbrB and Hpr, both shown to interact with the *sinI* promoter region) on *sinI* transcription in the presence of glucose.

- the *in vivo* examination of *sinR* multicopy and the $\Delta sinI$ mutation on the sporulation efficiency of GBS10 in the presence of glucose.
References


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