THE EFFECTS OF THE *crsA* MUTATION IN THE MAJOR VEGETATIVE SIGMA FACTOR σ^A ON THE REGULATION OF SPORULATION INITIATION IN *BACILLUS SUBTILIS*

by

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B.Sc., University of Alberta, 1991
M.Sc., University of Alberta, 1994

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

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Department of Microbiology and Immunology

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 2000

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Date October 11, 2000

Abstract

The *crsA* mutation is located within the gene for the major vegetative sigma factor of *Bacillus subtilis*, σ^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 *crsA* mutation on sporulation gene expression were examined.

The *in vivo* promoter activity of genes involved in the initial stages of sporulation was examined in the *crsA* mutant using promoter-*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 σ^A. The activity of genes encoding phosphorelay proteins was altered, suggesting the inappropriate activity of the sporulation sigma factor, σ^H; as well, both the expression of the operon encoding the transition state regulator SinR, and the expression of *spo* genes negatively regulated by SinR, were altered.

Analysis of *spo0A* promoter expression suggested that transcription from the vegetative promoter of *spo0A* was increased in *crsA* mutant strains. Analysis of both the expression from and the sporulation frequency of a *spo0A* promoter mutant supported this observation, and implicated altered *spo0A* expression in the glucose resistant sporulation phenotype of the *crsA* mutant. Comparative *in vitro* transcription assays were performed using wild type and *crsA* mutant RNA polymerases, providing evidence that transcription from the σ^A dependent *spo0A* promoter by the *crsA* mutant RNA polymerase was increased over that seen with the wild type enzyme.

The data presented herein suggested that the alteration of *spo0A* gene expression was a direct effect of the *crsA* mutation in σ^A. This increase in *spo0A* expression, combined with inappropriate σ^H activity and altered *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 σ^H activity are important in the mechanism of glucose inhibition of sporulation.

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Abbreviations and Symbols

0A box	nucleotide sequence at which Spo0A~P binds
abrB	gene encoding transition state regulator AbrB, a negative regulator of sporulation
alsA	allele of *ccpA*
Am^r	ampicillin resistance phenotype
amp	ampicillin resistance gene
BGSC	*Bacillus* Genetic Stock Center
bp	base pair
CAC	citric acid cycle
cAMP	cyclic adenosine monophosphate
cat	chloramphenicol resistance gene
ccp	genes *ccpA, ccpB,* and *ccpC*, genes encoding catabolite control proteins CcpA, CcpB, and CcpC
clp	*clpA, clpX, clpC* and *clpP*, genes encoding stress induced chaparonins/ ATPases (ClpA and ClpX) and proteases (ClpC and ClpP)
Cm^r	chloramphenicol resistance phenotype
CR	catabolite repression
cre	catabolite responsive element
CRP/CAP	catabolite repressor protein/cAMP activated protein
crs	catabolite resistant sporulation mutants; *crsA* allele resides in *sigA*
CsCl	cesium chloride
Δ	gene knockout
E	RNA polymerase core enzyme
EDTA	ethylenediamine tetraacetic acid
FDP	fructose-1,6-diphosphate
HCl	hydrochloric acid
Hepes	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid

hpr	gene encoding transition state regulator Hpr, a negative regulator of sporulation
HPr	PTS protein phosphorylated by enzyme I
IPTG	isopropyl-thio-β-D-galactoside
kan	kanamycin resistance gene
kb	kilobase pair
kinA	gene encoding major sporulation kinase KinA
Km^r	kanamycin resistance phenotype
lacZ	reporter gene used for analysis of promoter activity, gene encodes the enzyme β-galactosidase
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
P_{0A}	promoter from the *spo0A* gene of *Bacillus*
P_{A2}	promoter from ϕ29 phage A2 of *Bacillus*
P_S	σ^H specific sporulation promoter of *spo0A*
P_V	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

I would like to extend my appreciation to Dr. Spiegelman for the opportunity to work in his lab and for the guidance he offered as my supervisor. I would also like to thank my supervisory committee, Dr. W. Mohn, Dr. J. Kronstad, and Dr. J. T. Beatty, for their encouragement and advice. I must acknowledge the people in the laboratories of Dr. J. A. Hoch and Dr. I. Smith, who contributed to this work by providing me with both bacterial strains and useful discussion.

I had the privilege of working with some very interesting and knowledgeable people while in the Spiegelman lab, including Loverne Duncan, Maggie Cervin, Dean Rowe-Magnus, Megan Delehanty, Grace Lau, and Steve Seredick.

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.*,

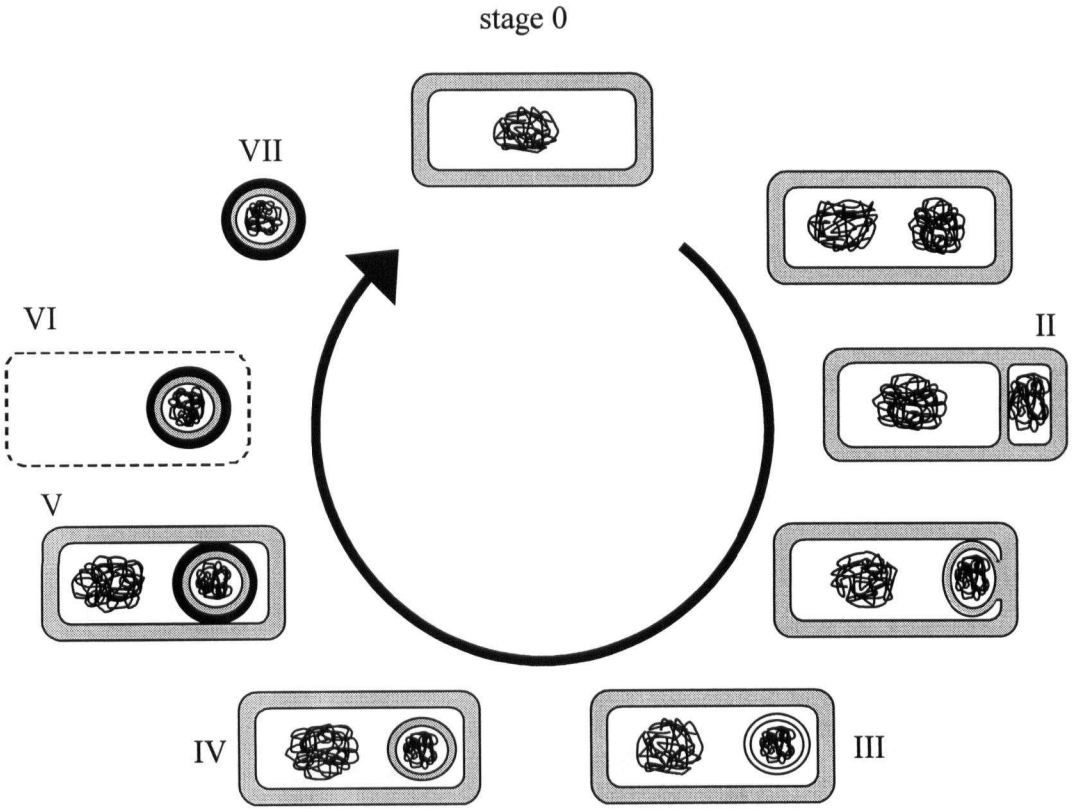


Figure 1. The stages of sporulation in *Bacillus subtilis*[a]. This picture depicts the morphologies of the seven stages of endospore formation in *B. subtilis*. The vegetative state of the cell cycle is defined as stage 0 (top). Sporulation can be initiated only after the completion of DNA replication. 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).

[a] Adapted from Losick, *et al.,* 1986.

1990; Stragier and Losick, 1996). 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 ($\alpha_2\beta\beta'$) 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 (*spoIIAC* 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 σ^K (*spoIVCB:spoIIIC* 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 (σ^E and σ^K), or is held inactive through complex formation with a second protein (σ^F and σ^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, $E\sigma^B$ transcribed genes are expressed at heightened levels during environmental stress, with many of these genes having promoters recognized by other holoenzymes (Haldenwang, 1995). $E\sigma^D$ 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). $E\sigma^L$ is involved in the transcription of a subset of degradative enzymes (Debarouille *et al.*, 1991a; 1991b). $E\sigma^X$ is thought to be involved in the regulation of peptidoglycan synthesis and turnover (Huang and Helmann, 1998), and $E\sigma^W$ is thought to be involved in stationary phase detoxification and/or synthesis of anti-microbial compounds (Huang *et al.*, 1999). *sigB, sigD,* or *sigL* null mutations do not appear to affect growth or sporulation in normal laboratory conditions (Haldenwang, 1995).

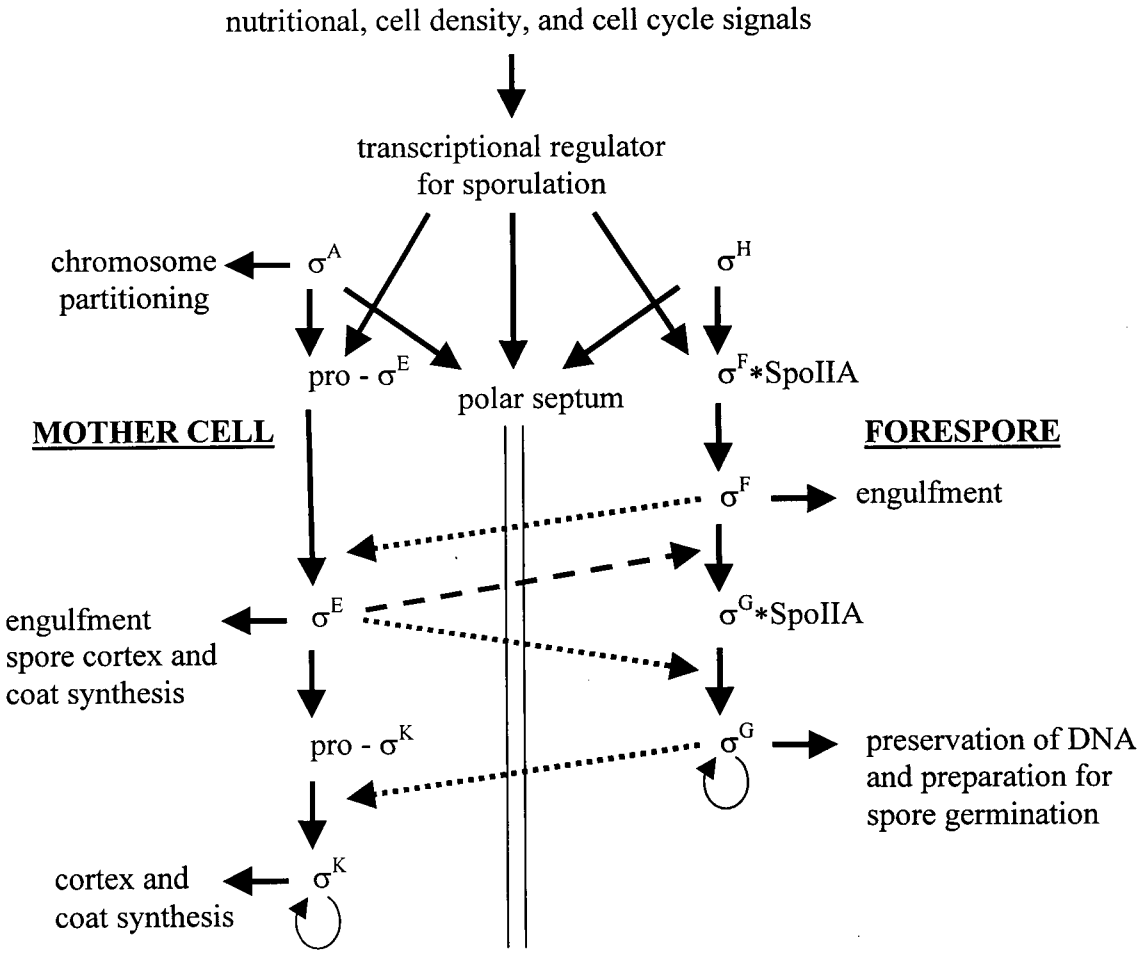


Figure 2. The regulation of sigma factor synthesis and activation[a]. Solid arrows indicate a dependence relationship between sigma factors and the gene products that bring about morphological changes. The two vertical lines represent the membrane partition between the mother cell and developing forespore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

[a] Adapted from Kroos, *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 *spo0A, spo0B, spo0E, spo0F, spo0H, spo0J, spo0K, spo0L,* and *spo0P* (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*

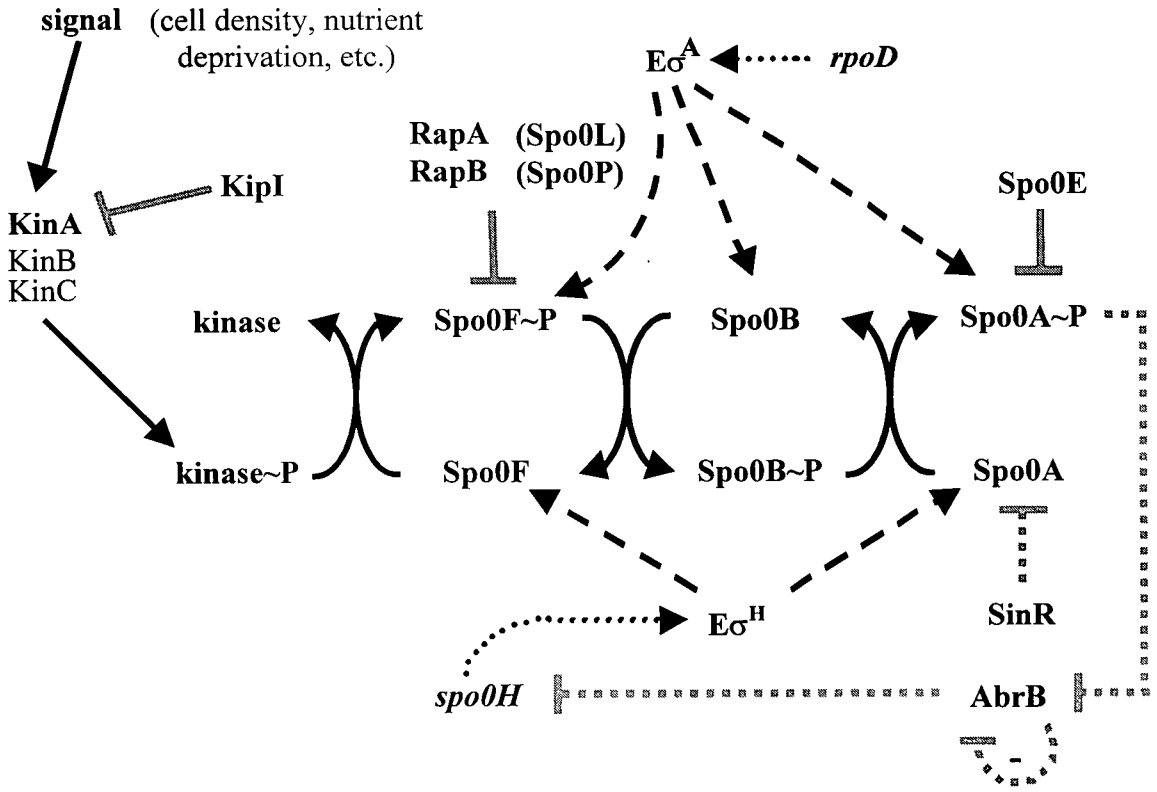


Figure 3. The regulation of the phosphorelay and phosphorylation of Spo0A. The phosphorelay is shown in the center of the figure. Solid black arrows represent the sporulation signal transduction through the phosphorelay. Dashed black arrows represent gene transcription mediated by a specific holoenzyme, 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. *spo0H* encodes the sigma factor σ^H. Spo0E, Spo0P, and Spo0L are phosphorelay phosphatases. KipI is the KinA kinase inhibitor. AbrB and SinR are transition-state regulators.

al., 1995). Spo0A~P is now known to bind DNA at the consensus sequence 5"- TGNCGAA-3" (Strauch *et al.*, 1990; Baldus *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 Spo0A~P for transcription, including *spoIIG, spoIIA,* and the *spo0A* gene itself (Spiegelman *et al.*, 1995). Details of how Spo0A~P activates and represses transcription can be found elsewhere (Spiegelman *et al.*, 1995).

The fact that Spo0A~P is required for the expression of both *spoIIG* and *spoIIA*, encoding sporulation specific sigma factors σ^E and σ^F, 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.

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). $E\sigma^H$, 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 P_S promoter produce very little Spo0A and are unable to sporulate (Strauch *et al.*, 1992; Siranosian and Grossman, 1994).

Transcription from the *spo0A* P_V 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* P_S 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* P_S 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 P_i-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 *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 *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 *citB* and *citZ* genes, as well as repressing those genes during anaerobiosis. The DNA sequence to which CcpC appears to bind is different from the *cre* sequence.

The link between CR and sporulation is poorly understood. The enzymes of the citric acid cycle (CAC) in *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, σ^E or σ^F 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 $E\sigma^{A47}$ 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 *crsA* mutation causes catabolite resistant sporulation.

The *crsA* mutation results in the production of an altered σ^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 *crsA* mutation resulted in an alteration in promoter utilization by RNA polymerase containing σ^{A47}, resulting in the inappropriate initiation of sporulation. *In vivo* and *in vitro* studies of the mutant RNA polymerase using different promoters involved in sporulation may indicate how the mutant phenotype occurs.

σ^A-dependent promoters that are either repressed or activated at the onset of sporulation include those upstream of the *spo0A, spo0F, spo0L, spo0P, spoIIG, sinR* and *abrB* genes. The mutation conferred by *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 σ^A-dependent promoters with unexplained changes in transcriptional activity *in vivo* were examined using *in vitro* techniques, to investigate the potential for $E\sigma^{A47}$ to be directly involved in the unusual expression of these genes.

σ^H-dependent promoters that are repressed or activated during sporulation initiation include those upstream of the *spo0A, spo0F, kinA, spoIIA, spoVG,* and *sinI* genes. σ^H-dependent transcription may be indirectly affected by the *crsA* mutation, either *via* a σ^{A47}-dependent activation of σ^H despite the presence of glucose, or as a consequence of alterations in σ^A-dependent *spo* gene expression. Such changes in σ^H-dependent expression may also permit either the inappropriate activation, or bypassing, of the phosphorelay.

By comparison of *in vivo* and *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 σ^A- and σ^H-directed transcription of several promoters were altered in the presence of the *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 σ^H, the unusually low transcription of the gene encoding the transition state regulator SinR, and an increase in the efficiency of transcription from the *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.

Strain or plasmid	Genotype, phenotype or description[a,b,c]	Source
Bacillus subtilis strains		
JH642	*trpC2 phe-1* (*sigA*$^+$)	J. Hoch.
GLU-47	*crsA strA*	BGSC[d]
JH12751	*trpC2 phe-1 amyE*::(*spo0A-lacZ* Km^r)	M. Perego
JH16304	*trpC2 phe-1 amyE*::(*spoIIG-lacZ* Km^r)	M. Perego
JH12604	*trpC2 phe-1 amyE*::(*abrB-lacZ* Cm^r)	M. Perego
JH12866	*trpC2 phe-1 amyE*::(*spo0P-lacZ* Km^r)	M. Perego
JH12981	*trpC2 phe-1 amyE*::(*spo0L-lacZ* Km^r)	M. Perego
JH12862	*trpC2 phe-1 amyE*::(*spo0F-lacZ* Cm^r)	M. Perego
JH16124	*trpC2 phe-1 amyE*::(*spoIIA-lacZ* Cm^r)	M. Perego
JH12664	*trpC2 phe-1 kinA*::(1.7 kb *kinA-lacZ* Cm^r)	M. Perego
JH12638	*trpC2 phe-1 kinA* W168::pJM8115 Cm^r	M. Perego
IS688	*leuA8 metB5 hisA1 spoVG*::(*spoVG-lacZ* Cm^r)	I. Smith
IS875	*leuA8 metB5 hisA1 ΔsinR*::Cm^r	I. Smith
IS423	*leuA8 metB5 hisA1 sinI*::(pIS135 Cm^r)	I. Smith
IS424	*leuA8 metB5 hisA1 sinIR*::(pIS142 Cm^r)	I. Smith
ZB456	*trpC2 pheA1* SPβ2Δ2::Tn*917*::*spoVG42-lacZ* Cm^r MLS^r	P. Zuber
GBS10	GLU-47 DNA ⟶ JH642	this study
GBS100	JH12751 DNA ⟶ GBS10	this study
GBS101	JH16304 DNA ⟶ GBS10	this study
GBS102	JH12604 DNA ⟶ GBS10	this study
GBS103	JH12866 DNA ⟶ GBS10	this study
GBS104	JH12981 DNA ⟶ GBS10	this study
GBS105	JH12862 DNA ⟶ GBS10	this study
GBS106	JH16124 DNA ⟶ GBS10	this study
GBS107	JH12664 DNA ⟶ GBS10	this study
GBS108	JH12638 DNA ⟶ GBS10	this study
GBS109	IS688 DNA ⟶ GBS10	this study
GBS110	IS688 DNA ⟶ JH642	this study
GBS111	IS875 DNA ⟶ GBS10	this study
GBS112	IS875 DNA ⟶ JH642	this study
GBS113	IS423 DNA ⟶ GBS10	this study
GBS114	IS423 DNA ⟶ JH642	this study

GBS115	IS424 DNA ⟶ GBS10	this study
GBS116	IS424 DNA ⟶ JH642	this study
GBS117	ZB456 DNA ⟶GBS10	this study
GBS118	ZB456 DNA ⟶ JH642	this study
GBS119	GBS107 Δ*spo0H*:: Km^r	this study
GBS120	JH12664 Δ*spo0H*:: Km^r	this study
GBS121	GBS100 *spo0H*::(pGBS-0H2 Cm^r)	this study
GBS122	JH12751 *spo0H*::(pGBS-0H2 Cm^r)	this study
GBS123	GBS10 *orfX*::(pGBS5 Km^r)	this study
GBS124	JH642 *orfX*::(pGBS5 Km^r)	this study
GBS125	GBS10 *amyE*::(*spo0AΔP$_S$-lacZ* Cm^r)	this study
GBS126	JH642 *amyE*::(*spo0AΔP$_S$-lacZ* Cm^r)	this study
GBS127	GBS10 *amyE*::(1.7 kb *kinA-lacZ* Cm^r)	this study
GBS128	JH642 *amyE*::(1.7 kb *kinA-lacZ* Cm^r)	this study
GBS129	GBS10 *kinA*::(780 bp *kinA-lacZ* Cm^r)	this study
GBS130	JH642 *kinA*::(780 bp *kinA-lacZ* Cm^r)	this study
GBS131	GBS10 *amyE*::(780 bp *kinA-lacZ* Cm^r)	this study
GBS132	JH642 *amyE*::(780 bp *kinA-lacZ* Cm^r)	this study
GBS133	GBS10 *kinA*::(700 bp *kinA-lacZ* Cm^r)	this study
GBS134	JH642 *kinA*::(700 bp *kinA-lacZ* Cm^r)	this study
GBS135	GBS10 *kinA*::(350 bp *kinA-lacZ* Cm^r)	this study
GBS136	JH642 *kinA*::(350 bp *kinA-lacZ* Cm^r)	this study
GBS137	GBS10 *amyE*::(350 bp *kinA-lacZ* Cm^r)	this study
GBS138	JH642 *amyE*::(350 bp *kinA-lacZ* Cm^r)	this study
GBS139	GBS10 *amyE*::(350 bp (variant) *kinA-lacZ* Cm^r)	this study
GBS140	JH642 *amyE*::(350 bp (variant) *kinA-lacZ* Cm^r)	this study
GBS141	GBS10 *amyE*::(125 bp *kinA-lacZ* Cm^r)	this study
GBS142	JH642 *amyE*::(125 bp *kinA-lacZ* Cm^r)	this study
GBS143	GBS10 *amyE*::(2.8 kb *kinA-lacZ* Cm^r)	this study
GBS144	JH642 *amyE*::(2.8 kb *kinA-lacZ* Cm^r)	this study
GBS145	GBS10 *spo0A::* (pJM103::P-*spo0AΔP$_S$* Cm^r)	this study
GBS146	JH642 *spo0A::*(pJM103::P-*spo0AΔP$_S$* Cm^r)	this study

Escherichia coli strains

DH5α	*hsdR*17 (r_k-, m_k+) *recA*1	NEB[e]
GM2163	*hsdR*2 (r_k-, m_k+) *recA*1 *dam*13::Tn*9* *dcm*-6	BRL[f]

Plasmids

pDH32	Am^r Cm^r promoter-*lacZ* fusion vector	J. Hoch
pJM103	Am^r Cm^r vector	J. Hoch
pJM8114	Am^r Cm^r P-*kinA*(-970 to +891)::*lacZ*	M. Perego
pGBS783	Am^r Cm^r pJM8114ΔP-*kinA*(-970 to +891)	this study
pGEM-T	Am^r commercial vector	Stratagene
pDG780	Am^r + Km^r cassette	BGSC[d]
pGBS-0H	Am^r Km^r pGEM-T::*spo0H*(+58 to +665)::Km^r	this study
pGBS-0H2	Am^r Cm^r pJM103::*spo0H* (+58 to +665)	this study

pBSK(-)	Am^r commercial vector	Stratagene
pGBS5	Am^r Km^r pBSK(-)::*orfX* (+1507 to +2085)::Km^r	this study
pJF1408	Am^r Cm^r pJH101Δ*tet*::P-*spo0A*(-856 to +750)	M. Perego
pJH14-M	Am^r Cm^r pJH101Δtet::P-*spo0AΔP$_S$* (deleted –104 to –29)	this study
pGBS14-M	Am^r Cm^r pJM103::P-*spo0AΔP$_S$* (-524 to +69)	this study
pGBS780	Am^r Cm^r pGBS783::P-*kinA*(-773 to –23)	this study
pGBS700	Am^r Cm^r pGBS783::P-*kinA*(-105 to +591)	this study
pGBS350	Am^r Cm^r pGBS783::P-*kinA*(-328 to +4)	this study
pGS17	Am^r Cm^r pDH32::P-*kinA*(-970 to +891)	this study
pGS780	Am^r Cm^r pDH32::P-*kinA*(-773 to –23)	this study
pGS350	Am^r Cm^r pDH32::P-*kinA*(-328 to +4)	this study
pGS350V	Am^r Cm^r pDH32::P-*kinA*(-328 to +4)	this study
pGS125	Am^r Cm^r pDH32::P-*kinA*(-102 to +4)	this study
pGS28	Am^r Cm^r pDH32::P-*kinA*(-2696 to +6)	this study

[a] Km^r, Cm^r, MLS, and Am^r refer to drug resistance of the bacterial strains. Km-kanamycin; Cm-chloramphenicol; MLS-erythromycin/lincomycin; Am-ampicillin.

[b] ⟶ -transformation with chromosomal DNA from the bacterial strain listed.

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

[d] *Bacillus* Genetic Stock Center

[e] New England BioLabs, Inc.

[f] Bethesda Research Laboratories

Table 2. PCR Primers used in this study.

Primer name	Primer sequence	Target region
1A	5' CGGAATTCTCATACAATCTGACTT 3'	*kinA*
1B	5' TGTCTAGACATTTTTGAATAAAAG 3'	*kinA*
2A	5' TTTCTAGATACCATAAGAATAGAAGGA 3'	*kinA*
2B	5' TCGGATCCACAGAATCCCTCCTTT 3'	*kinA*
OX5	5' GGAGAATTCTTTCGCTGATGCTTGC 3'	*orfX*
OX3	5' TCGAATTCCACAGAATCCCTCCTTT 3'	*orfX*
0H UP	5' CTGAGCTCACGAGCAGGTCATTGAA 3'	*spo0H*
0H DO	5' TAGCATGCTGCGTTTCACACGCTGA 3'	*spo0H*
UK5	5' ATGAATTCCTATTACAGCCAGTTTGGC 3'	*kinA*
UK3	5' ACGGATCCTTTTAGTTGTGCACCCTGT 3'	*kinA*
0A5	5' CGTGAATTCCGATATGGACACAAAG 3'	*spo0A*
0A3	5' TCGGATCCATGTCTTCCTGTCCTT 3'	*spo0A*

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 ψB 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 4 000 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 4 000 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. 1-2

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 *Bam*HI 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 *Pst*I (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 $E\sigma^A$ and $E\sigma^{A47}$ 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_{0A} template preparation.

The plasmid pUCA2trp was created by subcloning the fragment containing the A2 promoter from pKKA2 (Bird *et al.*, 1993) into the *Hind*III/*Bam*HI sites of the plasmid pUCIIGtrpA (Satola *et al.*, 1991), replacing the *spoIIG* promoter in that plasmid (Cervin *et al.*, 1998). When digested with *Pvu*II, 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 *Hind*III 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 μL 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 μL. The mixture also contained (unless otherwise stated) 0.4 mM ATP, 5 μM GTP, and 0.5 μCi of [α^{32}P]-GTP (800 Ci/mmol; NEN). Tubes containing this mixture were warmed to the appropriate temperature (usually 37°C) for 2 minutes prior to initiating the transcription reaction. Transcription was initiated by the addition of 2 μL 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 μL 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 μL 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 *in vitro* reaction. The 291 nt P_{0A} transcript was located relative to the 130 nt P_{A2} control transcript (Bird *et al.*, 1993; Cervin *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.

Strain / % glucose*	total cell count/ml	spore count**/ml	sporulation frequency (spores/total cells)
JH642			
0.0%	6.65×10^{8}	4.35×10^{8}	6.50×10^{-1}
0.1%	2.82×10^{8}	3.20×10^{4}	1.13×10^{-4}
0.5%	1.34×10^{8}	1.30×10^{4}	9.71×10^{-5}
1.0%	7.25×10^{7}	5.62×10^{3}	7.75×10^{-5}
2.0%	1.92×10^{7}	8.72×10^{2}	4.54×10^{-5}
GBS10			
0.0%	3.12×10^{9}	4.26×10^{9}	1.00×10^{0}
0.1%	2.76×10^{9}	2.09×10^{9}	7.57×10^{-1}
0.5%	8.14×10^{8}	7.91×10^{8}	9.72×10^{-1}
1.0%	3.51×10^{8}	4.02×10^{8}	1.00×10^{0}
2.0%	9.28×10^{7}	8.70×10^{6}	9.38×10^{-2}

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

** Spore counts were generated by treating total cell samples with 1/10 volume of choroform 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 *crsA* mutant strains while clearly inhibiting sporulation in JH642.

B. Investigation of the effects of the *crsA* mutation on the expression patterns of promoters involved in sporulation initiation.

Initially, the target gene or genes whose activity was affected by the *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 *lacZ*, and β-galactosidase activity throughout the growth period used as an indicator of promoter activity. 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.

Figure 4 shows the structure of pDH32, the plasmid used in most of the promoter-*lacZ* fusions. Prior to transformation into *Bacillus* strains, pDH32-based constructs containing a promoter insert were linearized with *Pst*I. Selection for chloramphenicol resistance would ensure that the promoter-*lacZ* fusions were recombined into the nonessential α-amylase gene (*amyE*) by a double crossover event. All transformants with pDH32 based promoter-*lacZ* fusions were confirmed to be *amyE*$^-$ as was described in Materials and Methods. One promoter-*lacZ* fusion, *kinA-lacZ*, was created elsewhere, using pJM783 (M. Perego, Scripps Institute), and the details are shown in Figure 5.

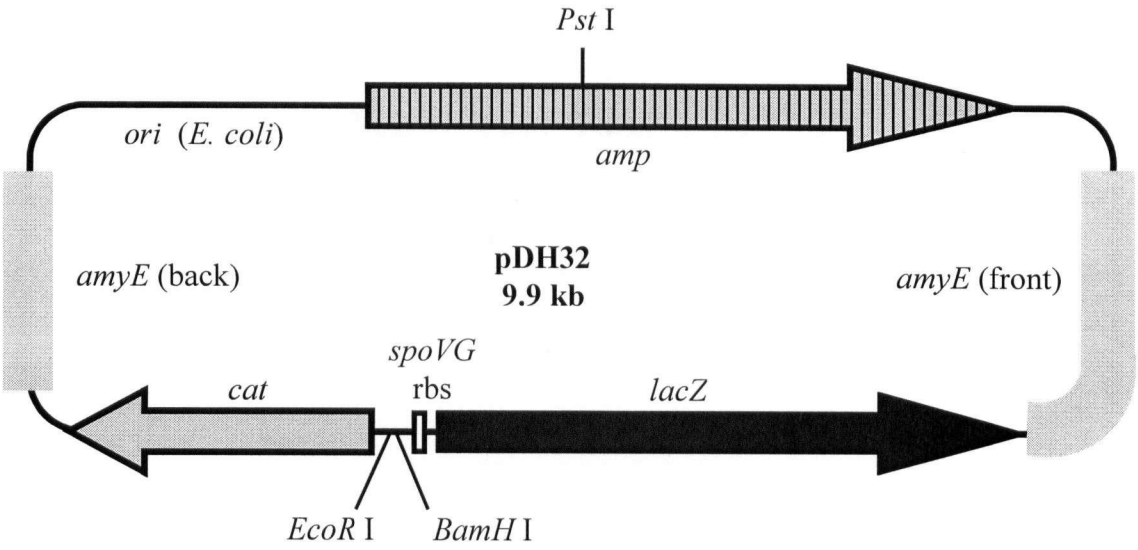


Figure 4. Structure of the *B. subtilis* promoter expression vector pDH32. The vector contains an *E. coli* origin of replication (*ori*) and an ampicillin resistance gene (*amp*) for growth and selection in *E. coli*. The chloramphenicol acetyltransferase gene (*cat*) allows for selection of the integrated 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.

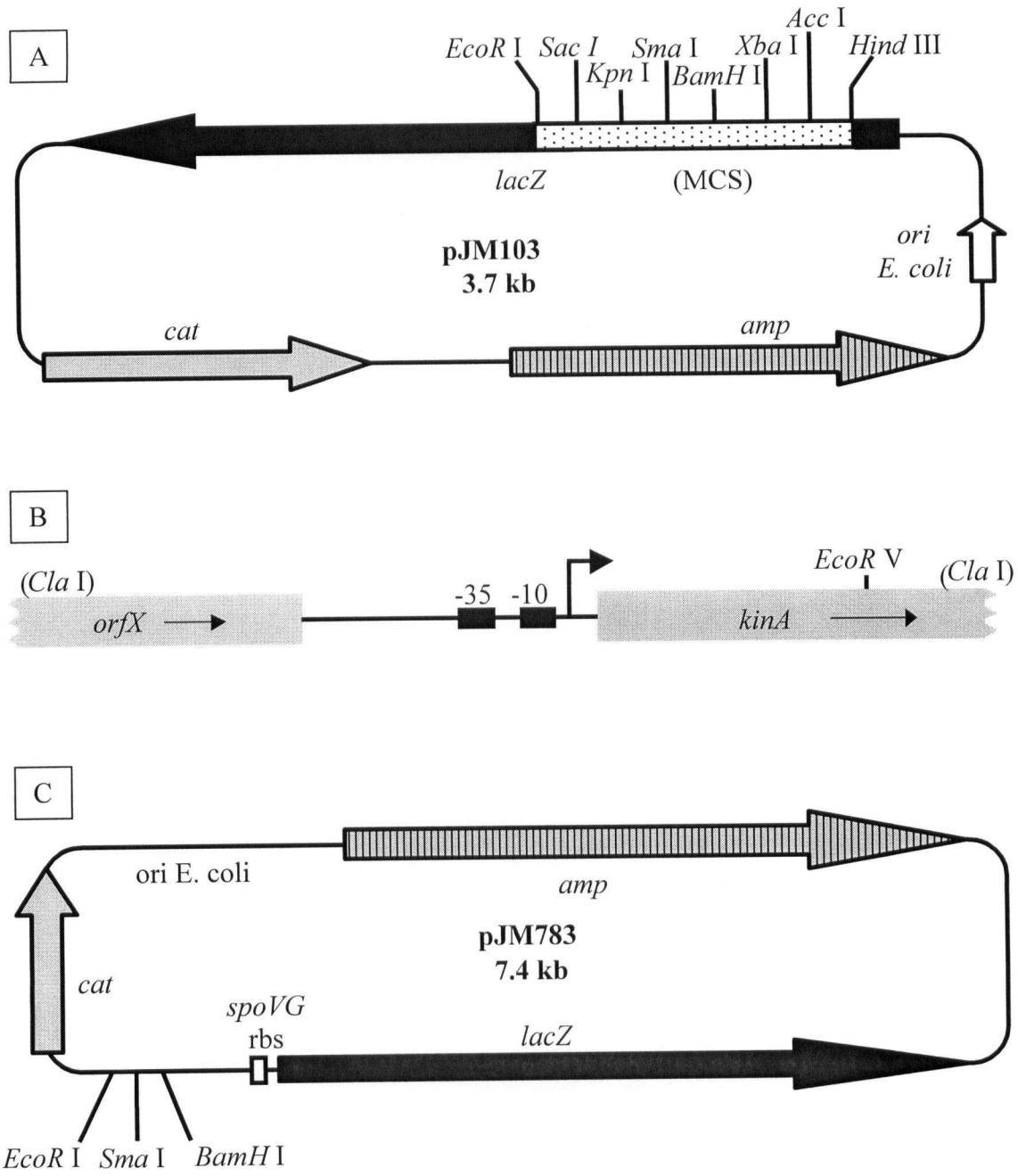


Figure 5. Creation of the 1.7 kb *kinA* promoter-*lacZ* reporter gene construct. The 1.7 kb chromosomal *Cla* I fragment containing the *kinA* promoter (represented in B) was obtained from a chromosomal DNA digest and cloned into the *Acc* I site of pJM103 (A). The 1.7 kb *EcoR* I / *EcoR* V fragment 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 (T_0) 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 T_0.

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 T_1. 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 T_0. Expression after T_0 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 T_0. 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 $T_{1.5}$ to T_2 at

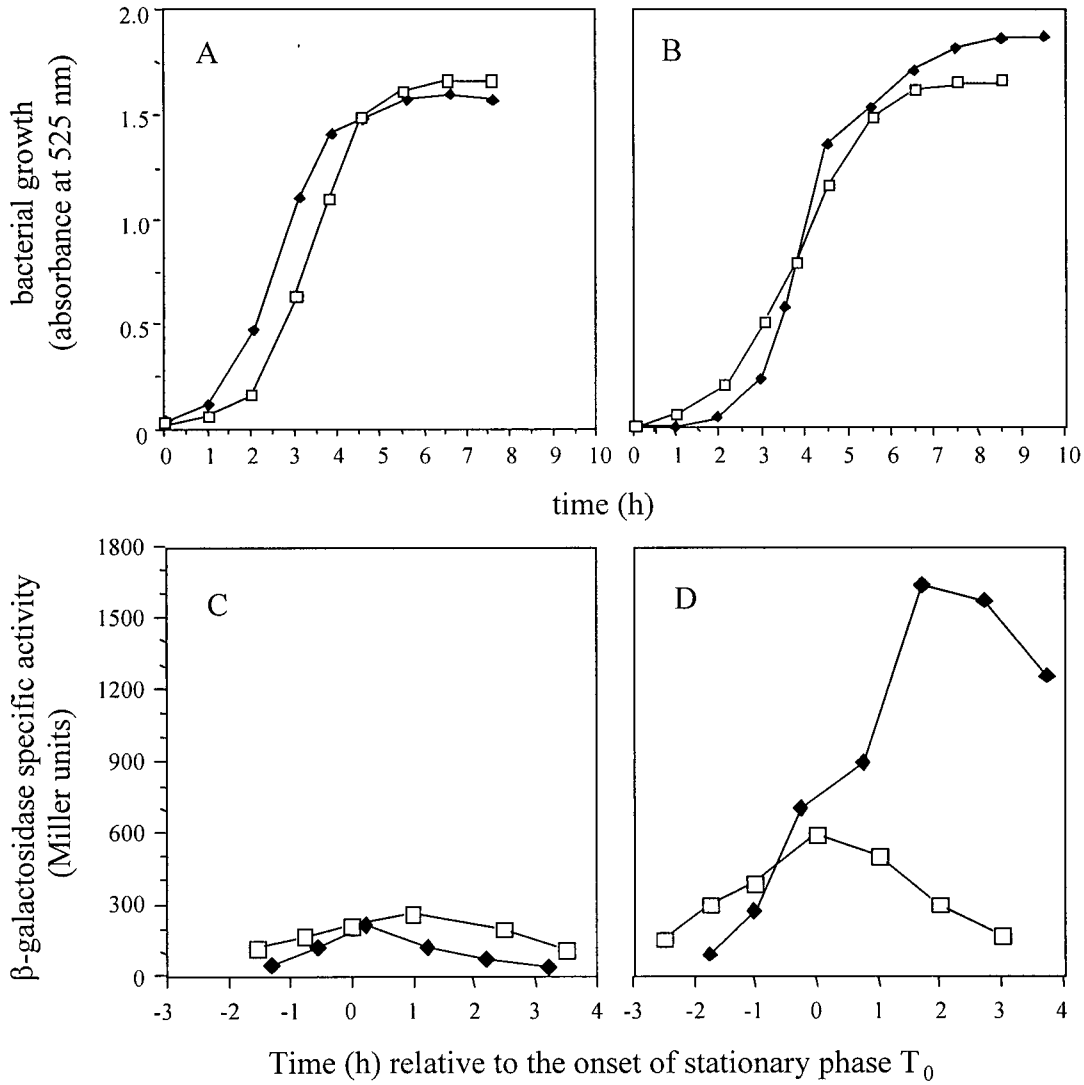


Figure 6. Growth of *B. subtilis* strains containing the *kinA* promoter-*lacZ* reporter gene fusion constructed in pJM783 and inserted in the *kinA* gene, and expression of the *kinA-lacZ* fusion. Strains are: (A and C) JH642; (B and D) GBS10. Y-axis values shown are the same for both (A) and (B), and 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 *kinA* promoter in GBS10 was unusual, as this promoter is known to be transcribed by $E\sigma^H$. Assuming that $E\sigma^{A47}$ cannot transcribe *kinA* itself, these results suggested the possibility of either unusual σ^H activity, or that the activity of a regulator of the *kinA* promoter was altered by the presence of the *crsA* mutation.

Figure 7 depicts the activity of the σ^A/σ^H dual *spo0F* promoter-*lacZ* fusion in JH642 and GBS10 strains. The *spo0F-lacZ* fusion was created using pDH32, inserted into the *amyE* gene of JH642 (to generate strain JH12862), and was generously provided by M. Perego (Scripps Institute). This *spo0F* promoter-*lacZ* fusion was introduced into GBS10 by transformation using chromosomal DNA from JH12862. In Figure 7A, the *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 *et al.*, 1992; Chibazakura *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, *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 *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 σ^A/σ^H *spo0A* promoter-*lacZ* fusion is shown in Figure 8. The *spo0A-lacZ* fusion was created using a pDH32-type vector, inserted into the *amyE* gene of JH642 (to generate strain JH12751), and was generously provided by M. Perego (Scripps

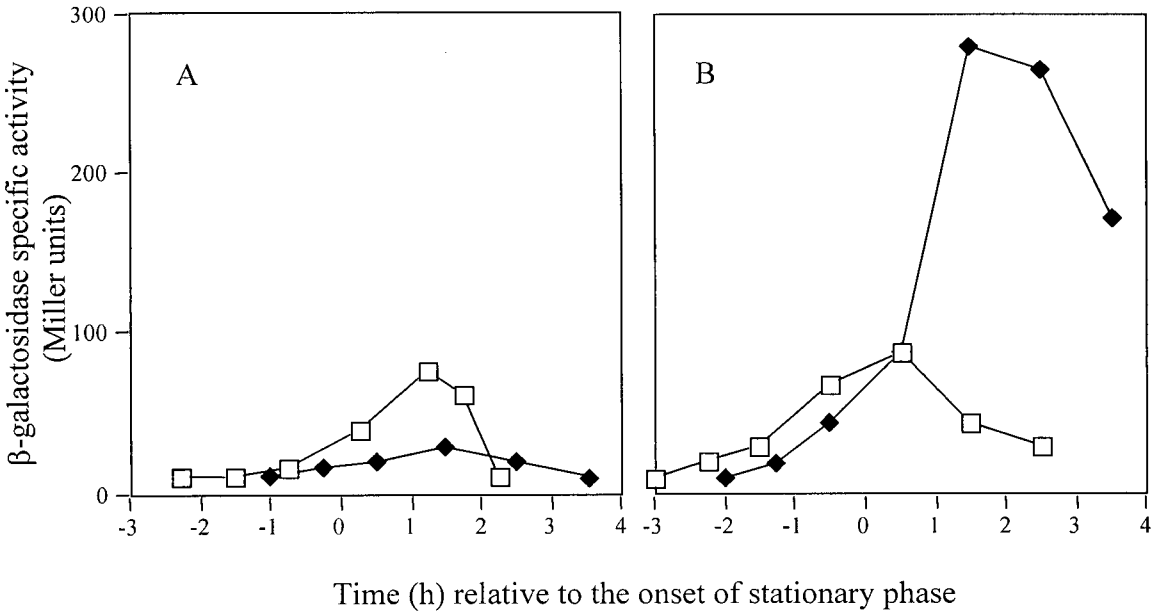


Figure 7. Expression of the *spo0F* promoter-*lacZ* reporter gene fusion. The promoter construct was inserted in the *amyE* gene in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) and without (open squares) 0.2% added glucose.

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.

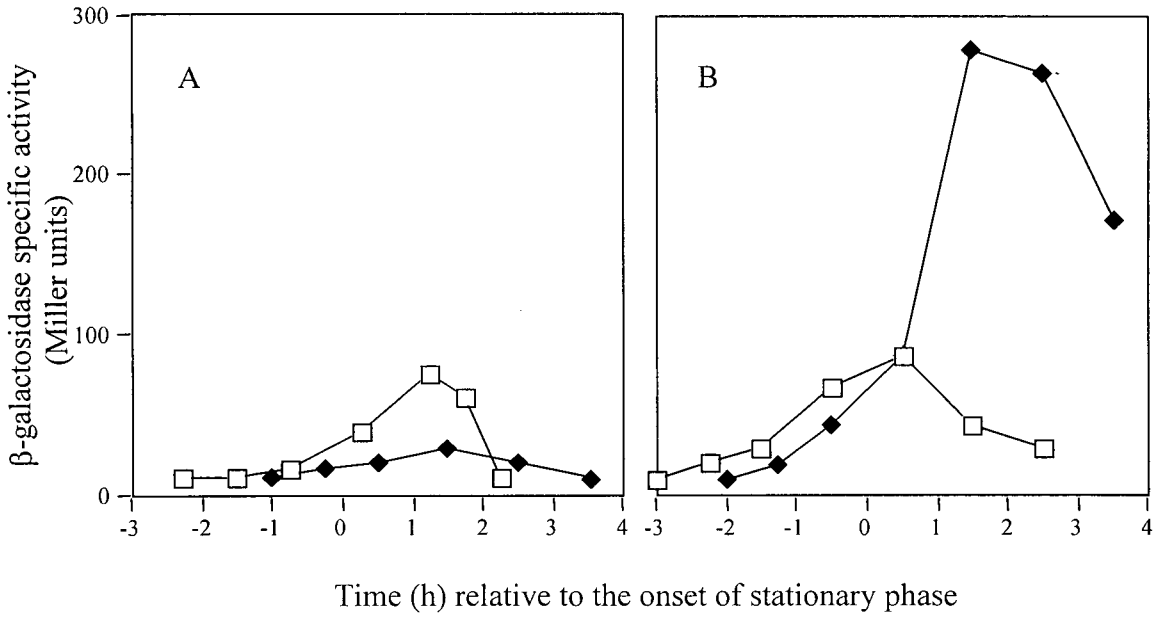


Figure 7. Expression of the *spo0F* promoter-*lacZ* reporter gene fusion. The promoter construct was inserted in the *amyE* gene in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) and without (open squares) 0.2% added glucose.

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 σ^E (mother cell specific, encoded by *spoIIGB*) and σ^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 σ^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

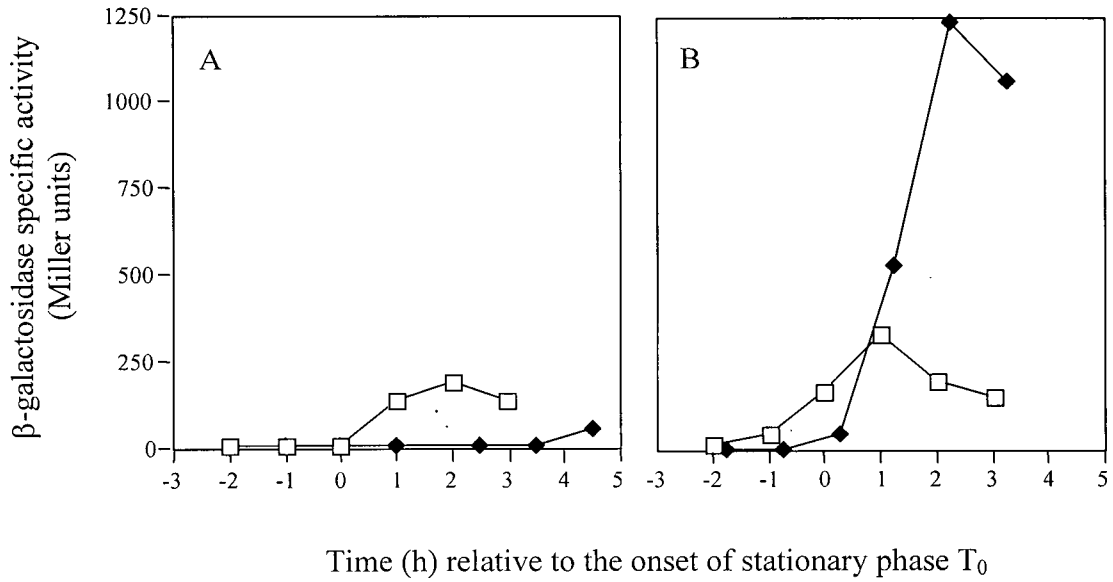


Figure 9. Expression of the *spoIIG* promoter-*lacZ* reporter gene fusion. The promoter construct was inserted in the *amyE* gene in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) or without (open squares) 0.2% added glucose.

roughly 5-times that seen in JH642 in the absence of glucose. The *spoIIG-lacZ* activity in GBS10 is interesting: in section III.B.1, transcriptional activity observed in GBS10 strains containing *kinA-*, *spo0F-*, and *spo0A-lacZ* fusions in the presence of glucose was also increased during late stationary phase. However, the *kinA*, *spo0F*, and *spo0A* promoters each have σ^H-dependent transcriptional activity, whereas the *spoIIG* promoter activity seen in GBS10 in Figure 9 is due strictly to σ^A-dependent transcription. A similar change in *spoIIG* promoter activity has been previously reported in cells containing a *sinR* null mutation, with peak *spoIIG* transcription levels 3.5- to 4-times higher than that observed for wild type cells (Mandic-Mulec *et al.*, 1992).

The activity of the σ^H-dependent *spoIIA* promoter-*lacZ* fusion is shown in Figure 10. The *spoIIA-lacZ* fusion was created using pDH32, inserted into the *amyE* gene of JH642 (to generate strain JH16124), and was generously provided by M. Perego (Scripps Institute). This *spoIIA* promoter-*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 *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 *et al.*, 1992; Baldus *et al.*, 1995). In the presence of glucose (closed diamonds), the *spoIIA* promoter was completely repressed. In GBS10 without glucose (Figure 10B, open squares) a low level of *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.

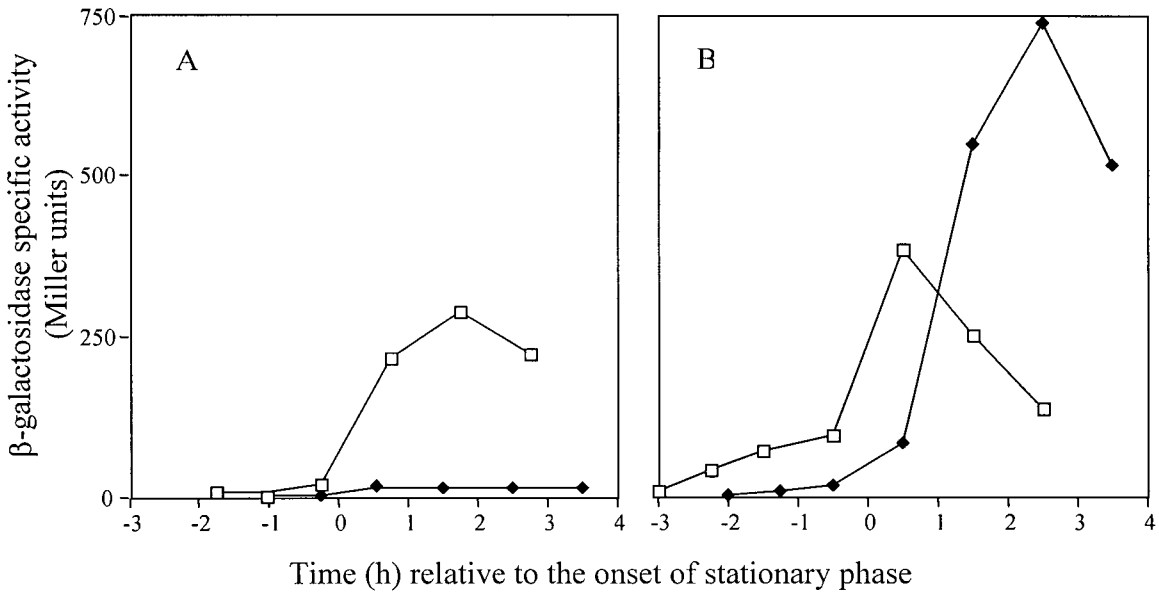


Figure 10. Expression of the *spoIIA* promoter-*lacZ* reporter gene fusion. The promoter construct was inserted in the *amyE* gene in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) or without (open squares) 0.2% added glucose.

The above results clearly indicate that the activity of both the *spoIIG* and *spoIIA* 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 σ^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 σ^H activity, as it is the only known σ^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 σ^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 T_1, 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 T_1 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 T_1 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 σ^H 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 σ^H, 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

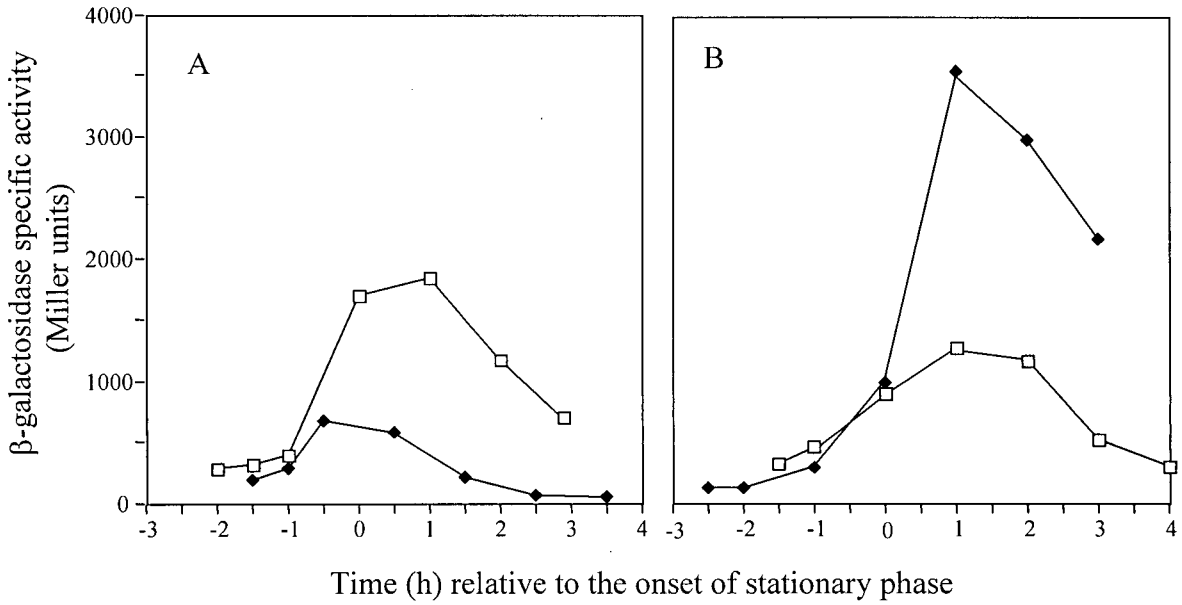


Figure 11. Expression of the *spoVG* promoter-*lacZ* reporter gene fusion. The promoter construct was inserted upstream of *spoVG*, in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) and without (open squares) 0.2% added glucose.

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 Spo0F 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 σ^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

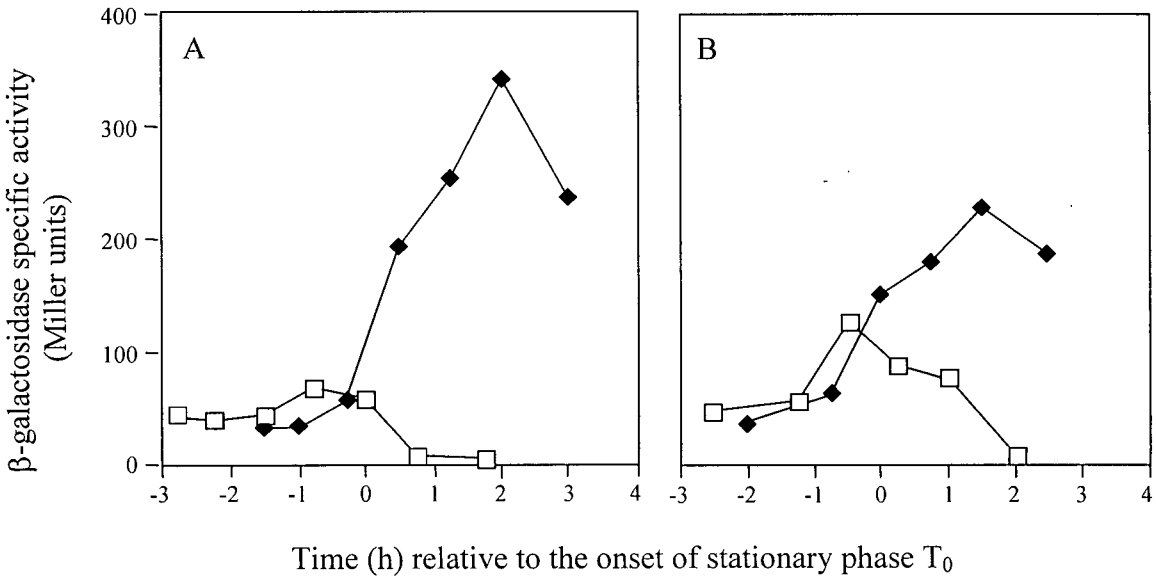


Figure 12. Expression of the *spo0P* promoter-*lacZ* reporter gene fusion. The promoter construct was inserted in the *amyE* gene in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) or without (open squares) 0.2% added glucose.

level slightly less than that seen in the wild type strain in the presence of glucose.

The results of the analysis of the σ^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

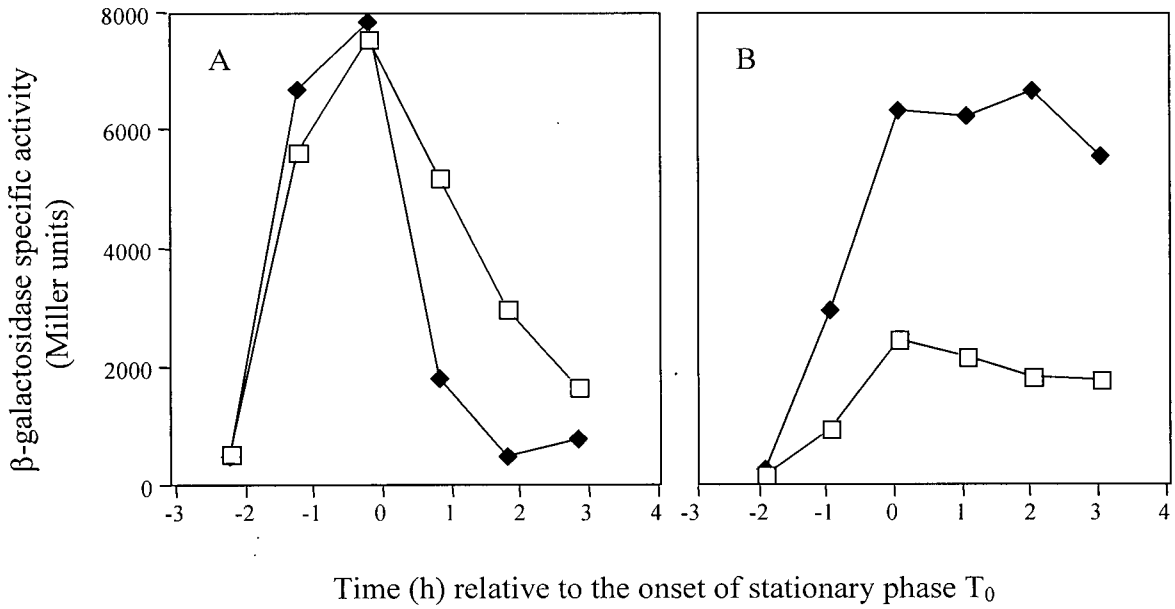


Figure 13. Expression of the *spo0L* promoter-*lacZ* reporter gene construct. The promoter construct was inserted in the *amyE* gene in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) or without (open squares) 0.2% added glucose.

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 *spo0A, 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

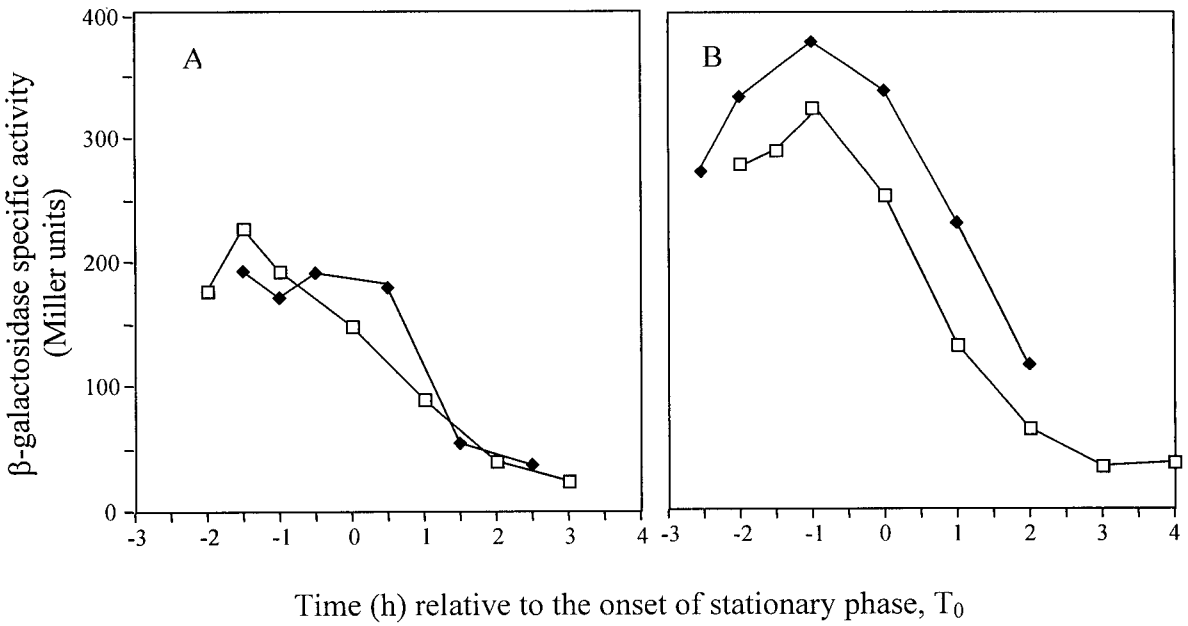


Figure 14. Expression of the *abrB* promoter-*lacZ* reporter gene fusion. The promoter construct was inserted in the *amyE* gene in strains JH642 (A) and GBS10 (B). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) or without (open squares) 0.2% added glucose.

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_{-1}), 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, Spo0A, 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 T_0. *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 T_0 and $T_{1.5}$. 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.

sinI promoter expression in GBS10 is shown in Figure 15B. In these cells, the transcriptional activity from the *sinI* promoter in the absence of glucose was lower than that seen in the wild type. *sinI-lacZ* activity was low throughout late logarithmic growth, rising only slowly to peak at $T_{0.5}$, at levels 25 to 30 percent of wild type. *sinI-lacZ* activity was also altered in GBS10 grown in the presence of glucose, with transcription rising from $T_{-1.5}$ to peak sometime after T_2 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 *sinI* and from the *sinR* promoter was greatly reduced from that seen in wild type cells, peaking at very low levels at roughly T_0 and decreasing after that. In the presence of glucose, transcription rose sharply from T_{-2} to peak at T_0 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 *sinI* promoter was observed. Thus, unlike in JH642 where the addition of glucose decreased the ratio of expression of *sinI:sinR*, in GBS10 the ratio of *sinI: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).

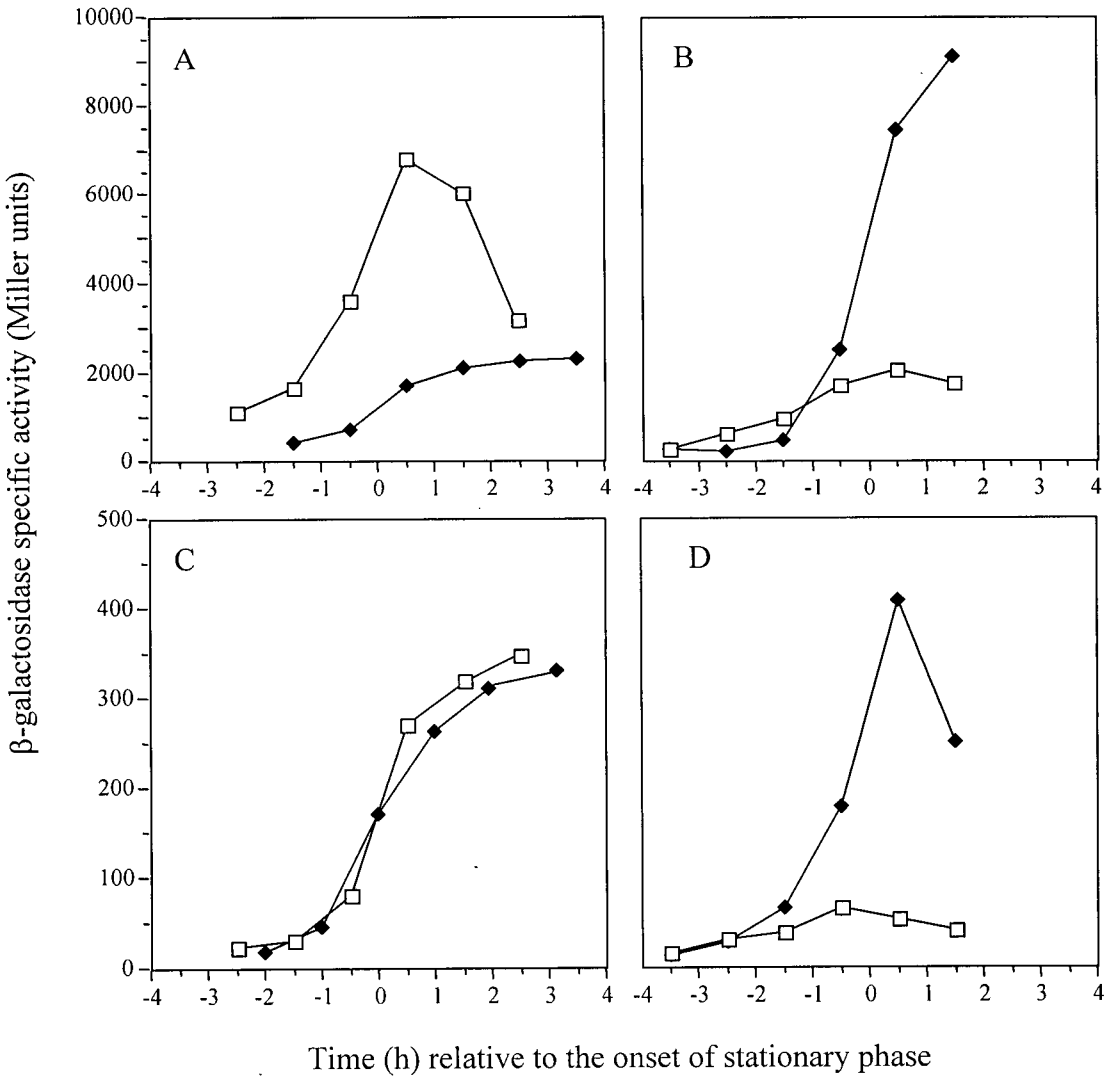


Figure 15. Expression of the *sinI* and *sinR* promoter-*lacZ* reporter gene fusions. The promoter constructs were inserted in the *sinI* gene (A and B), or *sinR* gene (C and D), in strains JH642 (A and C) and GBS10 (B and D). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) 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 *kinA* promoter *via* a single recombination event. As a result of the integration, the *kinA* promoter-*lacZ* clone is inserted into the *kinA* locus so that the 3' end of the promoter driving *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 *via* a double recombination event at the *amyE* locus, and the sequence of the promoter driving *lacZ* expression can be varied at both the 5' and 3' ends.

A schematic diagram of the *kinA* promoter is shown in Figure 16A, along with primer binding sites used to generate, *via* PCR, a 125 bp *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 *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, *cre*. Therefore, this sequence was altered by the PCR introduction of a *Xba*I site, to investigate its importance to *kinA* promoter expression. However, the mutation of the small palindrome did not affect *kinA-lacZ* activity (data not shown).

Figures 16A and 16B also detail the origins of the *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 *orfX* in GBS10 may influence *kinA* promoter activity. The sequence of *orfX* encodes a putative penicillin binding-like protein preceded by a σ^A-like promoter sequence (SubtiList

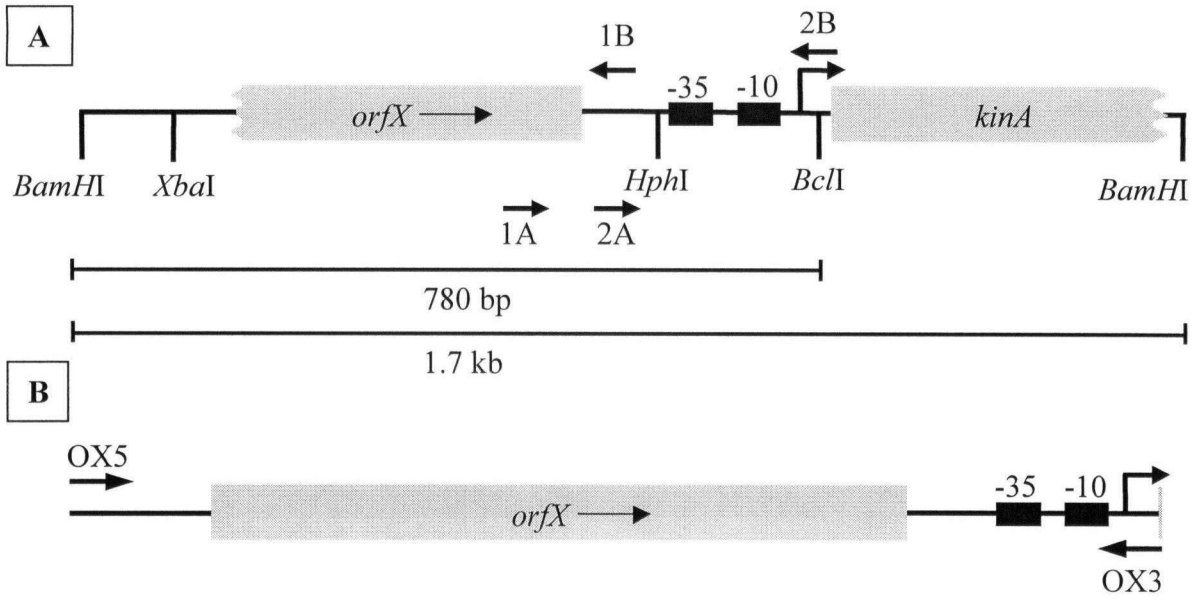


Figure 16. Creation of the 125 bp (pGS125), 350 bp (pGS350), 780 bp (pGS780), 1.7 kb (pGS17), and 2.8 kb (pGS28) *kinA* promoter-*lacZ* constructs in pDH32 (Figure 4). PCR with Vent polymerase (New England BioLabs) of pJM8114 (Figure 5) using primers listed below yielded two promoter fragments (A). To create the 350 bp promoter-*lacZ* fusion, primer pair 1A/2B was ligated directionally into *EcoR*I/*BamH*I 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 *Xba*I, purified using a Qiagen spin column, then ligated together at the *Xba*I ends, and amplified using primer pair 1A/2B. This variant 350 bp promoter was ligated directionally into *EcoR*I/*BamH*I digested pDH32. The 125 bp promoter clone was generated from the variant 350 bp promoter clone via digestion with *EcoR*I and *Xba*I, 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 *BamH*I fragment of pJM8114 (A) was removed and either cloned directly into *BamH*I digested pDH32 to generate the 1.7 kb promoter-*lacZ* fusion (A), or was digested with *Bcl*I to generate the 780 bp *kinA* promoter clone (A), which was ligated into the *BamH*I 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 *EcoR*I site of pDH32. The 350 bp *kinA* promoter was subcloned from pGS350 into the *EcoR*I/*BamH*I sites of pGBS783 (not shown; created from recircularization of the 5.7 kb *BamH*I fragment of pJM8114, see Figure 5), creating pGBS350. pDH32-derived plasmids were linearized with *Pst*I prior to transformation into JH642 and GBS10 strains. pGBS350 was transformed into GBS10 and JH642 intact, for integration into *kinA*.

1A: 5' CG**GAATTC**TCATACAATCTGACTT 3' (*EcoR*I)
1B: 5' TG**TCTAGA**CATTTTTGAATAAAAG 3' (*Xba*I)
2A: 5' TT**TCTAGA**TACCATAAGAATAGAAGGA 3' (*Xba*I)
2B: 5' TC**GGATCC**ACAGAATCCCTCCTTT 3' (*BamH*I)
OX5: 5' GGA**GAATTC**TTTCGCTGATGCTTGC 3' (*EcoR*I)
OX3: 5' TC**GAATTC**CACAGAATCCCTCCTTT 3' (*EcoR*I)
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

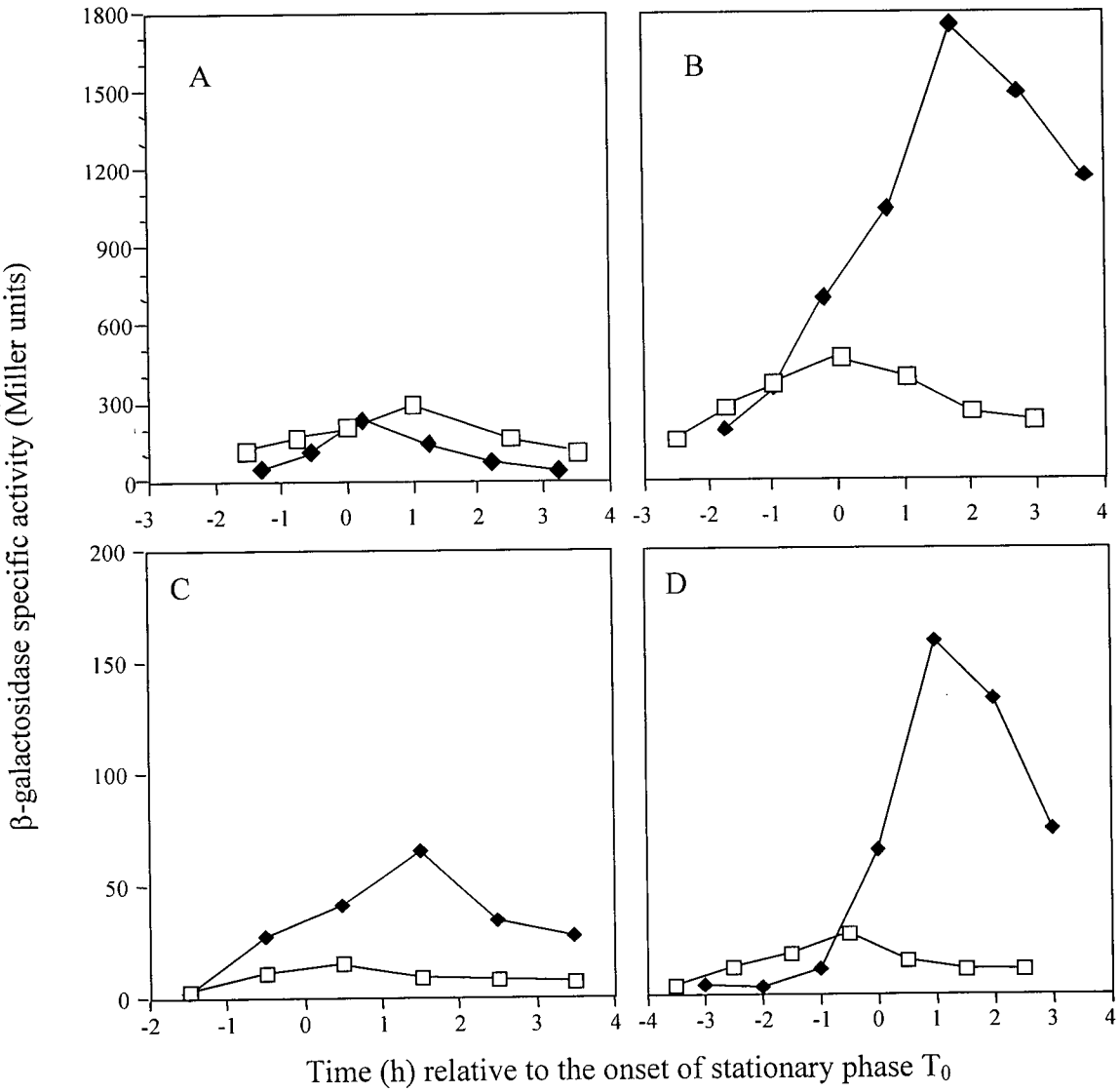


Figure 17. Expression of the 350 bp wild type kinA promoter-*lacZ* reporter gene fusion inserted in the *kinA* gene (A and B) and in *amyE* gene (C and D), in strains JH642 (A and C) and GBS10 (B and D). Strains were grown in Schaeffer's spore medium, pH 7.5, with (closed diamonds) or without (open squares) 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 $T_{0.5}$, 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 (T_1) at levels 6-times that seen for GBS10 cells grown in the absence of glucose, and maintained substantial activity beyond T_3. 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 σ^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 σ^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 σ^H required for sporulation of GBS10.

Figures 18 and 19 are diagrams of the creation of the *spo0H* and *orfX* knockout constructs. The *spo0H* knockout was created by inserting a kanamycin resistance cassette into the *spo0H* 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.*,

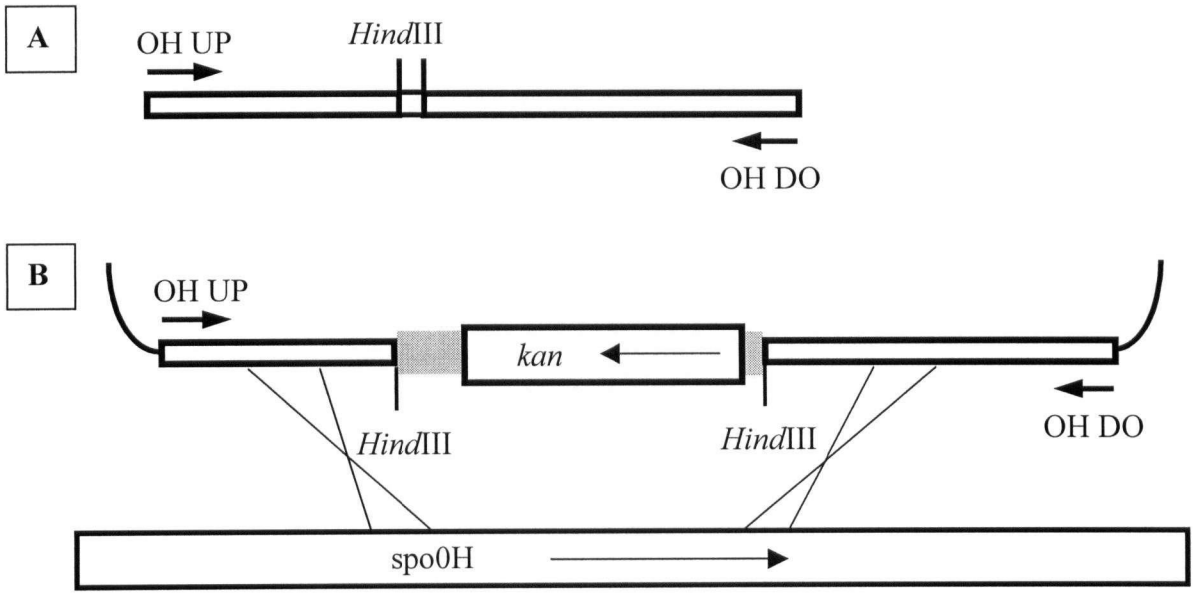


Figure 18. Creation of the clone used to assay sporulation in *spo0H*⁻ strains. The primers listed below were used to generate the 557 bp PCR product of the region internal to *spo0H* (A). This PCR product was ligated into pGEM-T (Stratagene, not shown), utilizing the A overhangs left by Taq polymerase (New England BioLabs), creating pGEM-0H (not shown). pGEM-0H was digested with *Hind*III, removing a 16 bp internal fragment of *spo0H*, and the 1.4 kb kanamycin resistance cassette from pDG780 (not shown; Guerout-Fleury *et al.*, 1995) was inserted, creating pGBS-0H (not shown). pGBS-0H was linearized with *Spe*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*I and *Sph*I (engineered into primer sequences), and the 557 bp *spo0H* fragment was ligated into *Sac*I/*Sph*I 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 *via* a single crossover event.

OH UP: 5' CT**GAGCTC**ACGAGCAGGTCATTGAA 3' (*Sac*I)
OH DO: 5' TA**GCATGC**TGCGTTTCACACGCTGA 3' (*Sph*I)

Note: Restriction sites engineered into primer sequences are shown in bold type.

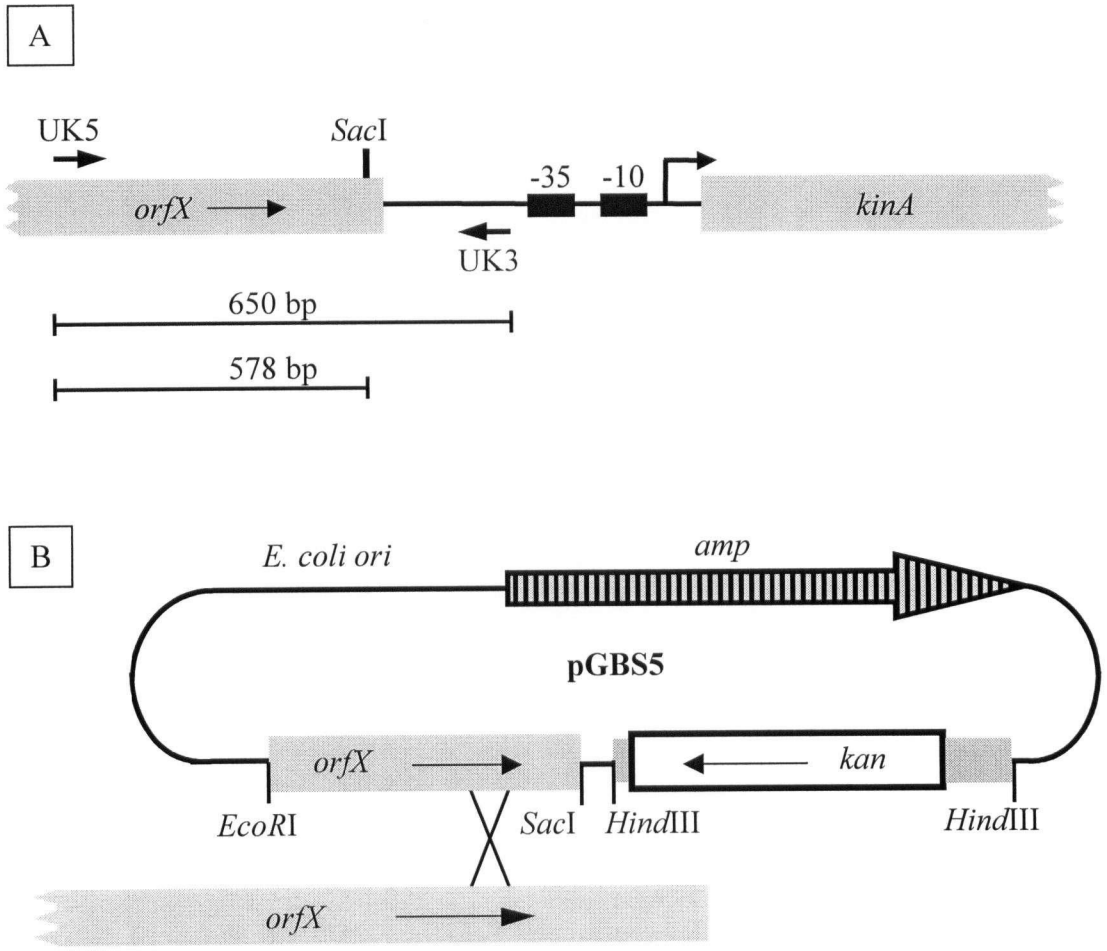


Figure 19. Creation of the clone used to assay sporulation in *orfX*⁻ strains. The primers listed below were used to generate the 650 bp PCR product of the region upstream of *kinA* (A). The PCR product was digested with *EcoR*I and *Sac*I, and the 578 bp fragment internal to *orfX* was ligated into *EcoR*I/*EcoR*V the multiple cloning site of pBSK(-) (Stratagene, not shown). The 1.4 kb *Hind*III 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' AT**GAATTC**CTATTACAGCCAGTTTGGC 3' (*EcoR*I)
UK3: 5' AC**GGATCC**TTTTAGTTGTGCACCCTGT 3' (*BamH*I)

Note: Restriction sites engineered into primer sequences are shown in bold type.

1995; Dartois *et al.*, 1996). In the presence of glucose, the sporulation frequency in *kinA*$^-$ cells mimicked that of *kinA*$^+$ cells, resulting in a 10^5-fold repression of sporulation, suggesting that glucose inhibition of sporulation is not achieved through a reduction in *kinA* expression. In the *crsA kinA* double mutant in the absence of glucose, the sporulation frequency was identical to that of the *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 *kinB* is known to be repressed by SinR (Dartois *et al.*, 1996), and the *sinI: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 *spo0H* knockout on sporulation in both JH642 and GBS10 strains. As was expected, the loss of the sporulation sigma factor σ^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 *spo0H* disruption was previously reported to be not suppressed by the presence of the *crsA* mutation (Boylan *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, *kinA* transcription levels were not affected by readthrough from the *orfX* promoter, even in the presence of the *crsA* mutation, and *kinA* expression was regulated by a means as yet undetermined. Lastly, the presence of σ^H was necessary for sporulation in both JH642 and GBS10.

D. Investigation of σ^H activity.

Table 4. The sporulation efficiencies of JH642 and GBS10 strains containing Δ*kinA*, Δ*orfX,* and Δ*spo0H* mutations.

genotype	sporulation efficiency*			
	JH642		GBS10	
	SSM **	SSMG ***	SSM	SSMG
strain only	6.5×10^{-1}	1.0×10^{-5}	9.5×10^{-1}	1.0×10^{0}
kinA^{-}	1.5×10^{-1}	2.0×10^{-5}	9.3×10^{-1}	1.2×10^{-1}
orfX^{-}	7.1×10^{-1}	8.5×10^{-5}	9.1×10^{-1}	8.8×10^{-1}
spo0H^{-}	$<7 \times 10^{-6}$	$<4 \times 10^{-7}$	$<9 \times 10^{-6}$	$<9 \times 10^{-7}$

* 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 σ^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 σ^H activity decreased in wild type *B. subtilis*. It was previously suggested that changes in the *sinI:sinR* ratio might result in lower levels of free SinR, which may result in the increased activity of some σ^H-dependent promoters, as SinR is known to negatively regulate the σ^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 *spo0F* 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 σ^H activity. Consequently, a more in depth examination of σ^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 σ^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 σ^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 Tn*917*, 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_1). 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 (T_1) 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 T_0 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

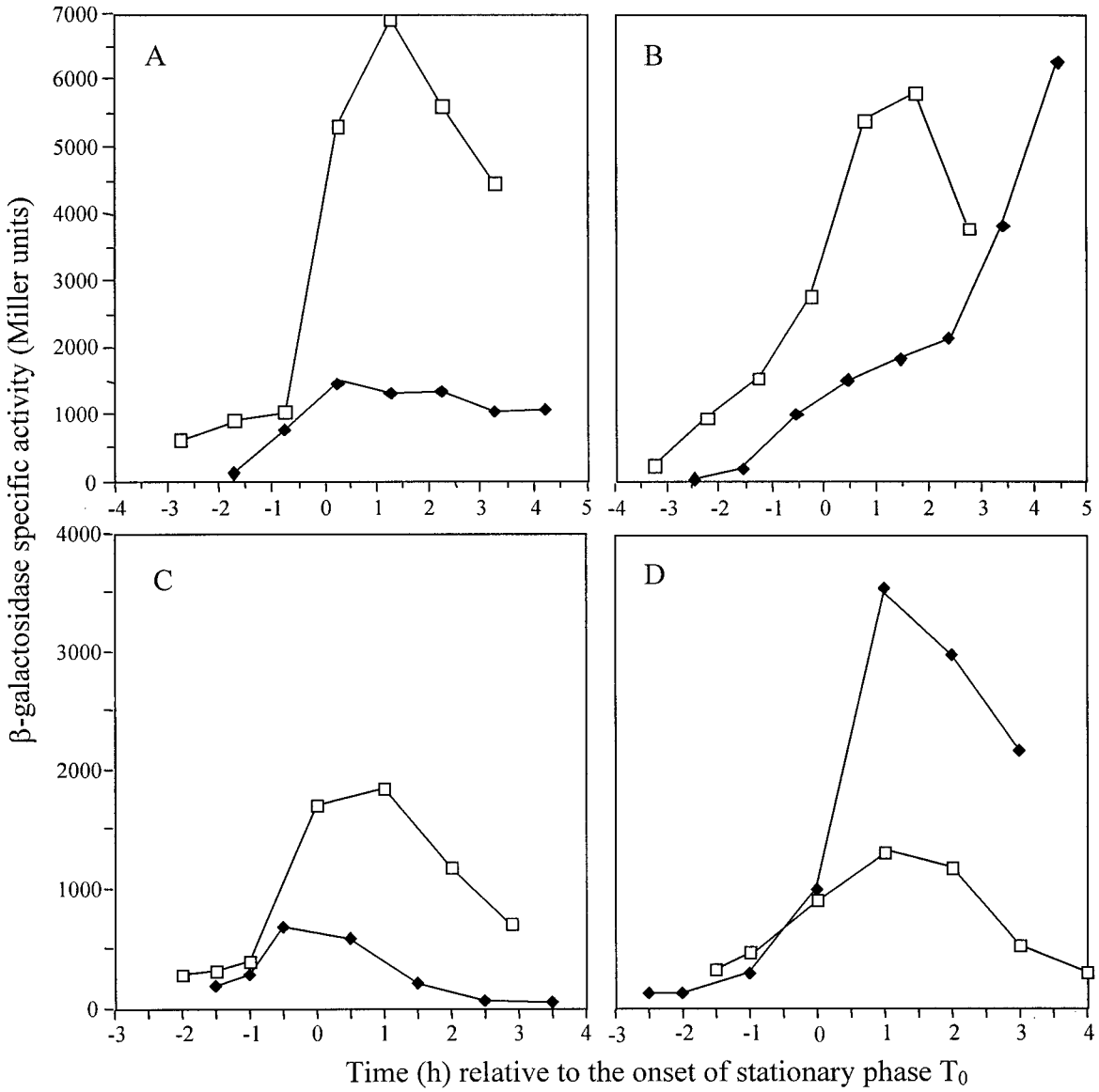


Figure 20. Expression of the *spoVG*42 promoter-*lacZ* reporter gene fusion inserted in the *amyE* gene (A and B), and of the *spoVG* promoter-*lacZ* reporter gene fusion inserted upstream of the *spoVG* (C and D), in strains JH642 (A and C) and GBS10 (B and D). Strains were grown in Schaeffer's spore 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 σ^H activity. A culture medium containing high levels of glucose, but buffered to a neutral pH, yielded cells with higher σ^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 σ^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 σ^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 σ^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

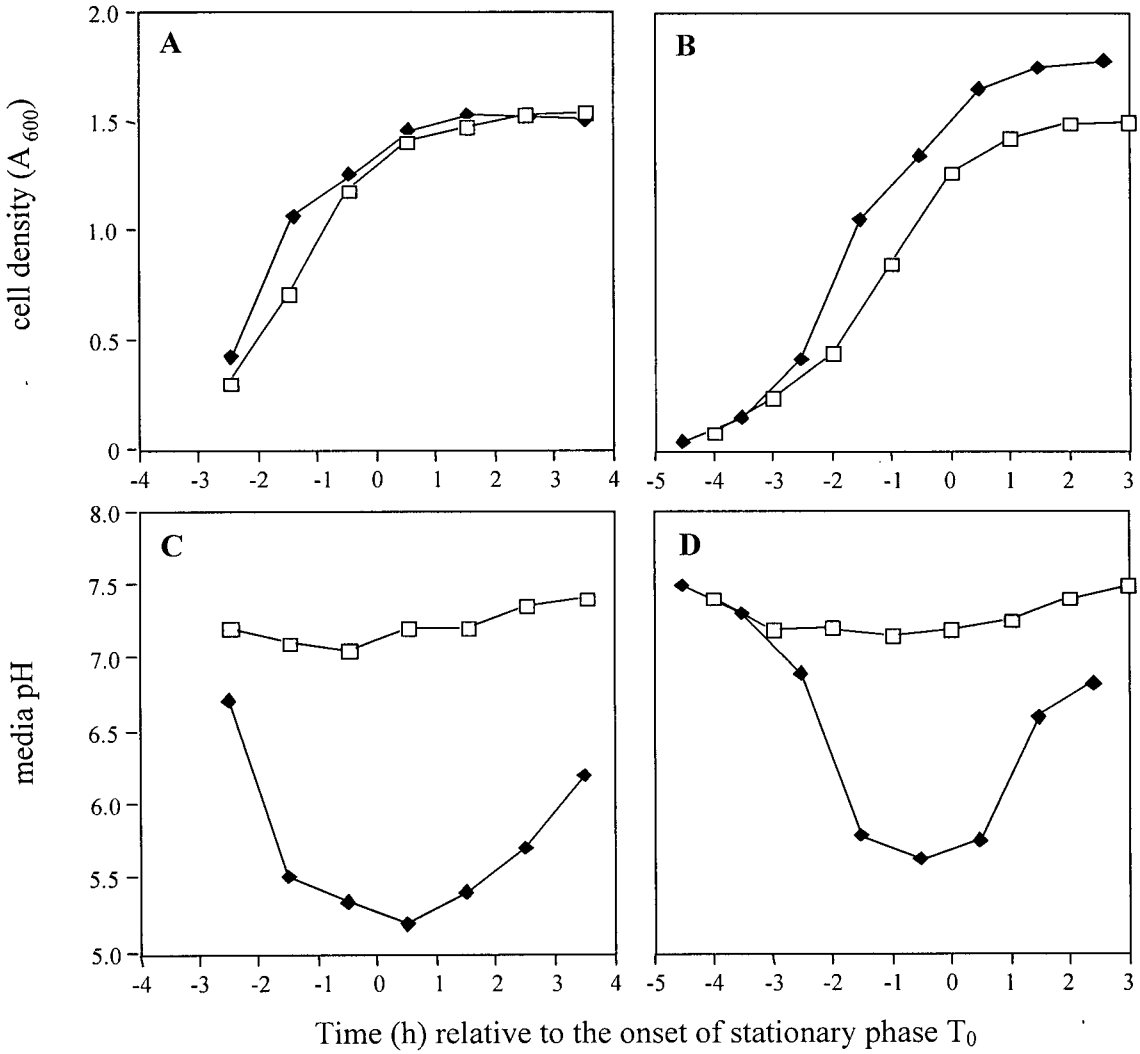


Figure 21. Growth pattern and pH profile of *B. subtilis* strains JH642 (A and C) and GBS10 (B and D). Strains were grown in unbuffered Schaeffer's spore medium, pH 7.5, with (closed diamonds) or without (open squares) 0.2% added glucose.

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 *kinA-lacZ* fusion (inserted in *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), *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 *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 *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 *kinA* promoter expression during the early phase of sporulation initiation than was seen in unbuffered medium, with peak activity unchanged. When glucose was added, *kinA-lacZ* activity increased sharply, regardless of pH, and the timing of expression was only marginally affected, with peak levels in unbuffered medium

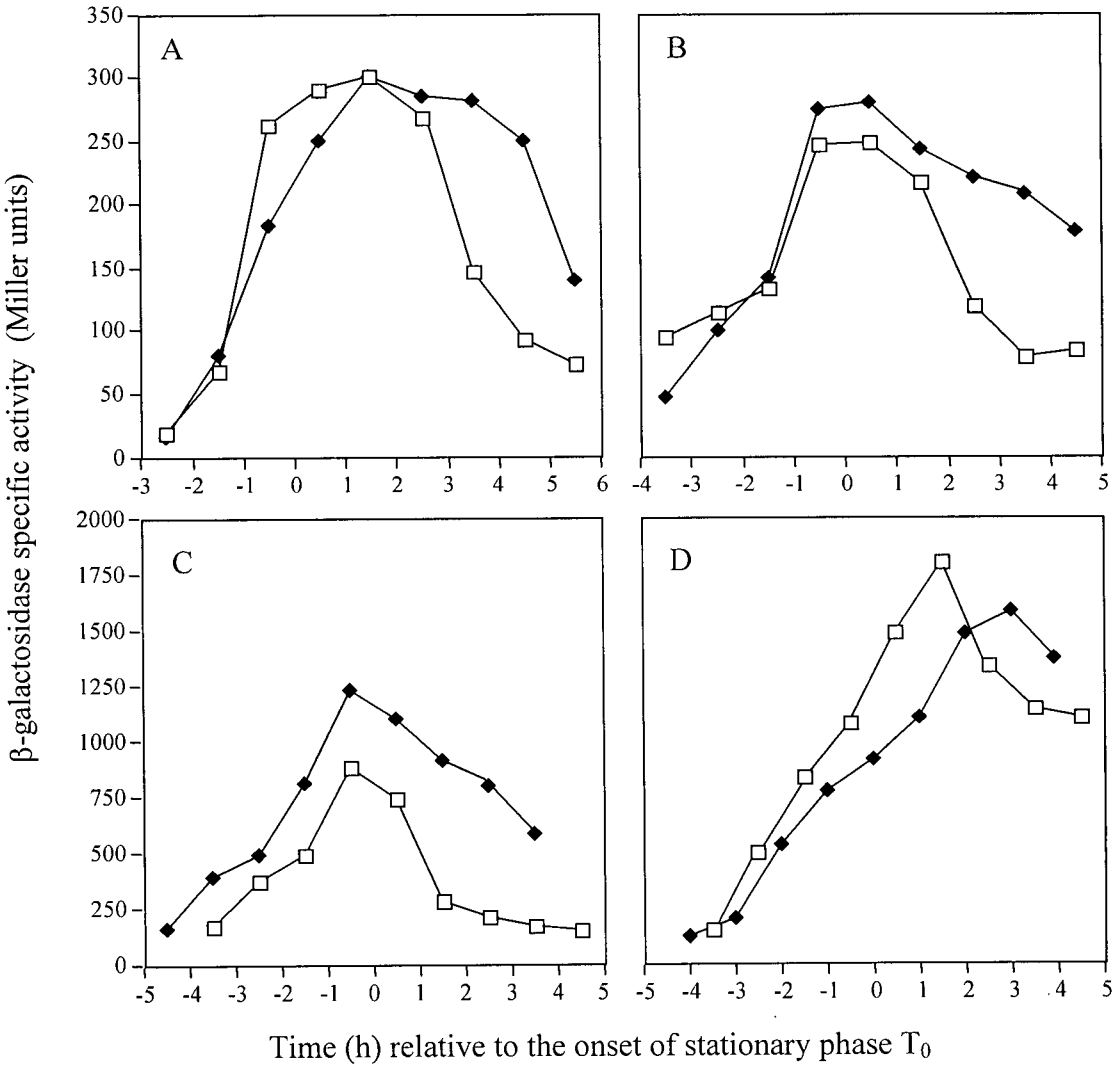


Figure 22. Expression of the *kinA* promoter-*lacZ* reporter gene fusion in the *kinA* gene in strains grown in media at different pH. Strains are JH642 (A and B) and GBS10 (C and D). Strains were grown in Schaeffer's spore medium at pH 5.2 (closed diamonds; used 70 mM MES buffer) or at pH 7.5 (open 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 σ^H, either in the presence or absence of glucose. Therefore, the increase in σ^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-*lacZ* fusion analyses in Results, section B concerned the unusual early expression from the *spo0A* dual σ^A/σ^H promoters in GBS10. This early expression was also seen in previously published data, in which the early derepression of the *spo0A* σ^A promoter was noted in a *crsA* mutant strain using an S1 nuclease protection assay (Chibazakura *et al.*, 1991). While that report stated that *spo0A* σ^H-dependent promoter activity was increased early in the presence of the *crsA* mutation, early P_V promoter activity and the high P_S promoter activity were not discussed (Chibazakura *et al.*, 1991).

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

1. Effect of a *spo0H* knockout on *spo0A* promoter activity.

The details of the creation of the *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 T_0.

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 T_0 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,

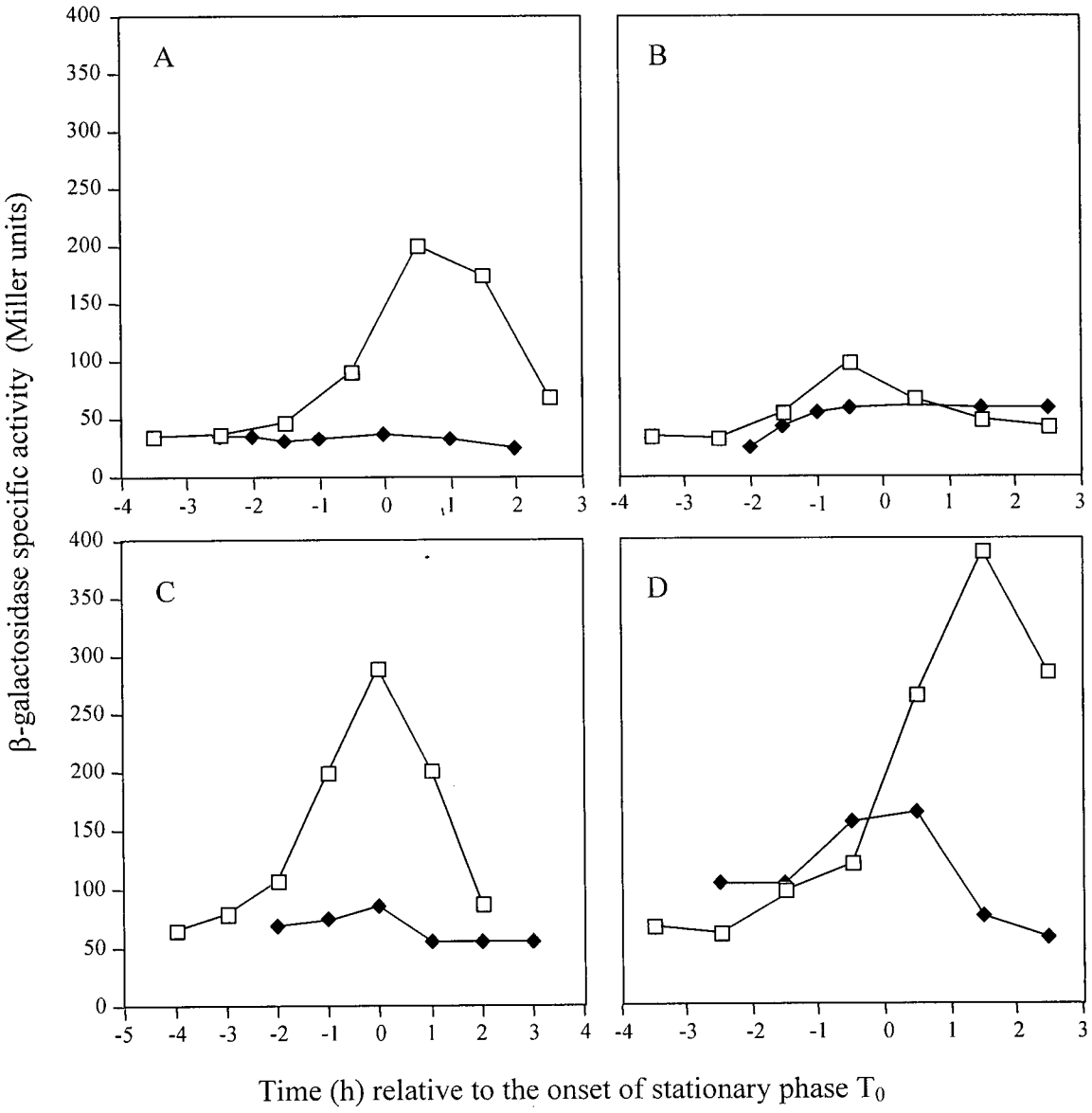


Figure 23. Expression of the *spo0A* promoter-*lacZ* reporter gene fusion in *spo0H*$^+$ and Δ*spo0H B. subtilis* strains. The promoter construct was inserted in the *amyE* gene in strains JH642 (A and B) and GBS10 (C and D) with (closed diamonds) and without (open squares) a kanamycin-linked *spo0H* 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 *spo0H*$^+$ strain, peaking at roughly 3-times the level seen in JH642 *spo0H*$^-$ in the presence of glucose. Transcription did not increase sharply at T_0, presumably due to the lack of σ^H. It is interesting to note that the level of transcription seen in the GBS10 *spo0H*$^-$ strain in the presence of glucose was roughly 80% of that seen in the JH642 *spo0H*$^+$ cells in the absence of glucose, and the timing of transcriptional activity was very similar.

The results seen in Figure 23 suggest that *spo0A* promoter activity in GBS10 *spo0H*$^-$ cells in the presence of glucose was roughly equivalent to the *spo0A* promoter activity in JH642 *spo0H*$^+$ cells in the absence of glucose. Clearly, the transcriptional regulation of this promoter was altered in GBS10; both σ^A- and σ^H-dependent expression of *spo0A* 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 *spo0A* P_S promoter deletion.

Because the loss of σ^H due to the *spo0H* gene disruption could have other effects which may impact on the regulation of stationary phase genes (such as *sinI*, *spo0F* and *kinA*), which in turn may affect the apparent activity of the *spo0A-lacZ* fusion, a second approach was used in examining the σ^A-dependent activity of the *spo0A* promoter. Figure 24 details the creation of the *spo0A*ΔP_S-*lacZ* construct for use in JH642 and GBS10 strains. This construct was created using the restriction enzymes *Ssp*I and *Hpa*I located 5' and 3' of the σ^H promoter (respectively, from bases –59 to +18 relative to the P_S transcription start site). This deletion simultaneously deleted both the σ^H promoter, and the SinR regulator binding site (from bases –23 to –3, relative to the P_S transcription start site) that overlaps the P_S promoter

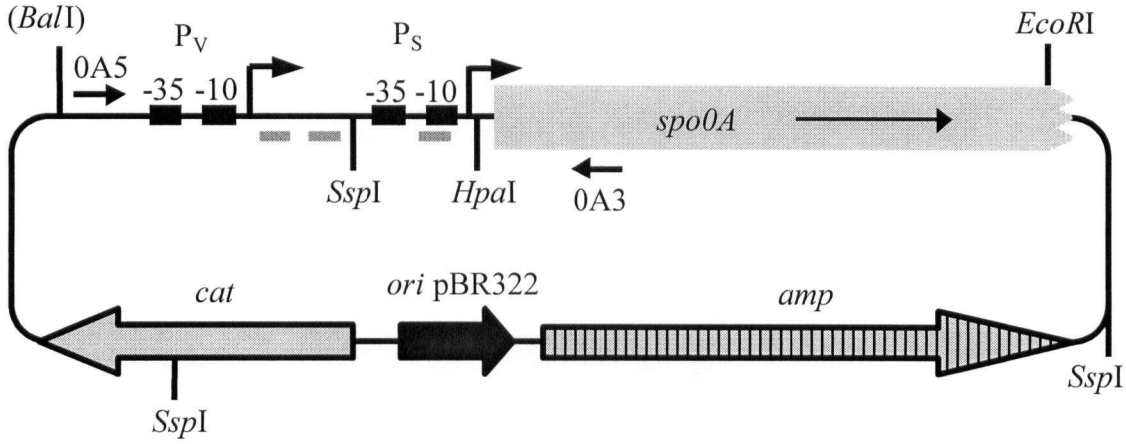


Figure 24. The plasmid pJH1408 and *spo0A* promoter-*lacZ* cloning strategy. To remove the sporulation promoter from the *spo0A* promoter fragment on pJH1408, the plasmid was first linearized by a 2 minute *Ssp*I digest, and then fully digested with *Hpa*I. The DNA was then recircularized in a dilute 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 *spo0A* promoter with engineered *EcoR*I/*BamH*I ends, which was then ligated directionally into *EcoR*I/*BamH*I digested pDH32 (see Figure 1).

0A5: 5' CGT**GAATTC**CGATATGGACACAAAG 3' (*EcoR*I)
0A3: 5' TC**GGATCC**ATGTCTTCCTGTCCTT 3' (*BamH*I)

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. (▬)

(Mandic-Mulec *et al.*, 1995). Because SinR is thought to repress transcription from the *spo0A* promoter by interfering with $E\sigma^H$ binding to the P_S promoter (Mandic-Mulec *et al.*, 1995), the deletion of the SinR binding site in the *spo0A*ΔP_S mutant was expected to lack the SinR-dependent negative affect on the *spo0A* P_V promoter transcriptional activity.

3. Effect of the *spo0A*ΔP_S deletion on *spo0A* promoter activity.

Figure 25 depicts the *spo0A*ΔP_S-*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 *spo0A*ΔP_S 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 *spo0A*ΔP_S promoter in the presence of glucose in both strains were, again, quite similar. In these cases, the *spo0A*ΔP_S promoter showed a large induction in media containing glucose prior to and at the onset of stationary phase. After T_0, promoter activity began to decline. The activity of the *spo0A*ΔP_S 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 *spo0A*ΔP_S promoter in both JH642 and GBS10 strains drop after T_{-1} to T_0, in the absence of glucose; secondly, what caused the induction from the *spo0A*ΔP_S promoter in both strains between T_{-2} and T_0 seen in the presence of glucose? Observations from a previously

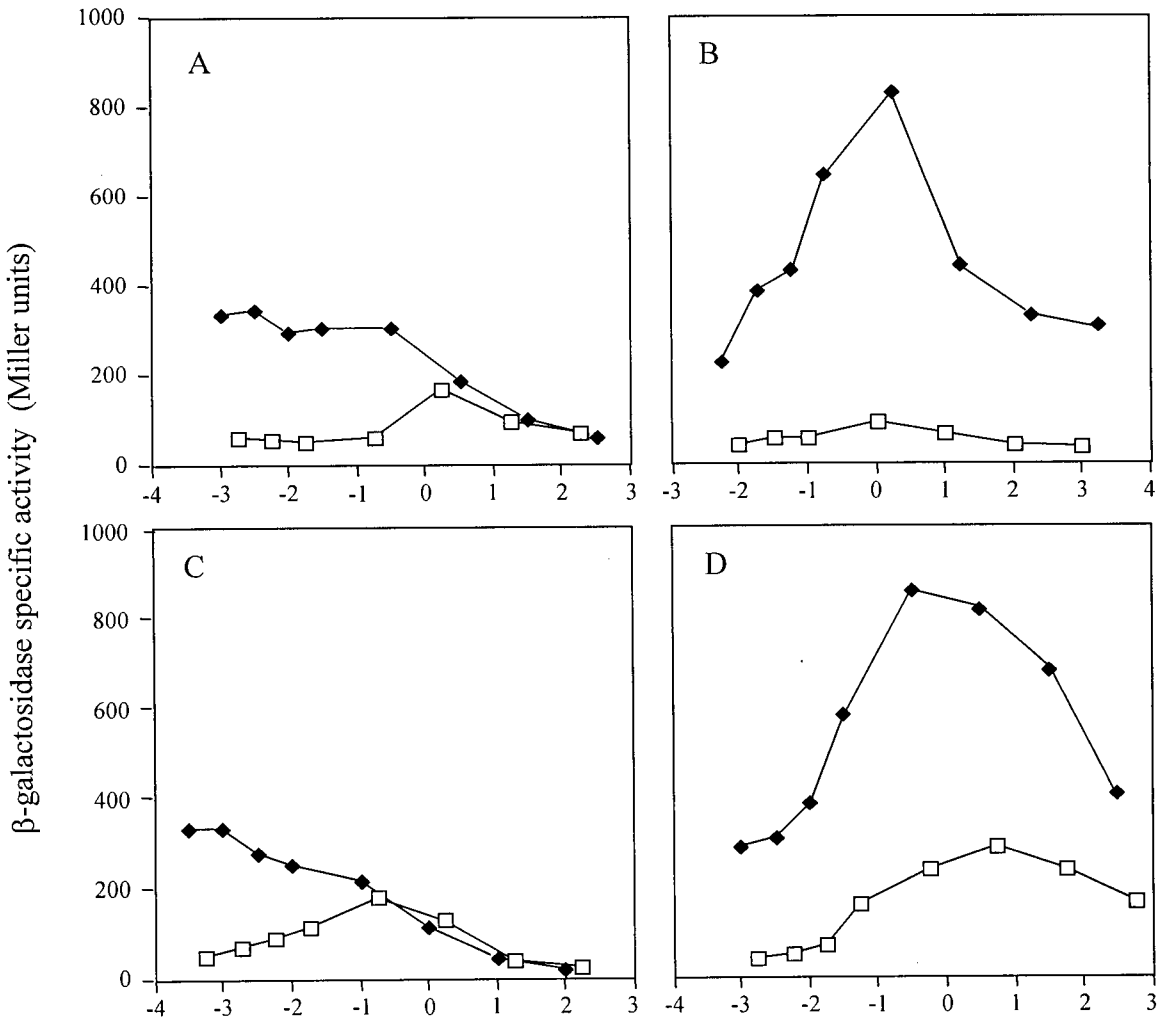


Figure 25. Expression of the *spo0A* promoter- and the *spo0A*ΔP_S promoter-*lacZ* reporter gene fusions (open squares and closed diamonds, respectively) in strains JH642 (A and B) and GBS10 (C and D). Strains were grown in Schaeffer's spore medium, pH 7.5, with (B and D) and without (A and C) 0.2% added glucose.

published paper may shed light on the first issue. Strauch, et al. (1992) created both the *spo0A*ΔP_S (identical to the mutant created in this thesis) and the *spo0A*ΔP_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~P inhibited transcription from the *spo0A* P_V promoter after T_0 and that Spo0A~P activated the *spo0A* P_S promoter after T_0. If this were the case, it would explain the decrease in transcription noted in Figures 25A-D in the *spo0A*ΔP_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_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 *spo0A*ΔP_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 $E\sigma^{A47}$ had a higher affinity for the *spo0A* P_V promoter than $E\sigma^A$, 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_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^H and a lack of SinR

negative regulation, is sufficient to allow sporulation in the presence of glucose.

To construct the *spo0A*ΔP_S knockout in JH642 and GBS10 cells, the *Eco*RI-*Bam*HI 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 Δ*sinR* 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, *spo0A*ΔP_S 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 spo0A*ΔP_S 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 spo0A*ΔP_S 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*ΔP_S and Δ*sinR* mutations.

genotype	sporulation efficiency*			
	JH642		GBS10	
	SSM**	SSMG***	SSM	SSMG
spo0A$^+$/*sinR*$^+$	6.5×10^{-1}	1.0×10^{-5}	9.5×10^{-1}	1.0×10^{0}
spo0A ΔP_S	2.5×10^{-5}	2.2×10^{-7}	6.0×10^{-1}	2.9×10^{-1}
sinR$^-$	7.4×10^{-1}	2.5×10^{-1}	ND[A]	ND

* 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* P_V 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 σ^H-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* P_V promoter (which is implicated in a glucose resistant phenotype, compare Figure 23 and Table 6). It is the 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 $E\sigma^{A47}$ may have a higher affinity for the *spo0A* σ^A promoter than $E\sigma^A$. As a direct test of this hypothesis, $E\sigma^{A47}$ was purified from GBS10 for examination in *in vitro* transcription assays.

1. Isolation of $E\sigma^{A47}$.

Figure 26A shows an SDS-PAGE of purified $E\sigma^{A47}$ 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 $E\sigma^{A47}$.

Figures 27 through 29 show preliminary characterizations of the activity of $E\sigma^{A47}$ on the *spo0A* σ^A 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

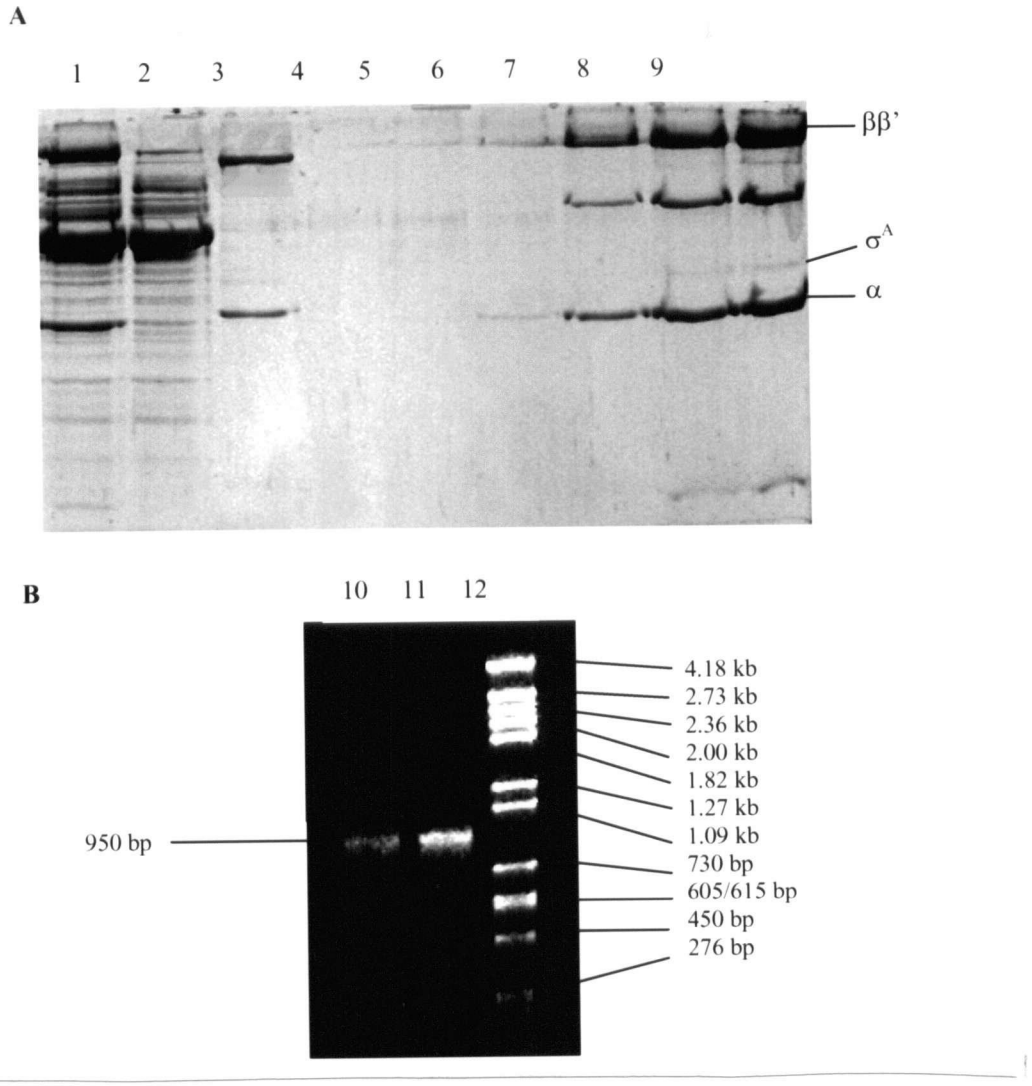


Figure 26. Purification of protein and DNA components of the transcription reaction. (A) Coomassie blue stain of a 12% SDS-PAGE gel of purified $E\sigma^{A47}$, with the RNA polymerase core enzyme components ($\alpha_2\beta\beta'$) and σ^A highlighted on the right. (B) Ethidium bromide stain of a 0.7% agarose gel of purified *spo0A* 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 $E\sigma^A$ 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 *spo0A* promoter fragment (60 ng; see Figure 24 for PCR details). 11-Purified *spo0A* promoter fragment (120 ng). 12-ϕ29 DNA digested with *Hind*III. Fragment sizes are listed to the right.

0A5: 5' CGT**GAATTC**CGATATGGACACAAAG 3' (*Eco*RI)
0A3: 5' TC**GGATCC**ATGTCTTCCTGTCCTT 3' (*BamH*I)

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 α^{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 Eσ^{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

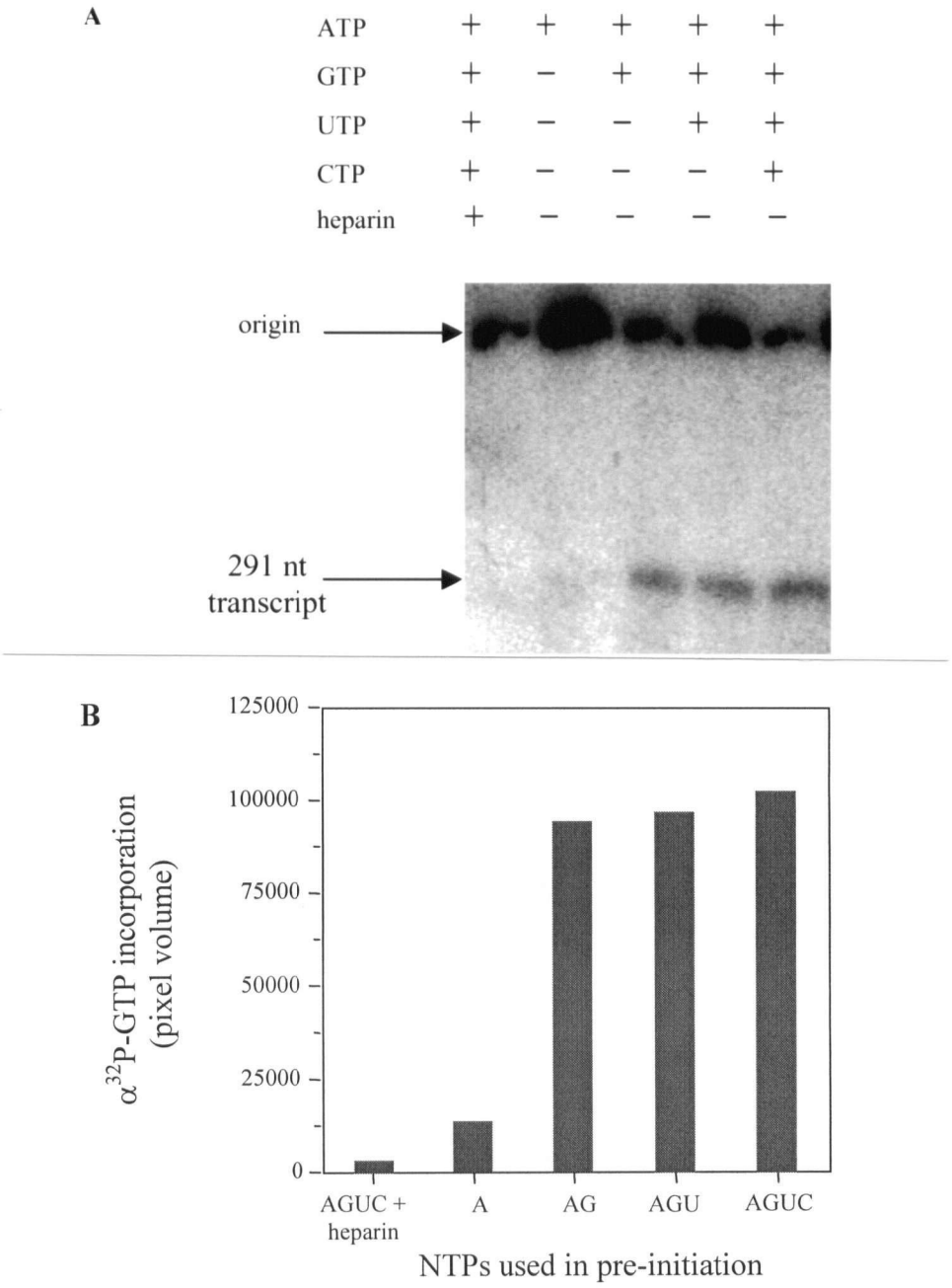


Figure 27. Nucleotide requirements for heparin resistance at the σ^A dependent *spo0A* promoter. (A) Autoradiograph of the gel used to separate the transcription products. (B) A graphical representation of the results shown in (A), using relative intensity calculated from a phosphorimager scan. Various combinations of nucleotides were preincubated with $E\sigma^{A47}$ 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 $E\sigma^{A47}$ 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.

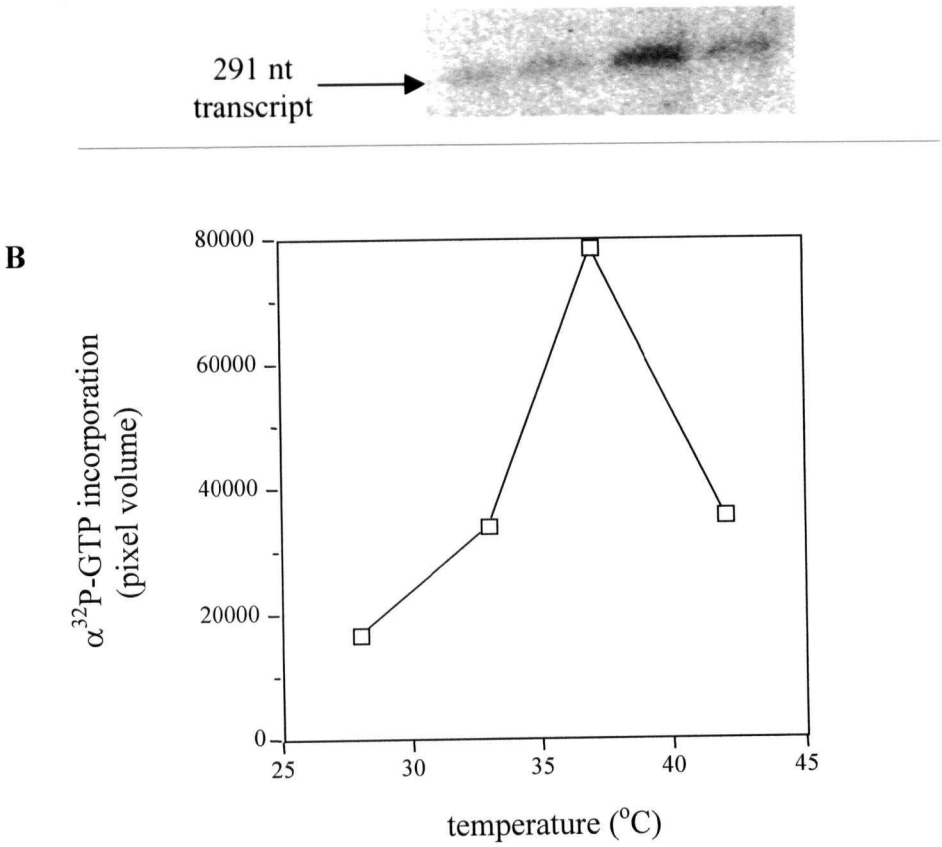


Figure 28. The effect of temperature on $E\sigma^{A47}$ transcription of the *spo0A* σ^A dependent promoter. All transcriptions included a 2 minute A+G pre-initiation. Pre-initiation, initiation and elongation were carried out at the indicated temperature, and the transcripts produced were separated 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 $E\sigma^{A47}$ and $E\sigma^{A}$. 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. $E\sigma^{A}$ transcription increased slightly between 50 and 95 mM salt, and began to drop at 110 mM potassium acetate. $E\sigma^{A47}$ 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 $E\sigma^{A47}$ was more salt-sensitive at the *spo0A* promoter at higher salt concentrations than was $E\sigma^{A}$. 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 *spo0A*ΔP_S promoter was transcribed *in vivo* at higher levels in GBS10 than in JH642. This suggested the possibility that $E\sigma^{A47}$ had a higher activity on the *spo0A* σ^{A} promoter than did $E\sigma^{A}$. In order to compare $E\sigma^{A47}$ and $E\sigma^{A}$ 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

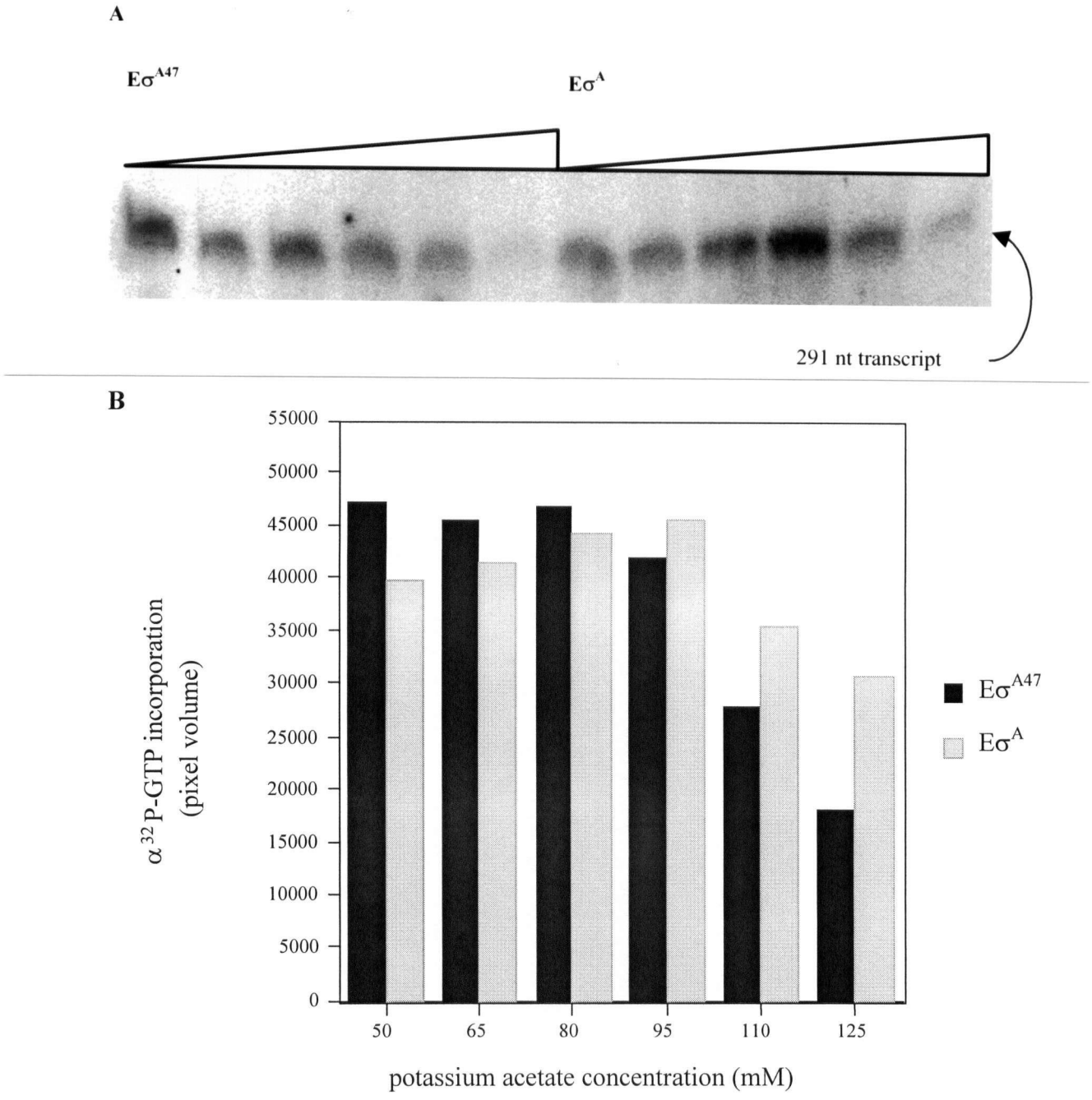


Figure 29. The effect of potassium acetate concentration on transcription from the *spo0A* promoter. (A) Autoradiograph of the gel used to separate the transcription products produced by $E\sigma^{A47}$ (left) and $E\sigma^{A}$(right). Potassium acetate concentrations, from left to right, are 50 mM, 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, $E\sigma^A$, and $E\sigma^{A47}$, 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 $E\sigma^A$ in Figure 30B, whereas Figure 30C had a slope of 65334 pixels/nM DNA for $E\sigma^{A47}$. 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 $E\sigma^A$ and $E\sigma^{A47}$ 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* P_V 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

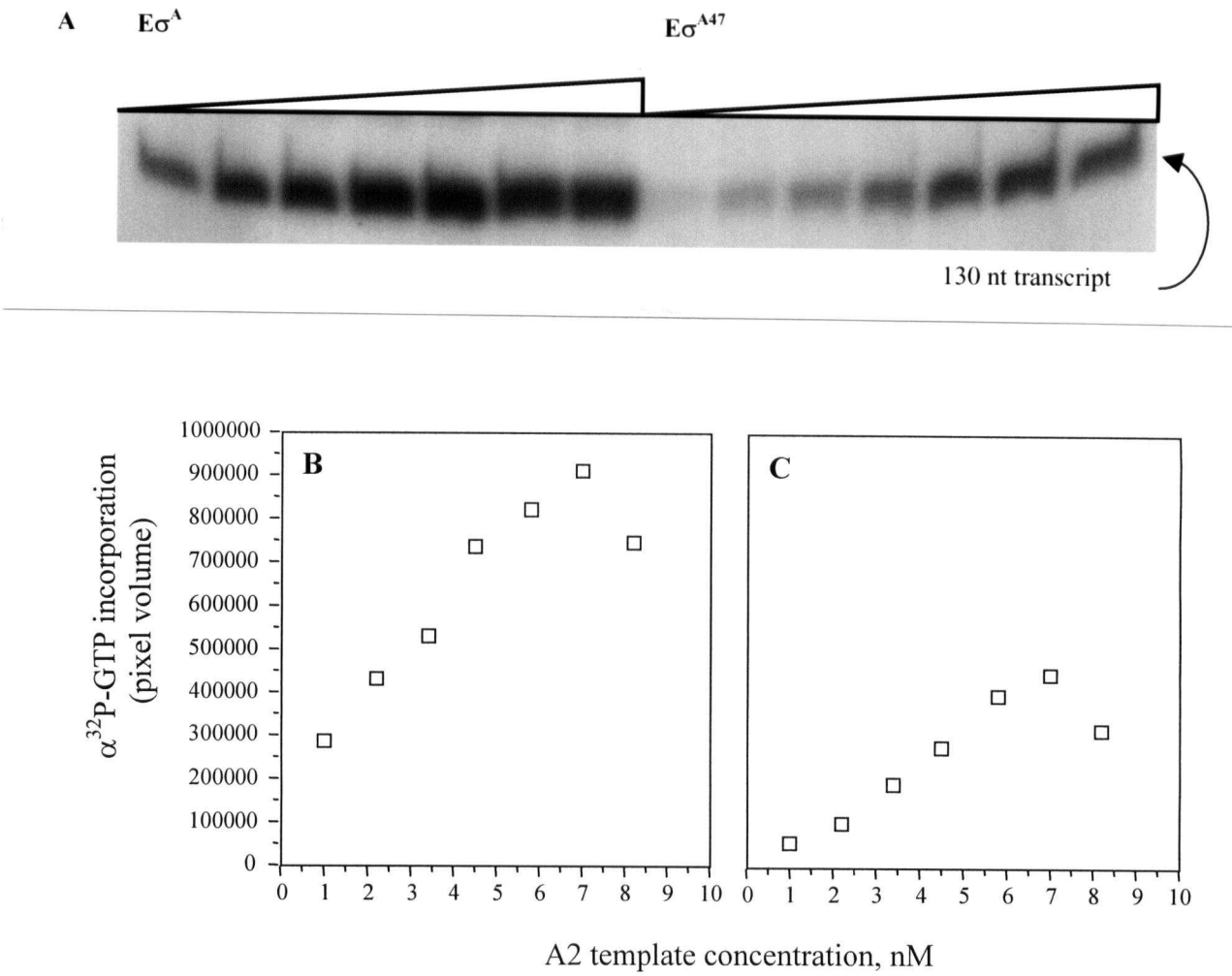


Figure 30. DNA input assay using the $\phi 29$ phage A2 promoter DNA. (A) Transcription from the A2 promoter using RNA polymerase isolated from JH642 (left) and GBS10 (right), and with increasing DNA concentrations. DNA levels from left to right are 1.0 nM, 2.2 nM, 3.4 nM, 4.6 nM, 5.8 nM, 7.0 nM, and 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.

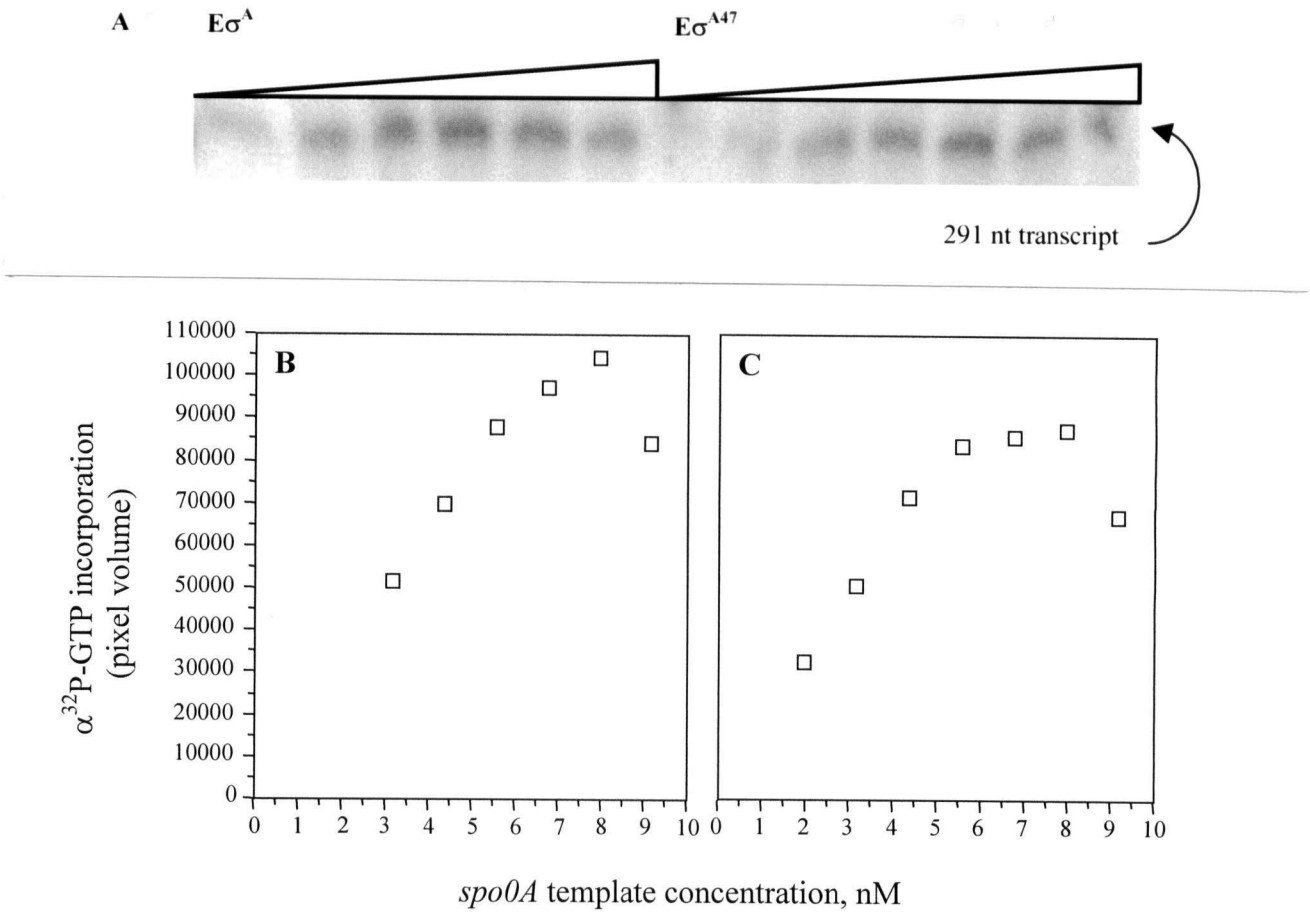


Figure 31. DNA imput assay using *spo0A* promoter DNA. (A) Transcription from the *spo0A* σ^A promoter using RNA polymerase isolated from JH642 (left) and GBS10 (right), and with increasing DNA concentrations. DNA levels from left to right are (for $E\sigma^{A47}$ only) 2.0 nM, 3.2nM, 4.4 nM, 5.6 nM, 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 $E\sigma^A$ and $E\sigma^{A47}$, based on the ϕ29 promoter as calculated in Figure 30, $E\sigma^{A47}$ appeared to have a two-fold higher activity on the *spo0A* promoter than $E\sigma^A$. 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 $E\sigma^{A47}$ and $E\sigma^A$ 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

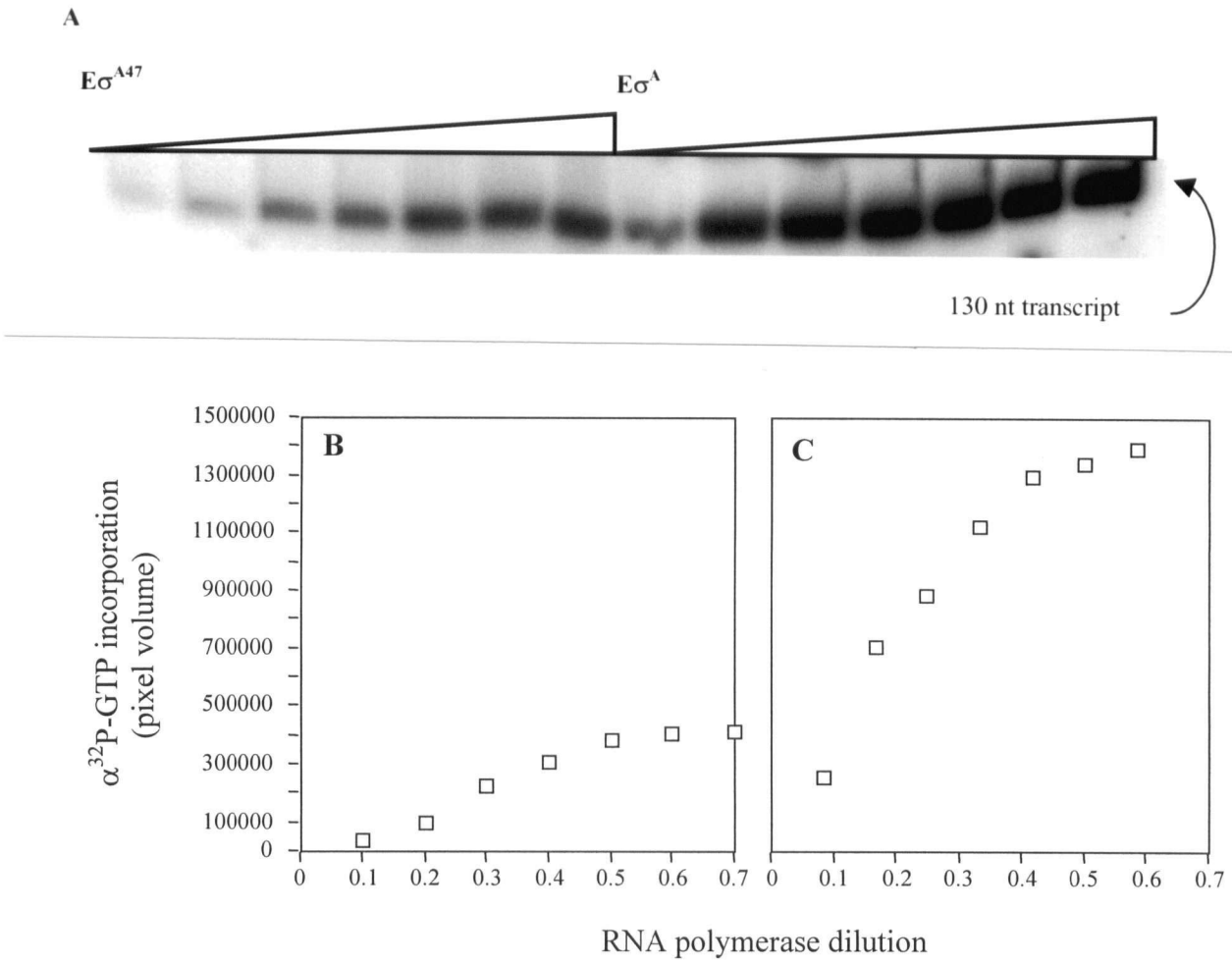


Figure 32. RNA polymerase input assay using 5.5 nM ϕ29 phage A2 promoter DNA. (A) Transcription from the A2 promoter using increasing concentrations of RNA polymerase isolated from GBS10 (left) and JH642 (right). (B and C) Intensities of the transcription bands were graphed vs. template concentration. (B) *crsA* mutant RNA polymerase. (C) wild type RNA polymerase.

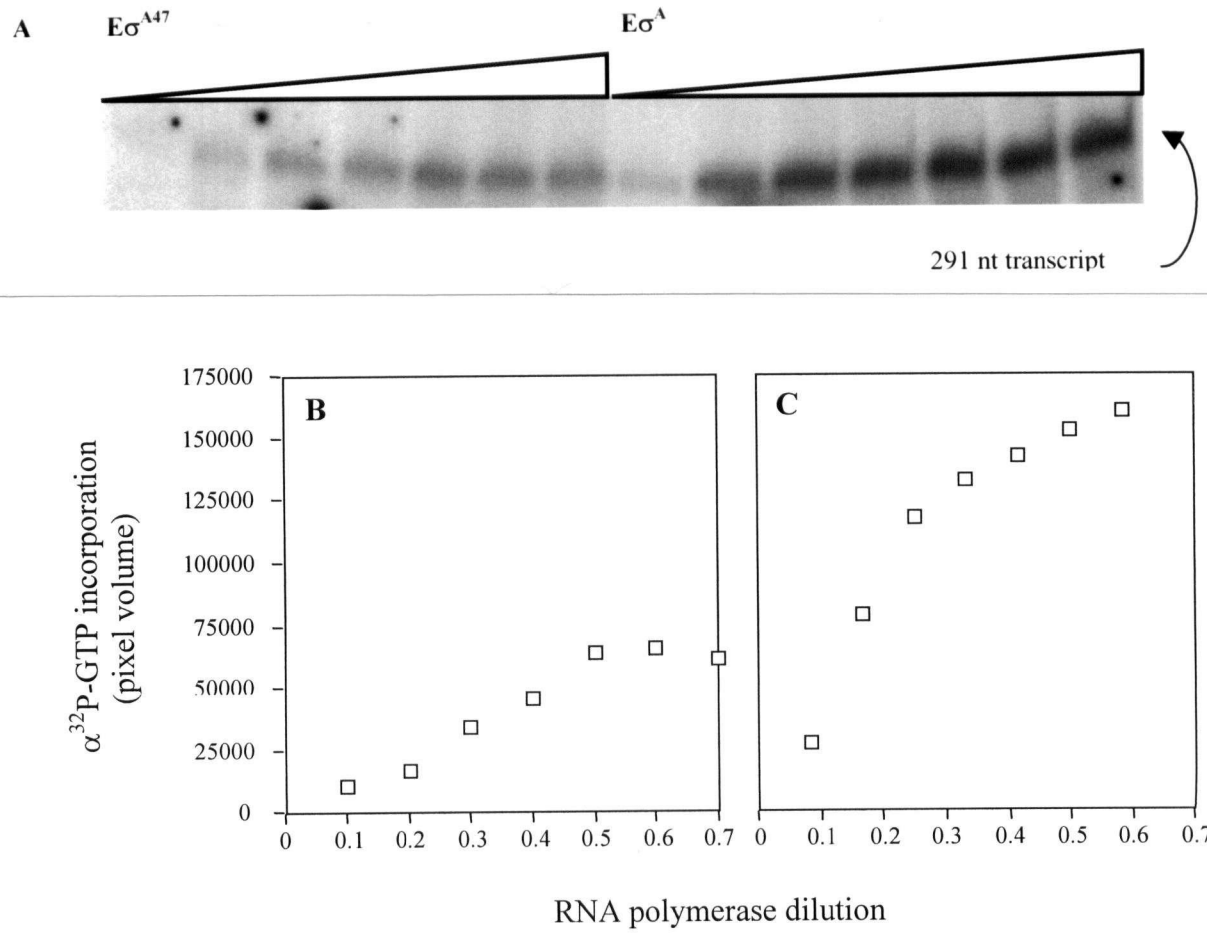


Figure 33. RNA polymerase imput assay using 5.5 nM *spo0A* promoter DNA. (A) Transcription from the *spo0A* promoter using increasing concentrations of RNA polymerase isolated from GBS10 (left) and JH642 (right). (B and C) Intensities of the transcription bands were graphed vs. template concentration. (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 $E\sigma^{A47}$ and $E\sigma^{A}$ 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, σ^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 σ^{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 σ^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 σ^A/σ^H *spo0A* promoter. Each of these effects was examined in some detail and is discussed below.

A. σ^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 σ^H, the protein product of the stage 0 gene *spo0H* (Carter and Moran, 1986; Dubnau *et al.*, 1988; Zuber *et al.*, 1989), and σ^A (Errington, 1993). σ^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, σ^A (Carter *et al.*, 1988) and other genes (Jaacks *et al.*, 1989), require σ^H for normal transcriptional activity during the *B. subtilis* growth cycle.

There is a poor correlation between the pattern of *spo0H* transcription, σ^H protein levels, and the expression of σ^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 σ^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 σ^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 σ^H in the transcription of several genes involved in sporulation initiation, it was hypothesized that either σ^H activity, or the activity of a regulator of σ^H-dependent promoters may be altered in the presence of the *crsA* mutation. Therefore, σ^H-dependent transcription was examined in some detail, to gain insight into whether σ^H activity or the regulation of σ^H-dependent promoters was altered in GBS10 in the presence of glucose, contributing to the glucose resistant sporulation phenotype of the mutant.

1. *spo0H* 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 *spo0H* 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 *spo0H* gene itself (reviewed in Grossman, 1995; Stragier and Losick, 1996).

The *spo0H* 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), *spo0H* 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 *spo0H* (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 σ^{H}. The mechanism of σ^{H} activation is discussed below, in section A.2.

Published observations show that σ^{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 σ^{H} activity and the presence of glucose are required for the development of competence in stationary phase cells. The need for σ^{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 (*sigA*$^{+}$ background) grown without glucose, the σ^{H}-dependent promoter fusions [Figures 6 (*kinA-lacZ*), 7 (*spo0F-lacZ*), and 11 (*spoVG-lacZ*)] exhibited a typical, transient σ^{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 (*crsA* 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 σ^H was activated earlier in the GBS10 transition phase than in JH642. However, these results did not suggest that σ^H activity was unusually increased during the early stationary phase of GBS10 cells grown without excess glucose.

The low level σ^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 σ^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 σ^H-dependent promoter expression was elevated in GBS10 cells grown in the presence of glucose

2. Possible mechanisms for σ^H activation.

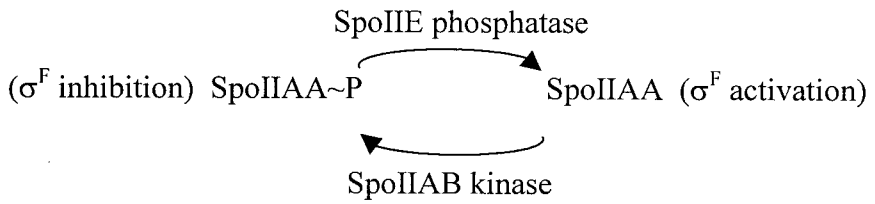
A number of observations have been made in recent years concerning the regulation of the activities of some sigma subunits in *B. subtilis.* Control of sigma factor activity has been demonstrated at many levels of expression, from transcription initiation of the sigma-encoding gene to the turnover of 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* σ^F and σ^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, 196; Helmann, 1999; Kroos *et al.*, 1999). Prior to asymmetric septation of cells, σ^F is held in an inactive complex, which is regulated as shown in the schematic below (adapted from Kroos *et al.*, 1999).

$$\text{SpoIIAA} + \text{SpoIIAB}\cdot\sigma^F \underset{\text{ATP}}{\overset{\text{ADP}}{\rightleftharpoons}} \text{SpoIIAA}\cdot\text{SpoIIAB} + \sigma^F$$



The anti-anti-sigma factor SpoIIAA can complex with either σ^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). σ^G has been shown to be held inactive as a result of binding to SpoIIAB (Kellner *et al.*, 1996); in this instance, σ^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 σ^H activity in *B. subtilis* is, or is not modulated *via* sigma factor binding to an anti-sigma factor. However, σ^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 σ^F (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 σ^E and σ^K are initially made as inactive precursors that must undergo proteolytic processing prior to becoming active; pro-σ^E cleavage involves the removal of 27 N-terminal amino acids (LaBell *et al.*, 1987; Miyao *et al.*, 1993), and pro-σ^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 σ^H activity in *B. subtilis* is controlled by a pro-sigma factor cleavage event, as the apparent molecular weight of σ^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 σ^H protein levels and therefore σ^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 σ^H, *via* proteolysis, in response to acid stress

(Liu *et al.*, 1999). The ClpX ATPase has been found to be required for activation of σ^H in media with neutral pH, in response to nutritional stress (Liu *et al.*, 1999). However, a mechanism for ClpX activation of σ^H has not been demonstrated.

Levels of σ^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 σ^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 σ^H activity observed in GBS10 cells seen in this thesis was not likely to be due to transcriptional regulation of *spo0H*. Differences in σ^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 σ^H activity (Liu *et al.*, 1999) have been observed to affect both the level and the timing of σ^H-dependent promoter expression, and so are good candidates for regulating σ^H activity.

3. σ^H-dependent transcription in the *crsA* mutant was deregulated.

The increase in σ^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 σ^H-dependent genes in GBS10. It is possible that the *spo0H* gene was more actively transcribed in GBS10 cells than in JH642 cells, resulting in higher σ^H protein levels. These higher levels cannot be due to decreased AbrB repression of *spo0H*, 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 σ^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 Δ*clpP* mutant (ClpP is implicated in σ^H proteolytic degradation; Liu *et al.*, 1999) exhibits earlier expression of the *spoVG42-lacZ* fusion than *clpP*$^+$ cells, although *spoVG42-lacZ* expression in the Δ*clpP* mutant was not increased beyond that seen in *clpP*$^+$ cells (Liu *et al.*, 1999). The transcription pattern of *spoVG42-lacZ* observed in Δ*clpP* cells during the late exponential phase of growth was similar to the pattern of σ^H-dependent promoter-*lacZ* expression seen in GBS10 in this thesis, suggesting that a premature increase in σ^H protein stability may be involved in the early σ^H-dependent transcription seen in GBS10 cells grown in the absence of glucose.

σ^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 σ^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 σ^H-dependent promoters observed in GBS10 cells grown in the presence of glucose is unclear. ClpX is implicated in σ^H activation at neutral pH; however, although Δ*clpX* mutants exhibit higher σ^H protein levels in the presence of excess glucose, σ^H-dependent promoter activity was not increased (Liu *et al.*, 1999). Lon proteases are implicated in σ^H degradation during acid stress; however, Δ*lon* mutants also do not exhibit dramatically high σ^H-dependent expression in the presence of excess glucose (Liu *et al.*, 1999). There are no currently published observations of abnormal σ^H activity with a pattern of σ^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 σ^{A47} resulted in increased σ^H activity in strains grown in the presence of glucose.

4. σ^H activation in the *crsA* mutant was not affected by reduction of pH.

Although σ^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 σ^H-dependent *spoVG* expression in stationary phase cells (Cosby and Zuber, 1997). The reduction in σ^H-dependent gene expression was explained by the reduced intracellular concentration of σ^H, which was associated with the continued presence of AbrB well past the onset of stationary phase. Stimulation of σ^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 σ^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 σ^H turnover was affected, because an earlier study showed different half-lives for σ^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 σ^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 σ^H-dependent *kinA* promoter activity despite the pH drop incurred through the metabolism of glucose (see Figures 21C and 21D). The persistence of σ^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 σ^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 σ^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 σ^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 σ^H activity: in a low pH, low glucose-containing medium, the effect on σ^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), σ^H activity was repressed (Cosby and Zuber, 1997; Liu *et al.*, 1999).

The above observations suggest that the published pH effect on σ^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 σ^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 σ^H activation. If this were true, however, then the mechanism behind the activation of σ^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 σ^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 σ^H-dependent promoter activity observed with the *kinA-lacZ* fusion in GBS10 in the presence of 0.2% glucose involved a σ^{A47}-mediated immunity to the effects of low pH.

5. The activity of the *kinA* promoter.

The high level of *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 *crsA* mutant. The observed increase in the expression from both *spo0F* and *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 *spo0F* and *spo0A* σ^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 *spo0P* and *spo0L* during the transition state (see Figures 12 and 13). The increase in *kinA* expression may result in sufficient KinA levels to overcome the activity of the phosphorelay phosphatases. Because the increase in *kinA* expression in GBS10 could not be explained by an increase in phosphorelay activity, the *kinA* promoter was examined in more detail to reveal potential regulatory sequences involved in *kinA* expression.

5.a. *kinA* transcription is independent of the phosphorelay.

The *kinA* gene is transcribed from a σ^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 σ^H activity at this time. Firstly, transcription of the *spo0H* gene (encoding σ^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, σ^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 σ^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 σ^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 σ^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 σ^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 σ^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 σ^H in these strains, and that there is no nutritional regulator affecting *kinA* expression.

6. σ^H activity in later stages of sporulation.

The loss of σ^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 σ^A/σ^B dual promoters during heat shock and stationary phase growth (Nanamiya *et al.*, 1998). Δ*clpC* strains overproduce a repressor of *clpP* transcription (the ClpP protease is implicated in σ^H degradation), and contain high levels of σ^H protein as late as T_4 in stationary phase. Furthermore, the activity of the *spo0A* P_S promoter in the Δ*clpC* 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 σ^H was more stable in the mutant than in wild type cells (Nanamiya *et al.*, 1998).

The σ^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 σ^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 σ^H began to decrease during this time, and that

the degradation of σ^H was not affected in GBS10. Therefore, the σ^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 σ^H degradation during late stationary phase.

One experiment presented in this thesis, the analysis of the *spoVG42-lacZ* fusion, contradicts the claim that σ^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 σ^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 σ^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 σ^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 σ^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 σ^H-dependent *sinI-lacZ* fusion in GBS10 in the presence of glucose (Figure 15B) paralleled that observed for the other σ^H-dependent promoters examined in this thesis, supporting the hypothesis presented above that σ^H 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 T_0 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 *sinI:sinR* transcriptional activity. For the following analysis of Table 6, two assumptions were made: firstly, that the ratio of *sinI:sinR* expression is directly related to the ratio of SinI:SinR protein levels present in the cell; secondly, that the smallest *sinI:sinR* expression ratio obtained in JH642 grown in the absence of glucose (20:1) represents the minimum ratio of SinI:SinR protein levels required to sequester SinR and permit sporulation initiation. Given these assumptions, three conclusions were made: 1) the SinI:SinR ratio prior to and during stationary phase in GBS10 cells grown in the absence of glucose would permit sporulation; 2) the SinI:SinR ratio prior to the onset of stationary phase in GBS10 cells grown in the presence of glucose would inhibit sporulation; and 3) the SinI: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 Δ*sinR* mutant (Table 5), which sporulated 25000-times better in the presence of excess glucose than the $sinR^+$ strain. The sporulation frequency of the Δ*sinR* 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), *spoIIG* and *spoIIA* (Mandic-Mulec *et al.*, 1992). It has been suggested that SinR interferes with *spoIIA* and *spoIIG* transcription by binding in the

Table 6. Relative transcriptional activities of the promoters of the *sin* operon in *B. subtilis*.

time (h) relative to the onset of stationary phase, T_0	ratio[a] of observed *sinI:sinR* transcription levels			
	JH642		GBS10	
	SSM[b]	SSMG[c]	SSM	SSMG
T_{-1}	50:1	10:1	20:1	11:1
T_0	35:1	5:1	40:1	22:1
T_1	20:1	8:1	40:1	26:1

[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 T_0 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 Δ*sinR* strain during post-exponential phase growth, although the patterns were different from those observed in this thesis (Mandic-Mulec *et al.*, 1992). In the Δ*sinR* 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 *sinR*$^+$ cells.

The patterns of the *spoIIG* and *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 *sinI* transcription observed in GBS10 cells grown in the presence of glucose (Figure 15), and with the *sinI:sinR* ratios shown in Table 6.

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

C. The activity of the *spo0A* promoter.

1. *spo0A* transcription is regulated by nutrient availability.

A current model for the transcription from the dual σ^A/σ^H *spo0A* promoters throughout the *B. subtilis* growth cycle is as follows (see also Chibazakura *et al.*, 1991; Strauch *et al.*, 1992; Hoch, 1993; Ireton *et al.*, 1993): 1) during exponential growth, the σ^A-dependent *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~P binds with high affinity to DNA target sites (0A boxes); 4) Spo0A~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 *spo0H* gene, causing an increase in σ^H protein production; 5) Spo0A~P and σ^H together induce the activity of the *sinI* σ^H-dependent promoter, resulting in increased SinI production, sequestration of SinR, and derepression of the σ^H-dependent *spo0A* promoter located downstream of the *spo0A* σ^A- dependent promoter; 6) binding of Spo0A~P at the 0A boxes upstream of the *spo0A* σ^H-dependent promoter is required for the activation of the $E\sigma^H$ transcription of *spo0A*, and results in amplification of Spo0A production; and 7) during this time, Spo0A~P and σ^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 σ^A/σ^H *spo0A* promoter has been associated with a lack of the σ^A (vegetative promoter, or P_V) to σ^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 σ^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 $E\sigma^{A47}$-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 *spo0A* promoter switch was analyzed in a *crsA* mutant by the use of an S1 nuclease protection assay (Chibazakura *et al.*, 1991). mRNA from the P_S promoter was shown to appear in wild type cells at roughly T_1, and was absent in cells grown in 2% glucose. In *crsA* mutant cells mRNA from the P_S promoter appeared regardless of glucose supplementation. The finding (discussed above) that SinR regulation of *spo* genes was altered in GBS10 cells, leads to the possibility that the abnormal *spo0A* promoter switch observed by Chibazakura *et al.* (1991) in *crsA* mutant cells in the presence of glucose occurred because of a decrease in or a lack of SinR repression of P_S. Similarly, the possibility that glucose repression of the *sinI* promoter resulted from low σ^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 *spo0A* transcription from the P_V promoter by $E\sigma^{A47}$ (raising the level of Spo0A), and abnormal σ^H activity (raising the level of phosphorelay components).

The effect of $E\sigma^{A47}$ on *spo0A* P_V expression was initially addressed by an analysis of *spo0A-lacZ* activity in Δ*spo0H* strains (Figure 23). Deletion of the *spo0H* gene has the potential to affect *spo0A-lacZ* expression in three ways: the loss of σ^H-dependent *sinI* expression; the loss of phosphorylation of Spo0F by KinB, through the repression of *kinB* transcription by SinR (Dartois *et al.*, 1996); and the loss of σ^H-dependent *kinA* and *spo0F* transcription. In JH642 Δ*spo0H*, *spo0A* P_V promoter activity was observed at low but constant levels throughout the growth cycle in the absence of glucose. Somewhat surprisingly, *spo0A* P_V promoter activity in JH642 Δ*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 *spo0A* P_V promoter was

noted by Chibazakura *et al.* (1991). In the absence of glucose, GBS10 Δ*spo0H* cells generated a higher level of *spo0A* P_V promoter activity prior to the onset of stationary phase than was seen in JH642 Δ*spo0H* cells. This result also suggests that $E\sigma^{A47}$ transcribed the *spo0A* P_V promoter better than did $E\sigma^{A}$. 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 Δ*spo0H* 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 Δ*spo0H* cells, but was not obvious because of the low transcriptional activity.

In the presence of glucose, GBS10 Δ*spo0H* 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 Δ*spo0H* cells. The observed decrease in transcription may be due to repression of P_V transcription by Spo0A~P, as was also suggested above.

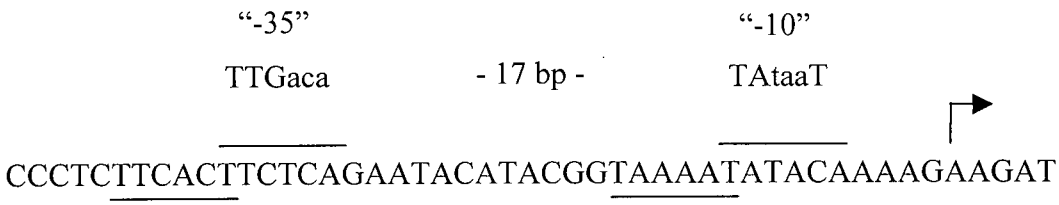
P_V promoter expression in *spo0H*$^+$ strains was examined using a *spo0A*ΔP_S promoter mutant, as another approach to examine RNA polymerase transcriptional activity (Figure 25). The deletion that created the *spo0A*ΔP_S promoter mutant removed both the P_S promoter and the SinR binding site (Mandic-Mulec *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 *spo0A*ΔP_S 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 *spo0A*ΔP_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 *spo0A*ΔP_S-*lacZ* promoter over that seen without glucose, with expression maintained until well into stationary phase. The molecular mechanism for the increase in *spo0A*ΔP_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. $E\sigma^{A47}$ transcribes the *spo0A* σ^A-dependent promoter more efficiently than $E\sigma^A$.

The *spo0A-lacZ* expression patterns in GBS10 suggested that $E\sigma^{A47}$ activity at the P_V promoter may be higher than that of $E\sigma^A$. 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.



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 σ^A promoters (Haldenwang, 1995). A conserved TG dinucleotide commonly found two bases upstream of the 5' end of the –10 promoter sequence in *B. subtilis* σ^A promoter sequences, known to be important for transcription from some weak promoters, is absent from both proposed *spo0A* σ^A 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 P_V promoter is indicated by an arrow. The distances between the putative –10 promoter sequences and the P_V 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* σ^A-dependent promoters, but distances of 5 and 10 bp have been shown to function in a small number of promoters (Helmann, 1995). The *spo0A* P_V 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 σ^A consensus promoter, the reason for the weak activity of $E\sigma^A$ 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

start site is 10 bases, a distance found in only 2 of 145 other described *B. subtilis* promoters (Helmann, 1995).

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 σ^{A47} is interesting, as both σ^{D} and *E. coli* σ^{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 $E\sigma^{A47}$ 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 σ^{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 $E\sigma^{A}$ and $E\sigma^{A47}$ on a standard template, the ϕ29 A2 promoter *versus* the *spo0A* promoter, by measuring the effects of both

template and enzyme inputs on transcript production, suggested that $E\sigma^{A47}$ did in fact transcribe the *spo0A* P_V promoter more efficiently than $E\sigma^A$, by a factor of approximately 2. The *in vitro* demonstration of higher activity of $E\sigma^{A47}$ at the P_V promoter than was seen for $E\sigma^A$ 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 *spo0A*ΔP_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 *spo0A*ΔP_S promoter mutant was several thousand-fold higher than that seen in JH642 *spo0A*ΔP_S cells, both in the presence and absence of glucose. The sporulation frequency seen in GBS10 *spo0A*ΔP_S cells strongly suggests that $E\sigma^{A47}$ 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. σ^H activation.

   The patterns of transcription of σ^H-dependent genes in cells grown in the presence and absence of glucose indicated that the regulation of σ^H activity was altered in *crsA* mutant cells. In GBS10 grown in the presence of glucose, inappropriately high σ^H activity was suggested by increased expression from σ^H-dependent promoter-*lacZ* fusions. The level of *abrB-lacZ* expression in GBS10 suggested that increased transcription of the primary transcriptional regulator of *spo0H*, AbrB, was not involved in the observed stationary phase increase in σ^H activity. Rather, this increase in σ^H activity is thought to be due to altered post-translational regulation of σ^H protein, which would occur as a result of changes in σ^{A47}-dependent gene expression. Because the mechanisms involved in the activation of σ^H are poorly understood, it is unknown whether σ^{A47} regulation of σ^H activity was direct, or indirect.

2. *spo0A* transcription.

   The presence of the *crsA* mutation had a direct effect on the expression of the *spo0A* gene. A higher level of exponential phase transcription from the *spo0A* P_V promoter was suggested from the analysis of *spo0A-lacZ* expression patterns *in vivo*, both in the presence and absence of an intact *spo0H* gene. The *in vitro* demonstration of a higher affinity of $E\sigma^{A47}$ for the *spo0A* P_V promoter than that seen with the wild type enzyme

confirmed that the *crsA* mutation resulted in increased expression of *spo0A* prior to the onset of stationary phase. Furthermore, the spo0AΔP_S deletion in GBS10 sporulated at high levels, indicating that the increased transcription of *spo0A* from the P_V promoter in GBS10 compensated for the lack of transcription from the P_S promoter, resulting in the attainment of threshold levels of Spo0A without the promoter switch. The observed sporulation efficiency of the *crsA*, *spo0A*ΔP_S double mutant contrasted with the *spo0A*ΔP_S deletion in *sigA*$^+$ cells, which showed a severe sporulation deficiency under all conditions tested. An increased *in vivo* transcriptional efficiency of $E\sigma^{A47}$ 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 σ^H activity (discussed above), would trigger activation of the phosphorelay (see Figure 3). At the onset of stationary phase, inappropriate activation and/or stabilization of σ^H would result in a high level of transcription of *kinA* and *spo0F*, and the resultant increase in phosphorelay components would increase the levels of phosphorylated Spo0A, despite *spo0L* and *spo0P* expression. Spo0A~P would repress *abrB* transcription, alleviating the transcriptional repression of *spo0H* and resulting in increased σ^H protein production. These events would trigger the *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 *et al.*, 1991), and would result in further accumulation of Spo0A~P in GBS10, despite the presence of glucose.

3. SinR regulation of transcription.

Because of the accumulation of Spo0A~P and active σ^H, the activity of $E\sigma^{A47}$ 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* σ^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 Δ*sinR* mutant in the presence of glucose supported the idea that SinR regulation was involved in the repression of sporulation by glucose. Given that σ^H has been shown to be somewhat active in stationary phase cells grown in the presence of glucose, SinR repression of the σ^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 σ^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 σ^H activation and Spo0A~P accumulation, but this has not been addressed directly, here or elsewhere. Other

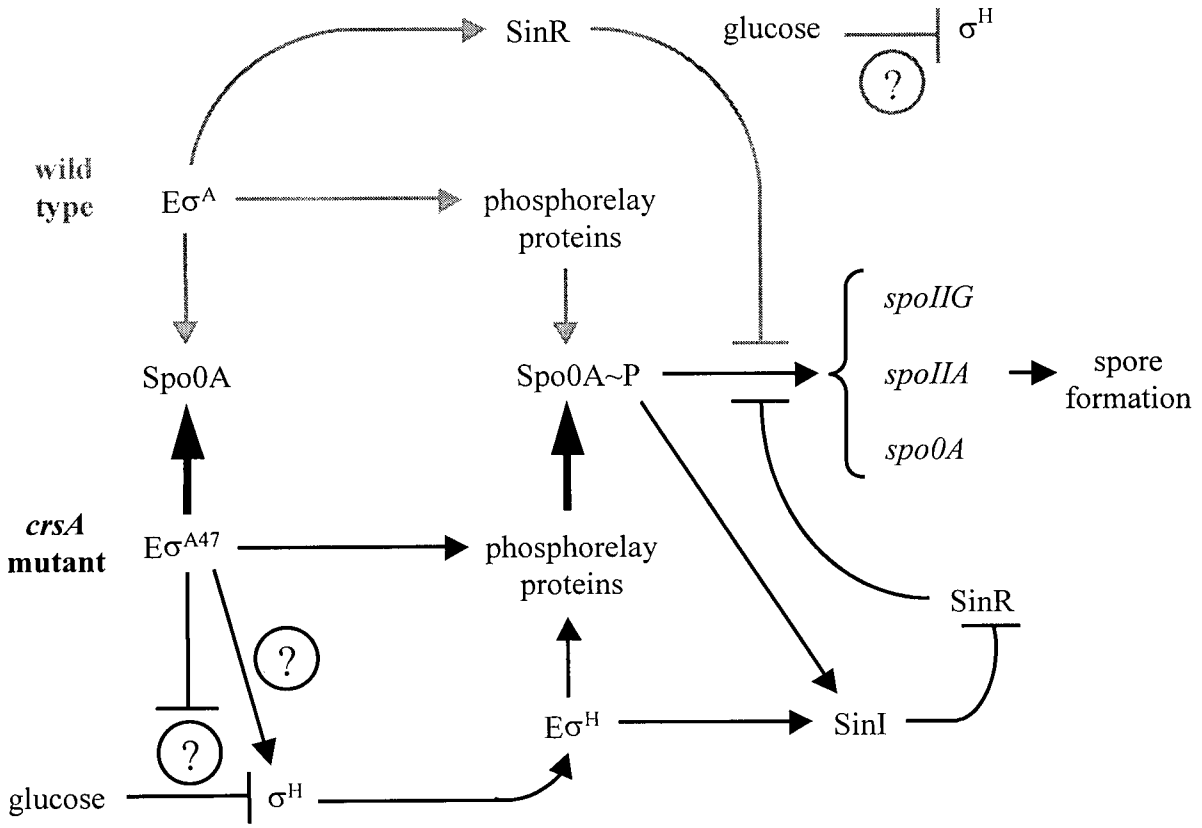


Figure 34. The effects of the *crsA* mutation on the sporulation initiation pathway. The accumulation and phosphorylation of Spo0A~P in the presence of glucose is shown at center. At the top (grey), $E\sigma^A$ in wild type cells interacts with promoters to result in low levels of phosphorelay proteins (Spo0F and Spo0B, not shown), including Spo0A. Minimal Spo0A~P accumulation, coupled with both persistence of SinR negative regulation, and no σ^H activation prevents sporulation from initiating. At the bottom (black), $E\sigma^{A47}$ in *crsA* mutant cells interacts with the *spo0A* P_V promoter more efficiently than is seen in wild type cells, resulting in increased Spo0A production. $E\sigma^{A47}$ also results in increased σ^H activity, through an unknown mechanism. High σ^H activity results in increased kinase production (not shown), resulting in increased Spo0A phosphorylation. High σ^H 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 σ^H 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 σ^H and high levels of Spo0A~P result in the activation of transcription from the *spo0A* P_S 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 $E\sigma^{A47}$. Secondly, the regulatory events surrounding the activation and stabilization of σ^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 σ^H activity seen in GBS10 cells. It is also possible that the presence of the *crsA* mutation affected σ^H protein activity more directly, *via* altered transcription of genes involved in the post-translational control of σ^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. σ^H protein levels and σ^H activity. What is the mechanism through which $E\sigma^{A47}$ results in increased σ^H-dependent transcription despite the presence of glucose?
   - the *in vivo* examination of increased σ^H protein levels using multicopy *spo0H*, and the potential of higher σ^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 $E\sigma^{A47}$ on σ^H activation, by the analysis of suppressor mutations that result in a decrease in σ^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 $E\sigma^{A47}$ *versus* $E\sigma^{A}$ on the *spo0A* P_V 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_V 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 σ^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 Δ*sinI* mutation on the sporulation efficiency of GBS10 in the presence of glucose.

References

Albano, M., J. Hahn, and D. Dubnau. 1987. Expression of competence genes in *Bacillus subtilis*. J. Bacteriol. **169:**3110-3117.

Antoniewski, C., B. Savelli, and P. Stragier. 1990. The *spoIIJ* gene, which regulates early developmental steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. J. Bacteriol. **172:**86-93.

Asai, K., F. Kawamura, H. Yoshikawa, and H. Takahashi. 1995. Expression of *kinA* and accumulation of σ^H at the onset of sporulation in *Bacillus subtilis*. J. Bacteriol. **177:**6679-6683.

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

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

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

Bird, T. H., J. K. Grimsley, J. A. Hoch, and G. B. Spiegelman. 1993. Phosphorylation of Spo0A activates its stimulation of *in vitro* transcription from the *Bacillus subtilis spoIIG* operon. Mol. Microbiol. **9:**741-749.

Bird, T. H., J. K. Grimsley, J. A. Hoch, and G. B. Spiegelman. 1996. The *Bacillus subtilis* response regulator Spo0A stimulates transcription of the *spoIIG* operon through modification of RNA polymerase promoter complexes. J. Mol. Biol. **256:**436-448.

Bourret, R. B., K. A. Borkovich, and M. I. Simon. 1991. Signal transduction pathways involving protein phosphorylation in prokaryotes. Annu. Rev. Biochem. **60:**401-441.

Boylan, S. A., K. T. Chun, B. A. Edson, and C. W. Price. 1988. Early-blocked sporulation mutations alter expression of enzymes under carbon control in *Bacillus subtilis*. Mol. Gen. Genet. **212:**271-280.

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

Carter, III, H. L. and C. P. Moran, Jr. 1986. New RNA polymerase sigma factor under *spo0* control in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **83:**9438-9442.

Carter, III, H. L., L.-F. Wang, R. H. Doi, and C. P. Moran, Jr. 1988. *rpoD* operon promoter used by σ^H-RNA polymerase in *Bacillus subtilis*. J. Bacteriol. **170:**1617-1621.

Cervin, M. A., R. J. Lewis, J. A. Brannigan, and G. B. Spiegelman. 1998. The *Bacillus subtilis* regulator SinR inhibits *spoIIG* promoter transcription *in vitro* without displacing RNA polymerase. Nucleic Acids Res. **26:**3806-3812.

Cervin, M. A. and G. B. Spiegelman. 1999. The Spo0A *sof* mutations reveal regions of the regulatory domain that interact with a sensor kinase and RNA polymerase. Mol. Microbiol. **31:**597-607.

Chambliss, G. H. 1993. Carbon source-mediated catabolite repression, p. 213-219. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-negative bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D. C.

Chauvaux, S., I. T. Paulsen, and M. H. Saier. 1998. CcpB, a novel transcription factor implicated in catabolite repression in *Bacillus subtilis*. J. Bacteriol. **180:**491-497.

Chibazakura, T., F. Kawamura, and H. Takahashi. 1991. Differential regulation of *spo0A* transcription in *Bacillus subtilis*: glucose represses promoter switching at the initiation of sporulation. J. Bacteriol. **173:**2625-2632.

Chibazakura, T., F. Kawamura, K. Asai, and H. Takahashi. 1995. Effects of *spo0* mutations on *spo0A* promoter switching at the initiation of sporulation in *Bacillus subtilis*. J. Bacteriol. **177:**4520-4523.

Collado-Vidas, J., B. Magasanik, J. D. Gralla. 1991. Control site location and transcriptional regulation in *Escherichia coli*. Microbiol. Rev. **55:**371-394.

Cooney, P. H., P. Fawcett Whiteman, and E. Freese. 1977. Media dependence of commitment in *Bacillus subtilis*. J. Bacteriol. **129:**901-907.

Coote, J. G. 1974. Comparative studies on induction of sporulation and synthesis of inducible enzymes in *Bacillus subtilis*. J. Bacteriol. **120:**1102-1108.

Cosby, W. M. and P. Zuber. 1997. Regulation of *Bacillus subtilis* σ^H (Spo0H) and AbrB in response to changes in external pH. J. Bacteriol. **179:**6778-6787.

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

Dartois, V., T. Djavakhishvili, and J. A. Hoch. 1996. Identification of a membrane protein involved in activation of the KinB pathway to sporulation in *Bacillus subtilis*. J. Bacteriol. **178:**1178-1186.

Dawes, I. W. and J. Mandelstam. 1970. Sporulation in *Bacillus subtilis* in continuous culture. J. Bacteriol. **103:**529-535.

Debarouille, M., I. Martin-Verstraete, F. Kunst, and G. Rapoport. 1991a. The transcriptional regulator LevR of *Bacillus subtilis* has domains homologous to both σ^{54} and phosphotransferase system-dependent regulators. Proc. Natl. Acad. Sci. USA **88:**2212-2216.

Debarouille, M., I. Martin-Verstraete, F. Kunst, and G. Rapoport. 1991b. The *Bacillus subtilis sigL* gene encodes an equivalent of σ^{54} from gram-negative bacteria. Proc. Natl. Acad. Sci. USA **88:**9092-9096.

deHaseth, P. L. and J. D. Helmann. 1995. Open complex formation by *Escherichia coli* RNA polymerase: the mechanism of polymerase-induced strand separation of double helical DNA. Mol. Microbiol. **16:**817-824.

Deutscher, J. and M. H. Saier. 1983. ATP-dependent, protein kinase-catalyzed phosphorylation of a seryl residue in HPr, the phosphoryl carrier protein of the phosphotransferase system in *Streptococcus pyogenes*. Proc. Natl. Acad. Sci. USA **80:**6790-6794.

Deutscher, J., E. Kuster, U. Bergstedt, V. Charrier, and W. Hillen. 1995. Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in Gram-positive bacteria. Mol. Microbiol. **15:**1049-1053.

Dion, P. and J. Mandelstam. 1980. Germination properties as marker events characterizing later stages of *Bacillus subtilis* spore formation. J. Bacteriol. **141:**786-792.

Dobinson, K. F. and G. B. Spiegelman. 1985. Nucleotide sequence and transcription of a bacteriophage ϕ29 early promoter. J. Biol. Chem. **260:**5950-5955.

Dobinson, K. F. and G. B. Spiegelman. 1987. Effect of the δ subunit of *Bacillus subtilis* RNA polymerase on initiation of RNA synthesis at two bacteriophage ϕ29 promoters. Biochemistry **26:**8206-8213.

Dubnau, E. J., K. Cabane, and I. Smith. 1987. Regulation of *spo0H*, an early sporulation gene in *Bacilli*. J. Bacteriol. **169:**1187-1191.

Dubnau, E. J., J. Weir, G. Nair, H. L. Carter, III, C. P. Moran, Jr., and I. Smith. 1988. *Bacillus* sporulation gene *spo0H* codes for σ^{30} (σ^{H}). J. Bacteriol. **170:**1054-1062.

Duncan, L., S. Alper, F. Arigoni, R. Losick, and P. Stragier. 1995. Activation of cell-specific transcription by a serine phosphatase at the site of asymmetric division. Science **270:**641-644.

Dunn, G., P. Jeffs, N. H. Mann, D. M. Torgensen, and M. Young. 1978. The relationship between DNA replication and the induction of sporulation in *Bacillus subtilis*. J. Gen. Microbiol. **108:**189-195.

Dunny G. M. and B. A. B. Leonard. 1997. Cell-cell communication in Gram-positive bacteria. Annu. Rev. Microbiol. **51:**527-564.

Errington, J., D. Foulger, N. Illing, S. R. Partridge, and S. M. Stevens. 1990. Regulation of differential gene expression during sporulation in *Bacillus subtilis*, p. 257-267. *In* M. Zukowski, A. T. Ganesan, and J. A. Hoch (ed.), Genetics and biotechnology of bacilli, vol. 3. Academic Press, Inc., San Diego, California.

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

Fabret, C., V. A. Feher, and J. A. Hoch. 1999. Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. J. Bacteriol. **181:**1975-1983.

Ferenci, T. 1999. Regulation by nutrient limitation. Cur. Op. Microbiol. **2:**208-213.

Ferrari, F. A., K. Trach, D. LeCoq, J. Spense, E. Ferrari, and J. A. Hoch. 1985. Characterization of the *spo0A* locus and its deduced product. Proc. Natl. Acad. Sci. USA **82:**2647-2651.

Ferrari, E., D. J. Henner, M. Perego, and J. A. Hoch. 1988. Transcription of *Bacillus subtilis* subtilisin and expression of subtilisin in sporulation mutants. J. Bacteriol. **170:**289-295.

Feucht, A., T. Magnin, M. D. Yudkin, and J. Errington. 1996. Bifunctional protein required for asymmetric cell division and cell-specific transcription in *Bacillus subtilis*. Genes Dev. **10:**794-803.

Fisher, S. H. and A. L. Sonenshein. 1991. Control of carbon and nitrogen metabolism in *Bacillus subtilis*. Annu. Rev. Microbiol. **45:**107-135.

Fisher, S. H., M. A. Strauch, M. R. Atkinson, and L. V. Wray, Jr. 1994. Modulation of *Bacillus subtilis* catabolite repression by transition state regulatory protein AbrB. J. Bacteriol. **176:**1903-1912.

Freese, E. 1981. Initiation of bacteria sporulation, p. 1-12. *In* H. S. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.

Frisby, D. and P. Zuber. 1994. Mutations in *pts* cause catabolite-resistant sporulation and altered regulation of *spo0H* in *Bacillus subtilis*. J. Bacteriol. **176:**2587-2595.

Gaidenko, T. A. and C. W. Price. 1998. General stress transcription factor σ^B and sporulation transcription factor σ^H each contribute to survival of *Bacillus subtilis* under extreme growth conditions. J. Bacteriol. **180:**3730-3733.

Gaur, N. K., K. Cabane, and I. Smith. 1988. Structure and expression of the *Bacillus subtilis sin* operon. J. Bacteriol. **170:**1046-1053.

Gonzy-Treboul, G., M. Zagorec, M.-C. Rain-Guion, and M. Steinmetz. 1989. Phosphoenolpyruvate:sugar phosphotransferase system of *Bacillus subtilis*: nucleotide sequence of *ptsX, ptsH,* and the 5'-end of *ptsI* and evidence for a *ptsHI* operon. Mol. Micrbiol. **3:**103-112.

Gould, G. W. 1984. Mechanisms of resistance and dormancy, p. 173-209. *In* A. Hurst and G. W. Gould (ed.), The bacterial spore, vol. 2. Academic Press Ltd., London.

Gralla, J. D. 1990. Promoter recognition and mRNA initiation by *Escherichia coli* $E\sigma^{70}$. Methods in Enzymology **185:**37-54.

Grossman, A. D. and R. Losick. 1988. Extracellular control of spore formation in *Bacillus subtilis*, Proc. Natl. Acad. Sci. USA **85:**4369-4373.

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

Guerot-Fleury, A.-M., K. Shazand, N. Franden, and P. Stragier. 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene **167:**335-337.

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

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557-569.

Harder, W. and L. Dijkhuizen. 1983. Physiological responses to nutrient limitation. Annu. Rev. Microbiol. **37:**1-24.

Hauser, P. M. and J. Errington. 1995. Characterization of cell cycle events during the onset of sporulation in *Bacillus subtilis*. J. Bacteriol. **177:**3923-3931.

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

Hecker, M., W. Schumann, and U. Volker. 1996. Heat-shock and general stress response in *Bacillus subtilis*. Mol. Microbiol. **19:**417-428.

Hecker, M and U. Volker. 1998. Non-specific, general and multiple stress resistance of growth-restricted *Bacillus subtilis* cells by the expression of the σ^B regulon. Mol. Microbiol. **29:**1129-1136.

Hederstedt, L. 1993. The Krebs citric acid cycle, p. 181-197. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D. C.

Helmann, J. D. and M. J. Chamberlin. 1988. Structure and function of bacterial sigma factors. Ann. Rev. Biochem. **57:**839-872.

Helmann, J. D., L. M. Marquez, and M. J. Chamberlin. 1988. Cloning, sequencing, and disruption of the *Bacillus subtilis* σ^{28} gene. J. Bacteriol. **170:**1560-1567.

Helmann, J. D. 1991. Alternate sigma factors and the regulation of flagellar gene expression. Mol. Microbiol. **5:**2875-2882.

Helmann, J. D. 1995. Compilation and analysis of *Bacillus subtilis* σ^A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. Nucleic Acids Res. **23:**2351-2360.

Helmann, J. D. 1999. Anti-sigma factors. Curr. Opin. Microbiol. **2:**135-141.

Helmann, J. D. and P. L. deHaseth. 1999. Protein-nucleic acid interactions during open complex formation investigated by systematic alteration of the protein and DNA binding partners. Biochemistry **38:**5959-5967.

Henkin, T. M., F. J. Grundy, W. L. Nicholson, and G. H. Chambliss. 1991. Catabolite repression of α-amylase gene expression in *Bacillus subtilis* involves a *trans*-acting gene product homologous to the *Escherichia coli lacI* and *galR* repressors. Mol. Microbiol. **5:**575-584.

Henkin, T. M. 1996. The role of the CcpA transcriptional regulator in carbon metabolism in *Bacillus subtilis*. FEMS Microbiol. Let. **135:**9-15.

Hitchins, A. D. and R. A. Slepecky. 1969. Bacterial spore formation as a modified prokaryotic cell division. Nature **223:**804-807.

Hoch, J. A. 1976. Genetics of bacterial sporulation. Adv. Genet. **18:**69-99.

Hoch, J. A., K. Trach, F. Kawamura, and H. Saito. 1985. Identification of the transcriptional suppressor *sof-1* as an alteration in the *spo0A* protein. J. Bacteriol. **161:**552-555.

Hoch, J. A. 1991. Genetic analysis in *Bacillus subtilis*, p. 305-320. *In* J. H. Miller (ed.), Methods in Enzymology, vol. 204, Bacterial Genetic Systems. Academic Press, Inc., San Diego, California.

Hoch, J. A. 1993. *spo0* genes, the phosphorelay, and the initiation of sporulation, p. 747-755. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-negative bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D. C.

Huang, X and J. D. Helmann. 1998. Identification of target promoters for the *Bacillus subtilis* sigma X factor using a consensus-directed approach. J. Mol. Biol. **279:**165-173.

Huang, X., A. Gaballa, M. Cao, and J. D. Helmann. 1999. Identification of target promoters for the *Bacillus subtilis* extracytoplasmic function sigma factor, sigma W. Mol. Microbiol. **31:**361-371.

Hueck, C. J. and W. Hillen. 1995. Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the Gram-positive bacteria? Mol. Microbiol. **15:**395-401.

Ireton, K., D. Z. Rudner, K. Jaacks Siranosian, and A. D. Grossman. 1993. Integration of multiple developmental signals in *Bacillus subtilis* through the Spo0A transcription factor. Genes Dev. **7:**283-294.

Ireton, K., S. Jin, A. D. Grossman, and A. L. Sonenshein. 1995. Krebs cycle function is required for activation of the Spo0A transcription factor in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **92:**2845-2849.

Jaacks, K. J., J. Healy, R. Losick, and A. D. Grossman. 1989. Identification and characterization of genes controlled by the sporulation-regulatory gene *spo0H* in *Bacillus subtilis*. J. Bacteriol. **171:**4121-4129.

Jarvis, E. D., R. L. Widom, G. LaFauci, Y. Setoguchi, I. R. Richter, and R. Rudner. 1988. Chromosomal organization of rRNA operons in *Bacillus subtilis*. Genetics **120:**625-635.

Jenal, U. and C. Stephens. 1996. Bacterial differentiation: sizing up sporulation. Curr. Biol. **6:**111-114.

Jenkinson, H. F., W. D. Sawyer, and J. Mandelstam. 1981. Synthesis and order of assembly of spore coat proteins in *Bacillus subtilis*. J. Gen. Microbiol. **123:**1-16.

Jiang, M., R. Grau, and M. Perego. 2000. Differential processing of propeptide inhibitors of Rap phosphatases in *Bacillus subtilis*. J. Bacteriol. **182:**303-310.

Jin, S. and A. L. Sonenshein. 1994. Transcriptional regulation of *Bacillus subtilis* citrate synthase genes. J. Bacteriol. **176:**5928-5932.

Jourlin-Castelli, C., N. Mani, M. M. Nakano, and A. L. Sonenshein. 2000. CcpC, a novel regulator of the LysR family required for glucose repression of the *citB* gene in *B. subtilis*. J. Mol. Biol. **295:**865-878.

Kallio, P. T., J. E. Fagelson, J. A. Hoch, and M. A. Strauch. 1991. The transition state regulator Hpr of *Bacillus subtilis* is a DNA-binding protein. J. Biol. Chem. **266:**13411-13417.

Kawamura, F., L.-F. Wang, and R. H. Doi. 1985. Catabolite-resistant sporulation (*crsA*) mutations in the *Bacillus subtilis* RNA polymerase σ^{43} gene (*rpoD*) can suppress and be suppressed by mutations in *spo0* genes. Proc. Natl. Acad. Sci USA **82:**8124-8128.

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

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

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

Kofoid, E. C. and J. S. Parkinson. 1988. Transmitter and receiver modules in bacterial signaling proteins. Proc. Natl. Acad. Sci. USA **85:**4981-4985.

Kraus, A., C. Hueck, D. Gartner, and W. Hillen. 1994. Catabolite repression of the *Bacillus subtilis xyl* operon involves a *cis* element functional in the context of an unrelated sequence, and glucose exerts additional *xylR*-dependent repression. J. Bacteriol. **176:**1738-1745.

Kroos, L., B. Zhang, H. Ichikawa, and Y.-T. Nicco Yu. 1999. Control of σ factor activity during *Bacillus subtilis* sporulation. Mol. Microbiol. **31:**1285-1294.

Kudoh, J., T. Ikeuchi, and K. Kurahashi. 1985. Nucleotide sequences of the sporulation gene *spo0A* and its mutant genes in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **82:**2665-2668.

Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, and A. Danchin. 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature **390:**249-56.

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

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

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

Lee, A. L., M. Malak, P. Louie, J. Arjomand, C. Ginther, and T. Leighton. 1992. Intergenic suppression of stage II sporulation defects by a mutation in the major vegetative sigma factor gene (*rpoD*) of *Bacillus subtilis*. Biochimie **74:**635-640.

Leirmo, S. and M. T. Record. 1990. Structural, thermodynamic and kinetic studies of the interaction of Es^{70} RNA polymerase with promoter DNA, p. 123-152. *In* R. Eckstein and D. M. J. Lilley (ed.), Nucleic acids and molecular biology, vol. 4. Springer-Verlag, Berlin, Heidelberg.

Leung, A., S. Rubinstein, C. Yang, L. Jing-Wei, and T. Leighton. 1985. Suppression of defective-sporulation phenotypes by mutations in the major sigma factor gene (*rpoD*) of *Bacillus subtilis*. Mol. Gen. Genet. **201:**96-98.

Lewis, P., T. Magnin, and J. Errington. 1996. Compartmentalized distribution of the proteins controlling the prespore-specific transcription factor σ^F of *Bacillus subtilis*. Genes Cells **1:**881-894.

Lewis, R. J., J. A. Brannigan, W. A. Offen, I. Smith, and A. J. Wilkinson. 1998. An evolutionary link between sporulation and prophage induction in the structure of a repressor:anti-repressor complex. J. Mol. Biol. **283:**907-912.

Liu, J., W. M. Cosby, and P. Zuber. 1999. Role of Lon and ClpX in the post-translational regulation of a sigma subunit of RNA polymerase required for cellular differentiation in *Bacillus subtilis*. Mol. Microbiol. **33:**415-428.

Lohman, T. M., deHaseth, P. L., and Record, M. T., Jr. 1978. Analysis of ion concentration effects on the kinetics of protein-nucleic acid interactions. Applications to *lac* repressor-operator interactions. Biophys Chem. **8:**281-294.

Lohman, T. M. 1985. Kinetics of protein-nucleic acid interactions: use of salt effects to probe mechanisms of interactions. CRC Crit. Rev. Biochem. **19:**191-245.

Louie, P., A. Lee, K. Stansmore, R. Grant, C. Ginther, and T. Leighton. 1992. Roles of *rpoD, spoIIF, spoIIJ, spoIIN,* and *sin* in regulation of *Bacillus subtilis* stage II sporulation-specific transcription. J. Bacteriol. **174:**3570-3576.

Losick, R. and J. Pero. 1981. Cascades of sigma factors. Cell **25:**582-584.

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

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

Magasanik, B. and F. C. Neihardt. 1987. Regulation of carbon and nitrogen utilization, p. 1318-1325. *In* F. C. Neihardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *salmonella typhimurium*; cellular and molecular biology. American Society for Microbiology, Washington, D. C.

Mandec-Mulec, I., N. Gaur, U. Bai, and I. Smith. 1992. Sin, a stage-specific repressor of cellular differentiation. J. Bacteriol. **174:**3561-3569.

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

Mandelstam, J. and S. A. Higgs. 1974. Induction of sporulation during synchronized chromosome replication in *Bacillus subtilis*. J. Bacteriol. **120:**38-42.

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

Margolis, P. S., A. Driks, and R. Losick. 1993. Sporulation gene *spoIIB* from *Bacillus subtilis*. J. Bacteriol. **175:**528-540.

Martin, J. F. and A. L. Demain. 1980. Control of antibiotic biosynthesis. Microbiol. Rev. **44:**230-251.

Matsuno, K., T Blais, A. W. Serio, T. Conway, T. M. Henkin, and A. L. Sonenshein. 1999. Metabolic imbalance and sporulation in an isocitrate dehydrogenase mutant of *Bacillus subtilis*. J. Bacteriol. **181:**3382-3391.

Matsuno, K. and A. L. Sonenshein. 1999. Role of SpoVG in asymmetric septation in *Bacillus subtilis*. J. Bacteriol. **181:**3392-3401.

Miller, J. H. 1972. Experiments in molecular genetics, p. 352-355. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

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

Mirel, D. B., and M. J. Chamberlin. 1989. The *Bacillus subtilis* flagellin gene (*hag*) is transcribed by the sigma 28 form of RNA polymerase. J. Bacteriol. **171:**3095-3101.

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

Monod, J. 1947. The phenomenon of enzymatic adaptation. Growth **11:**223-289.

Msadek, T., F. Kunst, and G. Rapoport. 1993. Two-component regulatory systems, p. 729-745. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D. C.

Msadek, T., V. Dartois, F. Kunst, M.-L. Herbaud, F. Denizot, and G. Rapoport. 1998. ClpP of *Bacillus subtilis* is required for competence development, motility, degredative enzyme synthesis, growth at high temperature and sporulation. Mol. Microbiol. **27:**899-914.

Msadek, T. 1999. When the going gets tough: survival strategies and environmental signaling networks in *Bacillus subtilis*. *Trends Microbiol.* **7:**210-207.

Nanamiya, H., Y. Ohashi, K. Asai, S. Moriya, N. Ogasawara, M. Fujita, Y. Sadaie, and F. Kawamura. 1998. clpC regulates the fate of a sporulation initiation sigma factor, σ^H protein, in *Bacillus subtilis* at elevated temperatures. Mol. Microbiol. **29:**505-513.

Ogasawara, N., S. Moriya, and H. Yoshikaya. 1983. The structure and organization of rRNA operons at the region of the replication origin of the *B. subtilis* chromosome. Nucleic Acids Res. **11:**6301-6318.

Perego, M. and J. A. Hoch. 1988. Sequence analysis and regulation of the *hpr* locus, a regulatory gene for protease production and sporulation in *Bacillus subtilis*. J. Bacteriol. **170:**2560-2567.

Perego, M., G. B. Spiegelman, and J. A. Hoch. 1988. Structure of the gene for the transition state regulator, *abrB*: regulator synthesis is controlled by the *spo0A* sporulation gene in *Bacillus subtilis*. Mol. Microbiol. **2:**689-699.

Perego, M., S. P. Cole, D. Burbulys, K. Trach, and J. A. Hoch. 1989. Characterization of the gene for a protein kinase which phosphorylates the sporulation-regulatory proteins Spo0A and Spo0F of *Bacillus subtilis*. J. Bacteriol. **171:**6187-6196.

Perego, M., C. F. Higgins, S. R. Pearce, M. P. Gallagher, and J. A. Hoch. 1991a. The oligopeptide transport system of *Bacillus subtilis* plays a role in the initiation of sporulation. Mol. Microbiol. **5:**173-185.

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

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

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

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

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

Perego, M. and J. A. Hoch. 1996b. Protein aspartate phosphatases control the output of two-component signal transduction systems. Trends in Genetics **12:**97-101.

Perego, M. 1997. A peptide export-import control circuit modulating bacterial development regulates protein phosphatases of the phosphorelay. Proc. Natl. Acad. Sci USA **94:**8612-8617.

Perego, M. 1998. Kinase-phosphatase competition regulates *Bacillus subtilis* development. Trends Microbiol. **6:**366-370.

Postma, P. W. 1987. Phosphotransferase system for glucose and other sugars, p. 127-141. *In* F. C. Neihardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *salmonella typhimurium*; cellular and molecular biology. American Society for Microbiology, Washington, D. C.

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

Price, C. W. and R. H. Doi. 1985. Genetic mapping of *rpoD* implicates the major sigma factor of *Bacillus subtilis* RNA polymerase in sporulation initiation. Mol. Gen. Genet. **201:**88-95.

Price, V. A., I. M. Feavers, and A. Moir. 1989. Role of sigma H in expression of the fumarase gene (*citG*) in vegetative cells of *Bacillus subtilis* 168. J. Bacteriol. **171:**5933-5939.

Reizer, J. S. L. Sutrina, M. H. Saier, Jr., G. C. Stewart, A. Peterofshy, and P. Reddy. 1989. Mechanistic and physiological consequences of HPr(ser) phosphorylation on the activities of the phosphoenolpyruvate:sugar phosphotransferase system in gram-positive bacteria: studies with site-directed mutants of HPr. EMBO J. **8:**2111-2120.

Roe, J.-H., R. R. Burgess, and M. T. Record, Jr. 1984. Kinetics and mechanism of the interaction of *Escherichia coli* RNA polymerase with the λ P_R promoter. J. Mol. Biol. **176:**495-521.

Rosenbluh, A., C. D. B. Banner, R. Losick, and P. C. Fitz-James. 1981. Identification of a new developmental locus in *Bacillus subtilis* by construction of a deletion mutation in a cloned gene under sporulation control. J. Bacteriol. **148:**341-351.

Rowe-Magnus, D. A. and G. B. Spiegelman. 1998. DNA strand separation during activation of a developmental promoter by the *Bacillus subtilis* response regulator Spo0A. Proc. Natl. Acad. Sci. USA **95:**5305-5310.

Sadaie, Y. and T. Kada. 1983. Formation of competent *Bacillus subtilis* cells. J. Bacteriol. **153:**813-821.

Saier, M. H. 1996. Regulation of carbon metabolism in bacteria. Res. Microbiol. **147:**439-448.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Satola, S., P. A. Kirchman, and C. P. Moran, Jr. 1991. Spo0A binds to a promoter used by σ^A RNA polymerase during sporulation in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **88:**4533-4537.

Satola, S. W., J. M. Baldus, and C. P. Moran, Jr. 1992. Binding of Spo0A stimulates *spoIIG* promoter activity in *Bacillus subtilis.* J. Bacteriol. **174(5):**1448-1453.

Schaeffer, P., J. Millet, and J. Aubert. 1965. Catabolic repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA **88:**4533-4537.

Schmidt, R., P. Margolis, L. Duncan, R. Coppolecchia, C. P. Moran, Jr., and R. Losick. 1990. Control of developmental transcription factor σ^F by sporulation regulatory proteins SpoIIAA and spoIIAB in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **87:**9221-9225.

Schyns, G., C. M. Buckner, and C. P. Moran, Jr. 1997. Activation of the *Bacillus subtilis spoIIG* promoter requires interaction of Spo0A and the sigma subunit of RNA polymerase. J. Bacteriol. **179:**5605-5608.

Shaner, S. L., P. Melancon, K. S. Lee, R. R. Burgess, and M. T. Record. 1983. Ion effects on the aggregation and DNA-binding reactions of *Escherichia coli* RNA polymerase. Cold Springs Harbor Symp. Quant. Biol. **47:**463-472.

Shimotsu, H. and D. J. Henner. 1986. Construction of a single-copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. Gene **43:**85-94.

Siranosian, K. J. and A. D. Grossman. 1994. Activation of *spo0A* transcription by σ^H is necessary for sporulation but not for competence in *Bacillus subtilis*. J. Bacteriol. **176:**3812-3815.

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

Smith, I., E. Dubnau, M. Predich, U. Bai, and R. Rudner. 1992. Early *spo* gene expression in *Bacillus subtilis*: the role of interrelated signal transduction systems. Biochimie. **74:**669-678.

Sonenshein, A. L. 1989. Metabolic regulation of sporulation and other stationary phase phenomena, p. 109-130. *In* I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of prokaryotic development. American Society for Microbiology, Washington, D.C.

Spiegelman, G., B. Van Hoy, M. Perego, J. Day, K. Trach, and J. A. Hoch. 1990. Structural alterations in the *Bacillus subtilis* Spo0A regulatory protein which suppress mutations at several *spo0* loci. J. Bacteriol. **172:**5011-5019.

Spiegelman, G. B., T. H. Bird, and V. Voon. 1995. Transcription regulation by the *Bacillus subtilis* response regulator Spo0A. *In* Two-component signal transduction, eds. J. A. Hoch and T. J. Silhavy, pp.159-179. American Society for Microbiology, Washington, DC

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

Stock, J. B., A. M. Stock, and J. M. Mottonen. 1990. Signal transduction in bacteria. Nature **344:**395-400.

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

Stragier, P. and R. Losick. 1990. Cascades of sigma factors revisited. Mol. Microbiol. **4:**1801-1806.

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

Strauch, M. A., G. B. Spiegelman, M. Perego, W. C. Johnson, D. Burbulys, and J. A. Hoch. 1989a. The transition state transcription regulator AbrB of *Bacillus subtilis* is a DNA binding protein. EMBO J. **8:**1615-1621.

Strauch, M. A., M. Perego, D. Burbulys, and J. A. Hoch. 1989b. The transition state transcription regulator AbrB of *Bacillus subtilis* is autoregulated during vegetative growth. Mol. Microbiol. **3:**1203-1209.

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

Strauch, M. A., K. A. Trach, J. Day, and J. A. Hoch. 1992. Spo0A activates and represses its own synthesis by binding at its dual promoters. Biochimie **74:**619-626.

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

Strauch, M. A. 1995a. Delineation of AbrB-binding sites on the *Bacillus subtilis spo0H, kinB, ftsAZ,* and *pbpE* promoters and use of a derived homology to identify a previously unsuspected binding site in the *bsuB1* methylase promoter. J. Bacteriol. **177:**6999-7002.

Strauch, M. A. 1995b. AbrB modulates expression and catabolite repression of a *Bacillus subtilis* ribose transport operon. J. Bacteriol. **177:**6727-6731.

Stryer, L. 1988. Chapter 2: protein structure and function. *In* Biochemistry, ed. A. Kudlacik, pp. 17-42. W. H. Freeman and Company, New York.

Stulke J. and W. Hillen. 1999. Carbon catabolite repression in bacteria. Cur. Op. Microbiol. **2:**195-201.

Takahashi, I. 1979. Catabolite repression-resistant mutants of *Bacillus subtilis*. Can. J. Microbiol. **35:**1283-1287.

Takahashi, I. and D. Sun. 1984. Effect of various inhibitors on sporulation of *Bacillus subtilis crs* mutants. Can. J. Microbiol. **30:**1337-1343.

Tatti, K., H. Luke Carter III, A. Moir, and C. P. Moran, Jr. 1989. Sigma-H directed transcription of *citG* in *Bacillus subtilis*. J. Bacteriol. **171:**5928-5932.

Trach, K., D. Burbulys, M. A. Strauch, J.-J. Wu, M. Perego, T. Bird, G. Spiegelman, C. Fogher, and J. A. Hoch. 1991. Control of the initiation of sporulation in *Bacillus subtilis* by a phosphorelay. Res. Microbiol. **142:**815-823.

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

Ullmann, A. and A. Danchin. 1983. Role of cAMP in bacteria. Adv. Cyclic Nucleotide Res. **15:**1-53.

Varon, D., M. S. Brody, and C. W. Price. 1996. *Bacillus subtilis* operon under the dual control of the general stress transcription factor s^B and the sporulation transcription factor s^H. Mol. Microbiol. **20:**3964-3971.

Vold, B. S. 1985. Structure and organization of genes for transfer ribonucleic acid in *Bacillus subtilis*. Microbiol. Rev. **49:**71-80

Voskull, M. I., K. Voepel, and G. H. Chambliss. 1995. The –16 region, a vital sequence for the utilization of a promoter in *Bacillus subtilis* and *Escherichia coli*. Mol. Microbiol. **17:**271-279.

Walter, G., W. Willing, P. Palm, and E. Fuchs. 1967. Initiation of DNA dependent RNA synthesis and the effect of heparin on RNA polymerase. Eur. J. Biochem. **3:**194-201.

Wang, L., R. Grau, M. Perego, and J. A. Hoch. 1997. A novel histidine kinase inhibitor regulating development in *Bacillus subtilis*. Genes Dev. **11:**2569-2579.

Warth, A. D. and J. L. Strominger. 1972. Structure of the peptidoglycan from spores of *Bacillus subtilis*. Biochemistry **11:**1389-1396.

Weikert, M., and G. H. Chambliss. 1990. Site-directed mutagenesis of a catabolite repression operator sequence in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **87:**6238-6242.

Weikert, M. and S. Adhya. 1992. A family of bacterial regulators homologous to Gal and Lac repressors. J. Biol. Chem. **267:**15869-15874.

Weir, J., E. Dubnau, N. Ramakrishna, and I. Smith. 1984. *Bacillus subtilis spo0H* gene. J. Bacteriol. **157:**405-412.

Weir, J., M. Predich, E. Dubnau, G. Nair, and I Smith. 1991. Regulation of *spo0H*, a gene coding for the *Bacillus subtilis* σ^H factor. J. Bacteriol. **173:**521-529.

Whipple, F. W. and A. L. Sonenshein. 1992. Mechanism of initiation of transcription by *Bacillus subtilis* RNA polymerase at several promoters. J. Mol. Biol. **223:**399-414.

Wray, Jr., L. V., F. K. Pettengill, and S. H. Fisher. 1994. Catabolite repression of the *Bacillus subtilis hut* operon requires a *cis*-acting site located downstream of the transcription initiation site. J. Bacteriol. **174:**2648-2658.

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

Wu, J.-J., P. J. Lewis, R. Allmansberger, P. M. Hauser, and J. Errington. 1995. A conjugation-like mechanism for prespore chromosome partitioning during sporulation in *Bacillus subtilis*. Genes Dev. **9:**1316-1326.

Youngman, P., J. B. Perkins, and R. Losick. 1984. A novel method for the rapid cloning in *Escherichia coli* of *Bacillus subtilis* chromosomal DNA adjacent to Tn*917* insertions. Mol. Gen. Genet. **195:**424-433.

Zahler, S. A., L. G. Benjamin, B. S. Glatz, P. F. Winter, and B. J. Goldstein. 1976. Genetic mapping of the *alsA, alsR, thyA, kauA,* and *citD* markers in *Bacillus subtilis*, p. 35-43. *In* D. Schlessinger (ed.), Microbiology – 1976. American Society for Microbiology, Washington, D.C.

Zuber, P. and R. Losick. 1983. Use of a *lacZ* fusion to study the role of the *spo0* genes of *Bacillus subtilis* in developmental regulation. Cell **35:**275-283.

Zuber, P. 1985. Localizing the site of *spo0*-dependent regulation in the *spoVG* promoter of *Bacillus subtilis*, p. 149-156. *In* J. A. Hoch *et al* (ed.), Molecular Biology of Microbial Differentiation. American Society for Microbiology, Washington, D.C.

Zuber, P. and R. Losick. 1987. Role of AbrB on *spo0A*- and *spo0B*- dependent utilization of a sporulation promoter in *Bacillus subtilis*. J. Bacteriol. **169:**2223-2230.

Zuber, P., M. Maraheil, and J. Robertson. 1988. Influence of *abrB* on the transcription of the sporulation-associated genes *spoVG* and *spo0H* in *Bacillus subtilis*, p123-127. *In* A. T. Ganesan and J. A. Hoch (ed.), Genetics and Biotechnology of Bacilli, vol. 2. Academic Press, Inc., San Diego, California.

Zuber, P., J. Healy, H. L. Carter III, S. Cutting, C. P. Moran Jr., and R. Losick. 1989. Mutation changing the specificity of an RNA polymerase sigma factor. J. Mol. Biol. **206:**605-614.