FUNCTION OF SPX AND ITS CONTROL BY PROTEOLYSIS

Ying Zhang
B.S., Fudan University, 2001

A dissertation submitted to the faculty of the
OGI School of Science & Engineering
at Oregon Health & Science University
in partial fulfillment of the
requirements for the degree

Doctor of Philosophy
in
Biochemistry and Molecular Biology

November 2007

The thesis “Function of Spx and its Control by Proteolysis” by Ying Zhang has been examined and approved by the following Examination Committee:

Peter Zuber, Ph.D., Thesis Advisor
Professor

Michiko Nakano, Ph.D.
Research Associate Professor

Petra Anne Levin, Ph.D.
Assistant Professor, Washington University in St. Louis

Jay L. Mellies, Ph.D.
Associate Professor, Reed College

ACKNOWLEDGMENTS

First I would like to express my sincere appreciation and gratitude towards my advisor, Dr. Peter A. Zuber, for giving me the chance to pursuit my Ph.D. degree in the field of molecular microbiology and for his continuous encouragement, support, patience and guidance, which have made my Ph.D. studies full of excitement and reward. His broad knowledge and logical way of thinking have been of great value for me. His technical advice and constructive comments have provided a good basis for this thesis.

My deeply thanks to Dr. Michiko Nakano for her continuous support and encouragement in my research work and life throughout my Ph.D. studies. My sincere thanks to Dr. Petra Anne Levin and Dr. Jay L. Mellies for their efforts and time to serve on my Ph.D. committee.

Thanks to other faculties of Environmental and Biomolecular system Dr. Matthew S. Sachs, Dr. Ninian Blackburn, Dr. Pierre Moënne Loccoz, Dr. James Whittaker and Dr. Gebretateos Woldegiorgis for their technical support, valuable courses and useful information.

I also want to thank Shunji Nakano for his guidance with my research project and for friendship and unconditional help. I would like to thank former and current lab members of Dr. Peter Zuber's lab, Dr. Michiko Nakano's lab, Dr. Matthew S. Sachs's lab and Dr. Ninian Blackburn's labs: Dr. Soon-Yong Choi, Dr. Montira Leelakriangsak, Dr. Dindo Reyes, Dr. Saurabh Garg, Dr. Hao Geng, Dr. Meray Baştürkmen, Cheng Wu, and Amanda Barry for their supports, technical assistances and generously sharing equipments and reagents.

Special thanks to department moms Nancy Christie and Amber Mullooly for all their help and kindness throughout my graduate studies.

I am forever indebted to my family especially my parents for their continuous love and encouragement, advice and support, which without, this thesis would not have been possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Function of Spx and its control by proteolysis

Ying Zhang

Ph.D., OGI School of Science & Engineering
Oregon Health & Science University
November 2007

Thesis Advisor: Dr. Peter A. Zuber

The Spx protein of *Bacillus subtilis* is a global transcriptional regulator that exerts both positive and negative control in response to oxidative stress by interacting with the C-terminal domain of the RNA polymerase (RNAP) alpha subunit (αCTD). One target of Spx-negative control is the ComPA signal transduction system, which activates the transcription of the *srf* operon at the onset of competence development. Previous genetic and structural analyses have determined that an Spx-binding surface resides in and around the α1 region of αCTD. Alanine-scanning mutagenesis of *B. subtilis* αCTD uncovered residues required for Spx function and ComA-dependent *srf* transcriptional activation. Analysis of *srf-lacZ* fusion expression, DNase I footprinting, and solid-phase promoter retention experiments indicated that Spx interferes with ComA-αCTD interaction and that residues Y263, C265, and K267 of the α1 region lie within the overlapping ComA- and Spx-binding sites for αCTD interaction. The requirement of the oxidized Spx for Spx-dependent activation of *trxA* and *trxB* expressions was demonstrated in previous work (Nakano *et al.*, 2005). Evidence is presented here that oxidized Spx, while enhancing interference of activator-RNAP interaction, is not essential for negative control.

Spx is under proteolytic control by the ATP-dependent protease, ClpXP. Previous studies suggested that the accumulation of Spx protein upon disulfide stress is due to the derepression of *spx* by PerR and YodB and the down-regulation of the ClpXP activity. The effect of disulfide stress on ClpXP activity was examined using the thiol-specific oxidant, diamide. ClpXP-catalyzed degradation of either Spx or a green fluorescent protein (GFP) derivative bearing an SsrA tag recognized by ClpXP was inhibited by diamide treatment *in vitro*. Spx is also a substrate for MecA/ClpCP-catalyzed proteolysis *in vitro*, but the same concentration of diamide that inhibited ClpXP had little observable effect on MecA/ClpCP activity. The derivative of transcriptional repressor HrcA bearing an SsrA tag is another ClpXP substrate *in vivo* and its degradation by ClpXP was reduced in the presence of diamide. ClpX bears a Cys4 Zinc-binding domain (ZBD), which in other Zinc-binding proteins is vulnerable to thiol-reactive electrophiles. Diamide treatment caused partial release of Zn from ClpX and the formation of high molecular weight species, as observed by electrophoresis through non-reducing gels. When two of the Zn-coordinating Cys residues of the ClpX ZBD were changed to Ser, Spx proteolysis was reduced *in vitro*, the Spx-dependent transcriptional controls were enhanced and the Spx protein accumulated *in vivo*. The results are consistent with the hypothesis that inhibition of ClpXP by disulfide stress is due to structural changes to the N-terminal ZBD of ClpX.

YjbH, a negative regulator of Spx, was examined in *B. subtilis*. Elevated Spx protein and enhanced Spx-dependent transcriptional control were observed in the cells bearing the *yjbH* insertion mutants. Thus, expression of *spx* was negatively affected by *yjbH* and this negative control was maintained when the *spx* was expressed from an IPTG-inducible promoter. The concentration of another ClpXP substrate HrcA-SsrA was not affected by YjbH *in vivo*, suggesting that YjbH is a specific negative regulator for Spx concentration. A mutation that changes the first cysteine residue of YjbH CXXC motif at the N terminus to alanine did not affect Spx-dependent transcriptional control and the control of Spx concentration in untreated and diamide-treated cells. Finally YjbH is proposed to post-tranlationally modulate Spx level in *B. subtilis*.

CHAPTER 1 INTRODUCTION

Bacillus subtilis is a Gram-positive bacterium commonly found in soil (Madigan & Martinko, 2005). As a member of the genus *Bacillus*, *B. subtilis* has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. It has also been called *Bacillus globigii*, Hay *Bacillus* or Grass *Bacillus*.

B. subtilis is not recognized as a human pathogen; it may contaminate food but rarely causes food poisoning (Ryan & Ray, 2004). It has been used in food industry for thousands of years in eastern Southeast Asia and Africa (Wang & Fung, 1996). Due to its ability to uptake exogenous recombinant DNA and secrete functional proteins, it is also widely used in industry for the enzyme synthesis such as amylases and proteases (Harwood, 1992).

Besides extensive application in industry, *B. subtilis* is used as a model organism of Gram-positive bacteria because the bacterium is amenable to genetic manipulation and there exists a wealth of available information that has made *B. subtilis* the principal paradigm for analysis of the physiology of Gram-positive bacteria; comparable to *Escherichia coli*, an extensively studied Gram-negative bacterium.

In our study *Bacillus subtilis* is used as the model organism to conduct research on gene expression and its regulation in response to environmental changes. This chapter summarizes the mechanisms of transcription initiation and proteolytic control in the oxidative stress response in *B. subtilis*. The transcriptional control exerted by the global regulator Spx and its control by proteolysis will be discussed in the following chapters of this thesis.

1.1 THE MOLECULAR MECHANISM OF TRANSCRIPTIONAL CONTROL IN *BACILLUS SUBTILIS*

An area of intensive investigation involving *B. subtilis* is the mechanisms of transcriptional control, which is at the center of gene regulation in prokaryotes. The process of transcription serves to transform the information of DNA to RNA, the synthesis of which requires a double-stranded DNA template and an enzyme complex, the DNA-dependent RNA polymerase to create a complementary RNA that is released to the cytoplasm for translation. Transcription can be divided to three steps: initiation, elongation and termination. This chapter will focus on the initiation of transcription, the process in which Spx participates.

1.1.1 Transcriptional initiation complex formation

Transcription begins with the binding of RNA polymerase to the promoter region in DNA. In *B. subtilis*, as in all bacteria, the RNA polymerase is composed of a core enzyme consisting of five subunits: 2 α subunits, 1 β subunit, and 1 β' subunit, and the ω subunit. At the start of initiation, the core enzyme is associated with a specific σ factor and in some cases other accessory proteins that aid in recognizing the appropriate -35 and -10 elements of the promoter by RNA polymerase. The accessory protein either causes conformation change of RNA polymerase to facilitate the DNA-RNA polymerase initiation complex formation or binding upstream cis-acting elements of the promoter to recruit RNA polymerase. The completed assembly of transcription factors and RNA polymerase bound to the promoter is called the *transcription initiation complex*.

Transcription initiation in prokaryotes is controlled at many levels and through a variety of protein-protein and protein-DNA contacts between RNA polymerase (RNAP), promoter DNA, and diverse regulatory factors.

1.1.2 Sigma factor of RNA polymerase holoenzyme

RNA polymerase sigma factor is largely responsible for promoter recognition in eubacteria. Usually cells have one essential housekeeping sigma factor and a variable number of alternative sigma factors that recognize different promoter DNA sequences. For example, σ^{70} of *E. coli* and σ^{A} of *B. subtilis* are “housekeeping” sigma factors that

direct most gene expression in the exponentially growing cells (Paget & Helmann, 2003). Alternative sigma factors are usually activated in response to environmental signals, which can rapidly reprogram gene expression, sometimes on a genome-wide scale. Accumulation and/or activation of the alternative sigma factor results in transcription of a set of specific genes through specific recognition of a promoter DNA sequence, leading to induction of cellular processes in response to a specific stress or to initiation of a developmental process (Gruber & Gross, 2003). For example, when cells enter stationary phase of the growth curve and facing nutrient deprivation, the alarmone guanosine 3', 5'-bispyrophosphate (ppGpp) accumulates in the cell (Cashel *et al.*, 1996). Accumulation of ppGpp can block DNA replication by directly inhibiting DNA primase (Wang *et al.*, 2007) and indirectly affect the GTP pool in *B. subtilis*. Since GTP is the initiation NTP for *B. subtilis* rRNA promoter such as *rrnO* and *rrnB* promoters (Krásný & Gourse, 2004), ribosomal RNA and ribosome production slows. In response to amino acid starvation in *E. coli*, ppGpp directly interacts with RNA polymerase to inhibit the expression of genes required for production of the translational apparatus and to induce the expression of genes whose products function in amino acid biosynthesis and protein hydrolysis, partially through down-regulation of σ^{70}-dependent promoters [review in (Cashel *et al.*, 1996)]. Additionally, alternative sigma factors σ^{S} (Bougdour & Gottesman, 2007), σ^{54} (Szalewska-Palasz *et al.*, 2007) and σ^{E} (Costanzo & Ades, 2006), required for expression of stationary-phase specific and stress-response gene expression, are activated.

σ^{70} family members when bound to core enzyme can form transcriptional open complex by their own, but another family of σ subunits, σ^{54}, require the aid of ATP, a DNA element called an enhancer, and an ATP-dependent enhancer binding protein for the holoenzyme to form the open complex (Buck *et al.*, 2000). σ^{54} is not present in high-GC, Gram-positive bacteria or in cyanobacteria.

Sigma factors are divided to four groups according to their phylogenetic relatedness to *E. coli* σ^{70} (Lonetto *et al.*, 1992). *B. subtilis* contains 18 σ^{70}-type sigma factors (Gruber & Gross, 2003). Group I sigmas are the housekeeping sigma factors such as σ^{70} of *E. coli* and σ^{A} of *B. subtilis*. Group II sigmas that include the stress response sigma factors such as σ^{S} of *E. coli* are closely related to the group I sigmas but are dispensable for growth. There are no group II sigmas in *B. subtilis*. Group III sigmas are

more divergent in sequence and can be divided into groups of evolutionarily related proteins with similar functions. In *B. subtilis* they are involved in the general stress response (SigB) (Haldenwang & Losick, 1980), heat-shock response [SigI (Zuber *et al.*, 2001)], flagella biosynthesis [SigD (Yang *et al.*, 1999)], sporulation [SigE, SigF, SigG, SigH, SigK, review in (Errington, 1991)], and early stationary phase growth [SigH, (Britton *et al.*, 2002)]. Group IV sigmas have distant sequence similarity to the other σ^{70} groups. They were originally called the extracytoplasmic function (ECF) family (Missiakas & Raina, 1998) because they were initially identified as sigmas that regulate some aspect of the cell surface or transport and were often found to be cotranscribed with a trans-membrane anti-σ. These include the sigma factors SigV, SigM, SigX, SigY, SigW, SigZ and YlaC in *B. subtilis*.

The σ^{70} family member contains four regions that function in RNA polymerase interaction and promoter DNA binding (Gross, 1996). Region 1.1 only exists in group I sigmas and functions as an autoinhibitory domain that interacts with the DNA-binding determinants (Nagai & Shimamoto, 1997). Region 2.3 might function in melting the duplex DNA. Region 2.4 recognizes the -10 element and region 4.2 targets the -35 element. Region 3.0 (formally region 2.5), when present, recognizes the extended -10 (Gross, 1996).

1.1.3 Crystal structures of RNA polymerase

High resolution crystal structures of *Thermus aquaticus* holoenzyme provide an insight to RNA polymerase, sigma and promoter DNA transcriptional initiation complex formation (Campbell *et al.*, 2002). The prokaryote RNA polymerase resembles a crab claw as the β and β′ subunits form the pincers. These pincers form a 27 Å wide internal channel, with the catalytic, Mg(II)-bound, site of the enzyme (where the RNA phosphodiester bond formation occurs). During elongation, downstream DNA reaches the active site via this channel; along its path into the enzyme the two strands separate to form the transcription bubble. Upon reannealing, the upstream duplex is at a right angle to the downstream DNA. The nascent RNA transcript follows the path of the template strand for several bases and then exits the polymerase underneath a flexible element of β called the flap domain (Kuznedelov *et al.*, 2002). In the crystal structure σ extends across

on one face of RNA polymerase, mainly in contact with β and β′ subunits. Region 3 occupies the tunnel through which the RNA exits the transcribing complex. All the promoter-recognition determinants in sigma are hydrophilic exposed regions in the holoenzyme structure. Conformation change of both core enzyme and sigma is required for promoter DNA binding and transcriptional initiation complex formation (Gruber & Gross, 2003).

1.1.4 C-terminal domain of RNA polymerase α subunit (RNA polymerase αCTD)

As the -35 and -10 sequence recognition by sigma factor is required for transcriptional initiation, the upstream promoter UP-element is also necessary for full promoter activity in some promoters. The UP element is an AT-rich region upstream of the -35 and directly interacts with the C-terminal domain of alpha subunit of RNA polymerase (Ross *et al.*, 1993). The UP-element was first identified in the *E. coli* ribosomal RNA operon promoter *rrnB* P1 and was also identified in the *B. subtilis* phage phi29 genome (Meijer & Salas, 2004) and a few other promoters (Banner *et al.*, 1983). The RNA polymerase alpha subunit consists of two separated N-terminal domains (αNTD) and the C-terminal domain (αCTD), connected by a flexible linker. αNTD is in contact with RNA polymerase, while two copies of αCTD can interact independently with one or more 9bp A/T rich UP-elements, contacting the DNA minor grove to increase the affinity of RNA polymerase for promoter DNA (Ross *et al.*, 1993).

αCTD also provides sites of contact for interaction with sequence specific DNA-binding transcriptional regulators that serve to recruit RNA polymerase to the promoter (Busby & Ebright, 1999; Ishihama, 1992; Ptashne & Gann, 1997). Such factors include the cyclic AMP receptor protein (CRP) (Igarashi & Ishihama, 1991), which can interact with both promoter DNA and an UP-element binding αCTD to recruit RNA polymerase to the promoter in the *E. coli* (Lloyd *et al.*, 2002). Another important class of transcriptional activators that interact with RNA polymerase are the DNA-binding members of the response regulator of two component regulatory proteins (Kenney *et al.*, 1995; Lacal *et al.*, 2006). In *B. subtilis* ComA, the response regulator of the ComP-ComA two-component signal transduction system, is essential for the transcriptional activation

of *srf* operon that is required for competence development (Hahn & Dubnau, 1991; Nakano *et al.*, 1991a; van Sinderen *et al.*, 1990). Two dimer of phosphorylated ComA interact with the two ComA box elements upstream of *srf* promoter DNA (Nakano & Zuber, 1993; Roggiani & Dubnau, 1993) to recruit αCTD. This ComA-dependent transcriptional activation through interaction with RNAP αCTD is interrupted by the protein Spx (Nakano *et al.*, 2003b; Zhang *et al.*, 2006).

Aside from transcriptional regulatory proteins, transcriptional regulation is also achieved by the direct interaction between small RNA and RNA polymerase. The small non-coding 6S RNA interacts with RNA polymerase to occupy the *E. coli* RNA polymerase active site and prevents RNA polymerase-DNA interaction. The 6S RNA bound to RNAP serves as a template for the synthesis of a 14- to 20-nucleotide product (pRNA) during outgrowth from stationary phase. This newly synthesized pRNA functions to interrupt the 6S RNA-RNA polymerase complex and thus release of pRNA-6S RNA hybrid and free RNA polymerase in response to nutrient status (Wassarman & Saecker, 2006) .

αCTD not only provides a binding site for activator, it also provides binding surface for other transcriptional regulators. *B. subtilis* RNA polymerase αCTD is composed of residues 246 to 311 and folds as an independent domain of five loosely packed α-helixes (Jeon *et al.*, 1995). The interaction between *B. subtilis* αCTD and protein Spx, the repressor of *srf* operon, was identified by yeast two-hybrid experiments (Nakano *et al.*, 2003b; Zuber, 2004). The residue Y263, which is necessary for the direct interaction of Spx and αCTD, is strictly conserved in all low G+C Gram-positive bacterial but not in Gram-negative organisms where the corresponding residue is often an alanine. The Y263 residue is located at the α helix1 exposed to the surface of the αCTD (Newberry *et al.*, 2005). Transcriptional regulator Spx will be further described in section 1.7.

1.1.5 Regulation of transcription termination

Transcription termination in bacteria has two mechanisms. The intrinsic termination requires the formation of a hairpin structure of intrastrand base-pairing within the nascent transcript followed by a stretch of poly U residues that is added by the transcription

felongation complex. This hairpin structure serves as a pause signal, and upon transcribing the poly U residues the RNA polymerase will terminate transcription and release both DNA and RNA. The factor dependent termination relies on Rho protein which functions as a hexamer that scans uncomplexed RNA towards the 3′ direction. When Rho encounters the RNA polymerase stopped at a transcriptional pause site, it will assist RNA polymerase to release the DNA and RNA.

Control of intrinsic termination sometimes involves the formation of alternative secondary structure called an antiterminator (Landick *et al.*, 1996), which shares complementarity with a sequence of a terminator helix. The competition between forming antiterminator or terminator, called transcription attenuation, will affect the transcription of downstream DNA. Different mechanisms are involved to affect this competition [for review (Henkin & Yanofsky, 2002)].

T-box mechanism is employed mainly in Gram-positive bacteria to control the expresses of gene involved in amino acid biosynthesis, transport and aminoacyl-tRNA synthesis. For instance, the induction of *B. subtilis tyrS* gene, which encodes tyrosyl-tRNA ($tRNA^{Tyr}$) synthetase is achieved by an uncharged $tRNA^{Tyr}$. The anti-codon of this $tRNA^{Tyr}$ can pair with a specific sequence within a secondary structure formed by the leader transcript upstream of the antiterminator. This interaction can facilitate the second pairing between the acceptor end of uncharged $tRNA^{Tyr}$ and a bulge region of the antiterminator (Grundy *et al.*, 1994). The interaction of uncharged tRNA and leader RNA prevents intrinsic termination of the elongation complex, thus allowing it to proceed to the coding regions of the T-box operon. If there is enough tyrosine in the cell, uncharged tRNA concentration decreases, resulting in an increase in termination events within the T-box leader region.

S-box was first identified in *B. subtilis* as a highly conserved motif in the leader sequence of 11 genes involved in cysteine and methionine biosynthesis which are induced upon methionine starvation (Grundy & Henkin, 1998). The regulator molecule S-adenosylmethionine (SAM) strongly binds to the leader RNA to form an anti-antiterminator structure containing the sequence required for antiterminator formation, thus stabilizing the terminator structure to block transcription of the downstream genes in the presence of methionine (McDaniel *et al.*, 2003). SAM also participates in another

regulator mechanism by directly blocking the binding of the 30S ribosomal subunit to the Shine-Dalgarno sequence to inhibit the translation of *metK* which encodes SAM synthetase in lactic acid bacteria (Fuchs *et al.*, 2007).

1.2 OTHER FORMS OF GENE REGULATION IN *BACILLUS SUBTILIS*

Gene regulation in *B. subtilis*, as is believed in the case of all cells, prokaryotic or eukaryotic, is achieved at four levels: transcriptional control, post-transcriptional control, translational control and post-translational control. As transcriptional control has been addressed above, this section will deal mainly with other levels of gene control.

1.2.1 The post-transcriptional controls

The post-transcriptional controls are exerted through mechanisms that include polyadenylation, non-coding small RNA and RNA binding proteins. First, the polyadenylation of RNA is a template-free addition of A residues at the 3′ end catalyzed by the enzyme called poly(A) polymerase I (or PAP I) encoded by the *pcnB* gene in *E. coli*. 3′ polyadenylation in bacteria yields a shorter poly-A sequence than what is found in eukaryotes, and, unlike in eukaryotes, promotes degradation of mRNA fragments (Dreyfus & Regnier, 2002). Secondly, small non-coding RNAs (sRNA) were known to occur in bacteria since the 1980s (Mizuno *et al.*, 1984). A recent study estimated that enterobacterial genomes with an average size of 4-5 mega base might contain 200 to 300 sRNA (Zhang *et al.*, 2004), approximately 5% of the total number of proteins. Functional analysis of sRNAs indicated that most of them are induced in response to different stress conditions to alter the stability of the corresponding mRNA (Gottesman, 2004; Wabiko *et al.*, 1988). Traditional anti-sense sRNA and its target RNA are cis-encoded by the same DNA segment from two opposite orientation on transposon, plasmid and phage genomes with a perfect complementary (Wagner *et al.*, 2002). Studies indicate many sRNAs act as anti-sense RNA on trans-encoded mRNA to change message stability. Usually the trans-encoding sRNA forms short and imperfectly base-paired regions with the 5′ untranslated region (UTR) of the target mRNA, which is often facilitated by the Sm-like protein Hfq (host factor for phage Qβ) (Valentin-Hansen *et al.*, 2004). Hfq is required for post-transcriptional regulation of *rpoS,* which encoded the stationary phase/general stress

sigma factor by sRNAs, DsrA and OxyS (Lease & Belfort, 2000; Zhang *et al.*, 1998). Hfq can protect DsrA sRNA from degradation by RNaseE through specific RNA binding which shields the RNaseE recognition site (Moll *et al.*, 2003). In low temperature, the riboregulator sRNA DsrA affects target RNA stability with two different mechanisms: it basepairs with the translational start and stop regions of *hns* RNA (which encoding HN-S transcriptional regulator) to expose the middle part of RNA to nuclease for degradation and also basepairs with *rpoS* RNA at a specific sequence that would otherwise form a translational inhibitory intramolecular structure (Lease & Belfort, 2000).

The synthesis of mRNA will lead to the process of translation, which synthesizes functional proteins or peptides according to the instructions provided by the mRNA templates. When a ribosome becomes stalled at the 3′ end of damaged mRNA or the region has acquired a rare codon, trans-translation takes place, which tags a prematurely terminated polypeptide with a short amino acid sequence that is encoded by the tmRNA (having the characteristics of both tRNA and mRNA and encoded by the *ssrA* gene). A 10 to 27 amino acid peptide tag with the C-termini nonpolar (Y/A)A(L/V)AA sequence can be recognized by the housekeeping proteases such as HlfB, ClpXP, ClpAP, and Tsp proteases in *E. coli* (Keiler *et al.*, 1996). Control of proteolysis will be described later in the introduction section.

1.2.2 The post-translational controls

Post-translational regulation includes phosphorylation, s-thiolation of the cysteine residue, as well as the turnover by proteolysis of protein or peptides that are no longer useful in a metabolic or developmental pathway, or become harmful to cells.

1.2.2.1 **Phosphorylation.**

Protein phosphorylation usually occurs at a hydroxyl group of the serine, tyrosine, threonine side chains, and is a reversible, post-translational mode of regulation that plays a key role in signaling cascades that have been widely studied in eukaryotes [reviewed in (Mukherji, 2005; Pawson & Scott, 2005)] Though there are a variety of phosphatases (that remove the phosphoryl group from a high-energy substrate such as ATP) and kinases (that transfer a high energy phosphate group to the substrate using ATP) that have

been predicted and identified in prokaryotic organism, studies of phosphorylation in bacterial systems were focused on the characterization of histidine and aspartic acid phosphorylation which are important in the bacterial signal transduction systems such as two-component regulation and the phosphotransferase (PTS) uptake systems (Beier & Gross, 2006; Deutscher *et al.*, 2006; Klumpp & Krieglstein, 2002). Recent study of phosphoproteome of *B. subtilis* identified hundreds of phosphorylation sites, most of which are on serine residues, with less on threonine and tyrosine residues. These phosphorylated proteins are involved in a wild range of metabolic processes, particularly in carbohydrate transport and metabolism (Macek *et al.*, 2007).

The phosphotransfer schemes also play key roles in signal transduction. Signal transduction systems exist in all living organisms and serve to monitor and transmit signals derived from intracellular and extracellular changes. In prokaryotes and in some lower eukaryotes, a common mechanism is the two-component signal transduction pathway. Two-component systems employ a histidine kinase to sense the environmental stimuli, to generate a signal in the form of a high-energy phosphoryl group and to transfer the phosphoryl group to an asparatate residue of the cognate response regulator to control the downstream effectors and activate specific responses.

The sporulation phosphorelay, which controls the initiation of sporulation is one such well-studied example of signal transduction involving two-component regulatory proteins. Sporulation is a process of cellular differentiation in which a daughter cell product of cell division differentiates into a dormant cell type known as the spore or endospore (Piggot & Coote, 1976; Stragier & Losick, 1996). Under poor growth conditions, *B. subtilis* will divide asymmetrically into two cell types, the mother cell and the forespore. After engulfment of the forespore, the mother cell eventually lyses and dies, and the forespore becomes a mature, highly stable spore. Once environmental conditions become favorable again, the spore will undergo germination followed by outgrowth that gives rise to a new, vegetatively growing *B. subtilis* cell.

Sporulation in *B. subtilis* is a multistage developmental process. The key regulator for initiation of sporulation is the response regulator, Spo0A, which is activated by a multicomponents phosphorelay (Burbulys *et al.*, 1991). The Spo0 phosphorelay (Burbulys *et al.*, 1991) involves phosphoryl group transfer requiring five histidine kinases

(KinA, KinB, KinC, KinD and KinE) and two phosphorelay proteins (the response regulator, Spo0F, and the histidine phosphotransferase, Spo0B) to activate the master regulator Spo0A. Two histidine kinases KinA and KinB, provide phosphate to Spo0F to initiate the signal transduction pathway. Accumulation of Spo0F~P is controlled by two regulatory aspartyl-phosphate phosphatases, RapA (Perego & Hoch, 1996) and RapB (Tzeng *et al.*, 1998). Transcription of the *rapA* gene is activated by ComA competence response regulator and the activity of RapA is modulated by PhrA, which is a pentapeptide inhibitor, the gene for which is cotranscribed with RapA (Ishikawa *et al.*, 2002). An export-import control circuit regulates the production of PhrA, its export and uptake of PhrA into the cell mediated by the Opp (peptide transporter) system (Perego, 1997). PhrA specifically represses RapA phosphatase activity to dephosphorylate Spo0F~P (Ishikawa *et al.*, 2002). The phosphate passed from Spo0F (Asp) to Spo0B (His), and finally reaches Spo0A (Asp) (Perego, 1998). Spo0E phosphatase is the last checkpoint to modulate the activity of Spo0A~P (Perego & Hoch, 1991). Spo0A (Jiang *et al.*, 2000), controls the genes required for initiation of the *Bacillus* sporulation process.

The examples listed above illustrate that phosphorylation is an important protein modification, which is required in signal transduction and enzyme activity involving several metabolic and developmental processes.

1.2.2.2 **S-thiolation**

S-thiolation is the disulfide exchange between the cysteines of protein and low molecular thiols such as glutathione to regenerate reduced proteins, such as thioredoxin and glyceraldehyde-3-phosphate dehydrogenase (Brune & Mohr, 2001) in eukaryotes and the oxidative stress specific transcription factor OxyR in *E. coli* (Hondorp & Matthews, 2004; Kim *et al.*, 2002; Zheng *et al.*, 1998). In *B. subtilis* cysteine serves as a major low-molecular weight thiol, but a new 398 Da substance may also serve as a biothiol in *B. subtilis* (Helmann, personal communication). The S-thiolation prevents proteins from irreversible damage caused by oxidative stress and facilitates protein redox control. This will be further addressed in the section describing the oxidative stress response.

1.3 COMPETENCE DEVELOPMENT IN *B. SUBTILIS*

When *B. subtilis* is confronted with a growth-restricting environment due to limited nutrient availability, oxidative stress, or high cell density, it will activate a complex network of interconnected signal transduction pathways to facilitate a "decision-making" process that allows *B. subtilis* to select one or more appropriate responses. These can include developmental processes involving cellular specialization such as sporulation and genetic competence, or establishment of motility, antibiotic production, aerobic/anaerobic growth and extracellular protease production.

Among the developmental programs operational in *B. subtilis*, genetic competence is a globally programmed physiological state, a semi-dormant condition, called the K state (Berka *et al.*, 2002) distinct from vegetative growth and sporulation. The hallmark of the competent cell is its ability to internalize exogenous high-molecular-weight DNA (Dubnau, 1999). In *B. subtilis*, competence develops post-exponentially and only a minority of the cells in a culture becomes competent.

Development of competence is also called the K state (Berka *et al.*, 2002) since it is tightly regulated via a complex regulatory system, centered around competence transcription factor ComK (Dubnau & Lovett Jr., 2002; Hamoen *et al.*, 2003). During exponential phase, competence development is prevented by maintaining a low concentration of ComK in the cell, which is achieved by both transcriptional and post-translational controls. The expression of *comK* is under a complex control that involves repression by directly binding of repressors AbrB (Hahn *et al.*, 1995; Hahn *et al.*, 1996), CodY (Ratnayake-Lecamwasam *et al.*, 2001; Serror & Sonenshein, 1996) and Rok to the *comK* promoter (Hamoen *et al.*, 2003a; Hoa *et al.*, 2002; Serror & Sonenshein, 1996) as well as positive control requiring DegU (Dahl *et al.*, 1992; Hamoen *et al.*, 2000) and ComK itself (van Sinderen & Venema, 1994; van Sinderen *et al.*, 1995). During exponential growth any synthesized ComK is bound by the adaptor protein MecA, which targets ComK for protease ClpCP-catalyzed degradation (Turgay *et al.*, 1998). During the transition from exponential phase to stationary phase, the cell responds to environmental changes, such as nutrient deprivation and increased cell densities, by relieving transcriptional repression of *comK* by AbrB and CodY (Hahn *et al.*, 1995a; Serror & Sonenshein, 1996) and by synthesis of small peptide (see below) which binds to MecA

and displaces ComK, resulting in the release of ComK from the proteolytic complex (Turgay *et al.*, 1997). Once ComK is free in the cell, it activates transcription of its own gene and the late competence genes, encoding the DNA-binding, -uptake and -integration machinery (van Sinderen & Venema, 1994).

1.3.1 ComP-ComA two-component signal transduction system activates *srf* operon

The *srf* operon encodes the small peptide ComS and surfactin synthetase which catalyzes the nonribosomal synthesis of the peptide antibiotic surfactin. ComS is transcriptionally regulated by the ComP-ComA two-component signal transduction system in response to external environmental changes. There are two *B. subtilis* extracellular peptide factors that accumulate in the medium as cells grow to high density and act via converging signal transduction pathways to activate the transcription of *srfA*. First is ComX, a modified peptide pheromone that mediates cell density-dependent control. ComP as a histidine kinase (Weinrauch *et al.*, 1990) in a two-component regulatory system (Parkinson, 1993), senses the presence of ComX by direct interaction, then autophosphorylates and donates its phosphoryl group to the cognate response regulator ComA (Weinrauch *et al.*, 1989; Weinrauch *et al.*, 1990). ComA, thus activated, is a transcription factor that binds to the *srfA* promoter. Another competence pheromone CSF (competence and sporulation stimulating factor) does not affect ComP but elevates the activity of ComA. CSF inhibits a phosphoprotein phosphatase RapC which otherwise dephosphorylates ComA (Turgay *et al.*, 1998).

Therefore both pheromone pathways regulate the level of ComA phosphorylation and phosphorylated ComA must bind to the promoter region of *srfA* (Dubnau, 1993; Nakano *et al.*, 1991a; Nakano *et al.*, 1991b; Nakano & Zuber, 1993) and activate the σ^A-dependent transcription of *srf* operon.

ComS was discovered by deletion analysis which revealed that a 569-bp fragment of *srfAB1* which encodes the valine-activating domain SrfAB1 is required for competence. When this fragment was fused to the *srfA* promoter, it complemented a *srfA* deletion mutation (Δ*srfA*) with respect to competence development (Parkinson, 1993). This fragment contains an open-reading-frame encoding 46 amino acids (orf46), which

encodes the *srfA*-associated competence regulatory factor ComS. ComS functions to release active ComK, which is sequestered by binding to a proteolytic complex of MecA and ClpCP (Lazazzera *et al.*, 1997; Solomon *et al.*, 1995). When ComS accumulates in cells, it interacts with MecA, causing ComK release from the inhibitory complex. ComK is now able to activate transcription of its own gene. When ComK is released from the complex, MecA and ComS become the targets for ClpCP-dependent proteolysis (Turgay *et al.*, 1998).

1.3.2 ComPA regulon

ComP-ComA also activates the transcription of *rapA*, which encodes an aspartate phosphatase. RapA prevents sporulation by dephosphorylating Spo0F~P and thus inhibiting the Spo0 phosphorelay that is required to initiate sporulation (Ishikawa *et al.*, 2002). Thus, the competence promoting ComPA system serves to down-regulate sporulation; sporulating cells do not become genetically competent and vice versa.

Recent microarray analysis of genes controlled by the small peptide ComX pheromone and ComPA signal transduction pathway indicated that three proteins control the same set of genes, which confirms that ComP is the only histidine kinase to accept the quorum signal from ComX, and that ComA is the sole response regulator to receive the phosphoryl group from and be activated by ComP (Comella & Grossman, 2005). The ComA regulon includes 20 directly controlled and 150 indirectly controlled genes including the genes required for competence development. Thus, the ComA-regulon functions in cell-cell communication and in the production of products that affect the extracellular environment. These activities enhance survival and colonization under conditions of high cell density (Comella & Grossman, 2005).

1.4 OXIDATIVE STRESS

Oxidative Stress (OS) is a general term used to describe the steady state level of oxidative damage in a cell, tissue, or organ. It is the result of three factors; increased generation of oxidant, decreased antioxidant protection and failure to repair oxidative damage (Sies, 1985). It is very important for organism to maintain the redox status for intracellular processes and metabolic pathways (Ritz & Beckwith, 2001).

1.4.1 Generation of reactive oxygen species (ROS)

Cell damage is induced by the reactive oxygen species (ROS) such as superoxide anion radicals (O_2-•), hydrogen peroxide (H_2O_2), peroxynitrite (ONOO−) and hydroxyl radicals (HO•), all of which are able to modify most cellular macromolecules including essential protein, lipid and DNA.

There are several cellular processes that lead to the production of ROS. Cellular aerobic respiration involving the reduction of molecular oxygen (O_2) to water via the electron transport chain is a major source of ROS *in vivo*. This reduction involves four one-electron reductions resulting in the formation of partially reduced and highly reactive intermediates, such as the superoxide anion radical (O_2-•),hydrogen peroxide (H_2O_2), and the hydroxyl radicals (HO•) that may act as prooxidants (Xia *et al.*, 1996). About 1 to 5% of these ROS might escape from the electron transport chain to damage cellular components (Punchard & Kelly, 1996). ROS are also produced by peroxisomal β-oxidation of fatty acids (Yamato *et al.*, 2007).

Microsomal cytochrome P450 metabolism is another important generator of ROS. NADPH-cytochrome P450 reductase changes xenobiotic compounds to free radical intermediates, which transfer an electron to O_2, producing superoxide anion radical (O_2-•), and regenerating the parent compound. This process occurs at the expense of cellular reducing equivalents, such as NADPH, which can have consequences that extend to other metabolic processes (Hanukoglu, 2006).

ROS are also generated by enzymes within cells. Superoxide anion radical (O_2-•) can be generated by tryptophan dioxygenase (Kurnasov *et al.*, 2003) and xanthine oxidase (Hille & Massey, 1981). Hydrogen peroxide (H_2O_2) can be generated by enzymes such as guanyl cyclase and glucose oxidase (Wu *et al.*, 1999). Similarly, under low arginine conditions nitric oxide synthase can generate superoxide anion radical (O_2-•) (Xia *et al.*, 1996). Extracellular superoxide anion radical (O_2-•) can also be produced by the leukotriene generator lipoxygenase (Baud *et al.*, 1983) and the prostaglandin generator cyclooxygenase (Leroyer *et al.*, 1987).

Metals, such as iron, copper, chromium, vanadium and cobalt, are also sources of ROS-generating activity. Metals are capable of accepting and donating single elections.

Two important reactions are Fenton's reactions, in which ferrous Iron (Fe^{2+}) is oxidized by hydrogen peroxide (H_2O_2) to ferric iron(Fe^{3+}), a hydroxyl radical (OH^-) and a hydroxyl anion (OH•). Fe^{3+} is then reduced back to Fe^{2+}, a peroxide radical (OOH•) and a proton by the same hydrogen peroxide (H_2O_2).

(1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$

(2) $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\bullet + H^+$

1.4.2 ROS-induced cell damage

ROS have been implicated in disease states, such as Alzheimer's disease, Parkinson's disease, cancer, and aging since they target macromolecules to cause peroxidation of polyunsaturated fatty acids in membrane lipids, mutagenic lesions and strand breaks in DNA and protein oxidation to a number of different amino acid side-chain conjugates.

ROS can cause irreversible side chain and backbone modifications of proteins and can lead to their unfolding, aggregation and premature degradation (Imlay, 2003). Oxidation of protein includes 1) carbonylation, which is an irreversible process that targets lysine, arginine, proline and threonine, 2) nitration of tyrosine 3) oxidation of methionine to methionine sulphoxide (Stadtman, 1993) and oxidation of cysteines.

Oxidation of cysteine residues include intramolecular and intermolecular disulfide bond formation which can be reduced by thioredoxin. Another consequence of cysteine oxidation is mixed disulfide bond formation with low molecular weight thiols such as glutathione or cysteine, which can be reduced by thioredoxin, glutathionine and glutaredoxin. Other products of oxidized cysteine such as S-nithiosothiols (SNO) can be reduced by glutathionine, thioredoxin and ascorbate, while sulphenic acids (SOH) can be reduced by glutathionine and thioredoxin (Watson *et al.*, 2004). Sulphinic acids (SO_2H) and Sulphonic acids (SO_3H) are irreversibly oxidized products of cysteine [reviewed in (Ghezzi, 2005)].

Thioredoxin present at micromolar concentration are the major mechanism for detoxification of cysteine oxidation through disulfide exchange. Disulfide bond formation in oxidized thioredoxin could be reduced by thioredoxin reductase, whose reduced form is restored by NADPH, yielding oxidized NADP. In *B. subtilis*, protein Spx is required

for transcription of genes *trxA* (encoding thioredoxin) and *trxB* (encoding thioredoxin reductase) in response to disulfide stress (Nakano *et al.*, 2003a).

1.4.3 Antioxidant protection

To counteract the reactive oxygen species, all aerobically growing organisms express a set of proteins and synthesize various small molecules (such as glutathione, cysteine, Coenzyme A) that eliminate ROS and reverse some of the oxidative protein modifications.

Enzymes that function in antioxidant protection include superoxide dismutase, which converts superoxide anions into hydrogen peroxide and oxygen, whereas catalase, glutathione peroxidase, and peroxiredoxins reduce and detoxify peroxides into alcohols or H_2O. Disulfide reductases like thioredoxin and glutaredoxin, on the other hand, reduce unwanted disulfide bonds in cytosolic proteins, while methionine sulfoxide reductases reduce methionine sulfoxides. All of these proteins, while constitutively expressed, can be upregulated in response to different kinds of oxidative stress.

1.4.3.1 S-glutathionylation

Glutathione, the major low-molecular weight thiol present in eukaryote and eubacteria, reduce H_2O_2 to H_2O through glutathione peroxidase via oxidation of GSH to GSSG. Glutathione GSSG can also form mixed disulfide bonds with reduced cysteines of protein (PSH) to prevent irreversible oxidation of protein via thiol/disulfide exchange to generate PSSG and GSH. Oxidized Glutathione GSSG can be reduced to GSH by glutathione reductase.

S-glutathionylation also functions in redox control of thioredoxin and Glyceraldehyde-3-phosphate dehydrogenase in eukaryotes and methionine synthase (MetE) (Hondorp & Matthews, 2004) and PAPS reductase (CysH) (Russel *et al.*, 1990) in *E. coli.*

1.4.3.2 **S-cysteinylation**

Glutathione is one of the most abundant intracellular non-protein thiol in biological systems and glutathionylation is the main form of S-thiolation in most organisms. In the extracellular environment, such as in plasma, glutathione is present in very low concentration. Cysteine now becomes the predominant extracellular low-molecular weight thiol.

In *B. subtilis* and many other Gram-positive bacterial species, glutathione is not synthesized. Cysteine is a major low molecular weight thiol present at over hundred micromolar range (Newton *et al.*, 1996). During thiol-specific oxidative stress, cysteine is the only amino acid whose biosynthesis is highly induced (Leichert *et al.*, 2003), and cysteine-based S-thiolation is an important mechanism for protein protection against oxidative stress and redox control in *B. subtilis* (Hochgrafe *et al.*, 2007).

1.5 DIAMIDE-INDUCED THIOL-SPECIFIC OXIDATIVE STRESS

Among the oxidative stress-inducing agents selected, diamide [diazenedicarboxylic acid bis (*N*, *N*-dimethylamide)] is a thiol-oxidizing agent. Diamide treatment results in rapid oxidation of GSH to GSSG, causing a GSH/GSSG redox imbalance in *E. coli*, and inducing disulfide bond formation in *B. subtilis*. As a specific oxidant for thiols, diamide reacts with free thiols to form disulfide bonds and a hydrazine derivative. So thiol-specific oxidative stress induced by diamide is also called disulfide stress.

Disulfide bonds play a critical role in stabilizing protein architecture, and extracellular proteins are especially dependent on disulfide bonds to maintain their structure (Bardwell, 1994). On the other hand, nonnative disulfide bond formation may lead to protein misfolding. In some enzymes, the transient disulfide bridges also serve as part of the catalytic cycle, as in reactions catalyzed by ribonucleotide reductase, methionine sulfoxide reductase, alkylhydroperoxide reductase, and arsenate reductase (Deneke, 2000). Other proteins possess cysteines as molecular redox switches that control their activity, such as in the transcriptional factor NF-κB in higher eukaryotes, Yap1p in *Saccharomyces cerevisiae*, OxyR and the chaperone Hsp33 in *E. coli* (Jakob *et*

al., 1999.; Kang *et al.*, 1999; Kuge *et al.*, 2001; Schumann *et al.*, 2002; Zheng *et al.*, 1998).

Disulfide stress in *B. subtilis* by treatment with 1 mM diamide significantly induces (at least three-fold) oxidative stress genes under the control of the global repressor PerR and heat-shock genes controlled by the global repressor CtsR. Other genes that were strongly induced encode putative regulators of gene expression and proteins protecting against toxic substances and heavy metals. Many genes were substantially repressed by disulfide stress, among them most of the genes controlled by the stringent response (Leichert *et al.*, 2003). The response to disulfide stress seems to be a complex combination of different regulatory networks, indicating that redox-sensing cysteines play a key role in different signaling pathways sensing oxidative stress, heat stress, toxic element stress, and growth inhibition.

1.5.1 Diamide-induced *spx* expression

In *B. subtilis* the concentration and activity of the global transcriptional regulator Spx increase in cells treated with diamide (Nakano *et al.*, 2003a). Recent studies in our lab indicates *spx* expression is controlled by its σ^A-dependent P3 promoter, which is induced upon diamide treatment (Leelakriangsak & Zuber, 2007). Transcription from the P_3 promoter is repressed by PerR and YodB, which bind independently at the *spx* P3 promoter during normal growth conditions (Leelakriangsak *et al.*, 2007), and are released from their operators after diamide treatment.

Since Spx was identified as the substrate for ClpXP (Nakano *et al.*, 2002b), the effect of the thiol-specific oxidant diamide on proteolytic control of Spx is one of my thesis topics, which will be discussed in the following chapters.

1.6 PROTEOLYSIS IN *B. SUBTILIS*

Proteolysis is an important mechanism used by bacteria to rapidly modulate protein levels during adaptive responses to changing environmental conditions, during cell cycle progression, and during development (Dubnau & Lovett Jr., 2002). It controls the turnover of the short-lived proteins controlling rate-limiting steps in regulatory pathways to limit the lifetime and amount of key regulators. It also removes the mis-

folding or truncated peptides generated by abortive translation that may interfere with metabolic processes. These irreversibly damaged proteins are produced continuously through events such as biosynthetic error, spontaneous denaturation and production of products encoded by loci that have accumulated mutations. Proteolytic elimination of abnormal protein is therefore crucial for cell homeostasis and optimal metabolic activities. Proteases are particularly vital during stresses that exacerbate the occurrence of damaged proteins (Gottesman, 1996)

1.6.1 AAA+ protease subunit

In bacteria, most of the short-lived and abnormal protein substrates are targeted for proteolytic degradation in the cytoplasm by a family of ATP-dependent proteases belonging to the AAA+ (ATPases associated with a variety of cellular activities) superfamily [for review (Neuwald *et al.*, 1999)]. These ATP-dependent proteases are usually composed of an ATPase component and the proteolytic component. The ATPase domain functions to recognize, unfold, and thread substrates into its attached proteolytic chamber [for review (Wickner *et al.*, 1999)]. The Clp proteolytic complex consisting of a proteolytic core flanked by an ATPase is highly conserved in bacteria. ClpP possesses only peptidase activity, but when paired with a member of the Clp ATPase family, the Clp Complex has serine protease activity (Maurizi *et al.*, 1990). The first substrate of ClpP found to be degraded *in vitro* was casein, hence, Clp stands for caseinolytic protease (Katayama-Fujimura *et al.*, 1987).

In *B. subtilis*, there are five ATP-dependent proteases, including representatives from each of the four subfamilies found in many bacterial species: LonA, LonB, FtsH, HslV and ClpP. The LonA, LonB and the membrane associated, FtsH proteins contain both the AAA+ ATPase and protease domains on one polypeptide (Liu *et al.*, 1999; Wehrl *et al.*, 2000). The ClpP and HslV proteins have only the proteolytic domain. HslV (ClpQ, CodW) requires its cognate AAA+ ATPase HslU (ClpY, CodX) to form an active protease complex (Kang *et al.*, 2001). There are three different AAA+ ATPases subunits: ClpX, ClpC and ClpE can associate with ClpP protease to form active proteolytic complexes with different but potentially overlapping specificities, which will be further described below.

1.6.1.1 **ClpC**

ClpC in *B. subtilis* is directly involved in solubilization and degradation of damaged and aggregated proteins, the accumulation of which is toxic for the cell (Kruger *et al.*, 2000). Expression of *clpC* is under control of both σ^A and σ^B promoters under normal growth conditions. During heat-shock response, transcription is induced primarily from the σ^A-dependent promoter. The σ^B-dependent promoter is activated at the end of exponential growth and turned-off at an early stage of sporulation, an event that requires the early sporulation stage sigma subunit σ^H. *clpC* expression is negatively controlled by CtsR, encoded by the first gene of the *clpC* operon and CtsR is also required for repression of other Class III heat-shock genes (Kruger & Hecker, 1998).

Class I heat-shock genes including *dnaK*, *groE*, *grpE*, which are most efficiently induced upon heat-shock, are controlled by the σ^A-dependent promoter and negatively regulated by HrcA, through its interaction with the inverted repeat CIRCE element at their promoters (Reischl *et al.*, 2002), and GroE which regulates HrcA activity (Mogk *et al.*, 1997). Class II genes are activated by other stresses and cell starvation. This class is controlled by alternative sigma factor σ^B (Boylan *et al.*, 1993; Haldenwang, 1995; Hecker & Volker, 1998). Class III heat-shock genes are general stress response genes controlled be vegetative promoter and do not require σ^B, CIRCE and HrcA. The class III genes include those encoding some ATP-dependent proteases and their subunits such as *lon* (Riethdorf *et al.*, *1994*), *clpC* (Kruger *et al.*, 1994), *clpP* (Gerth *et al.*, 1998), *clpX* (Gerth *et al.*, 1996) *and ftsH* (Deuerling *et al.*, 1995; Hecker *et al.*, 1996). Thioredoxin gene *trxA* (Scharf *et al.*, 1998) and alkylhydroperoxide reductase operon *ahpCF* (Antelmann *et al.*, 1996) are also members of this group.

ClpCP is an ATP-dependent protease that sometimes requires an adaptor or molecular chaperone for substrate recognition. In *B. subtilis* MecA is one of the molecular chaperones for ATP-dependent proteases, which facilitate the proteolysis of protein ComK by ClpCP protease. The *mecA* gene is located in the vicinity of *spx* in the *B subtilis* genome. MecA targets ComK, the competence regulator, in exponential phase cells to facilitate its turnover by ClpCP, and in response to environmental changes such

as high cell density. MecA binds to the small peptide ComS (encoded by the *srf* operon) which is activated by the ComPA two-component signal transduction system upon receiving high cell density signals through the extracellular signaling peptides ComX and CSF (Lazazzera & Grossman, 1998). ComS-MecA interaction results in the release of ComK from the ClpCP proteolytic complex (Turgay *et al.*, 1998). ComK exerts transcriptional autoregulation and controls the transcription of late competence genes (Dubnau, 1999) to establish the competent cell state or K-state.

YpbH is a paralog of MecA, which can interact with ClpC and increases competence gene expression and blocks sporulation when overproduced (Nakano *et al.*, 2002b; Persuh *et al.*, 2002). It can serve as a molecular chaperone for ClpCP-dependent degradation of ComK *in vitro* (Nakano *et al.*, 2002b).

1.6.1.2 **ClpE**

ClpE is similar to ClpC and is identified as a new member of the Hsp100 Clp ATPase family, whose expression is also induced by heat-shock and translation interruption by puromycin treatment (Derre *et al.*, 1999a). Its expression is negatively controlled by global heat-shock response regulator CtsR [which also negatively controls its own expression and other class III heat-shock genes such as *clpC* and *clpP* (Kruger & Hecker, 1998)]. CtsR directly binds to the *clpE* gene promoter region (Derre *et al.*, 1999b). ClpE is involved in disaggregation of insoluble heat-denatured proteins and ClpEP was found to degrade CtsR with different kinetics compared with that of ClpCP *in vivo* and *in vitro* after heat stress. So CtsR autoregulates through proteolytic control by ClpEP (Miethke *et al.*, 2006).

1.6.1.3 **ClpX**

ClpX is the ATP-dependent substrate-binding subunit of ClpXP protease and directly functions in the degradation of mis-folded proteins (Kruger *et al.*, 2000). ClpX is required for the expression of Class III heat-shock proteins and is also essential for both competence and sporulation. The *clpX* mutant strain displays chains of elongated cells and exhibits impaired viability under stress conditions and starvation. It is involved in the

response to nutritional stress, in sporulation, and pH stress through post-translational control of σ^H activity (Liu *et al.*, 1999).

ClpX and ClpP orthologs are found in most bacteria, mitochondria, and chloroplasts. Intensive studies have focused on *E coli* ClpX. Through proteomic studies, five distinct degradation signals were identified, including three sequence motifs at the N termini of natural substrates and two sequence motifs found at the C termini (Flynn *et al.*, 2003). The C-terminal residues of MuA transposase, the N-terminal residues of the lambda phage O protein are known as recognition signals for ClpXP degradation (Gonciarz-Swiatek *et al.*, 1999; Levchenko *et al.*, 1997).

In *Bacillus subtilis* the C-terminal residues LAN of Spx are required for its degradation by ClpXP (Nakano *et al.*, 2002b; Nakano *et al.*, 2003a; Nakano *et al.*, 2003b). This sequence shows similarity to the ssrA-tag (AANDENYALAA) protein, which is another substrate recognition motif for ClpXP in both *E coli* and *B. subtilis* (see below)(Gottesman *et al.*, 1998; Wiegert & Schumann, 2001).

Except for the direct sequence recognition by ClpX, some substrates require an additional adaptor protein to tether substrates to the ATP-dependent unfoldase. The response regulator RssB in *E coli* can act like an anti-σ factor by recognizing the stationary phase sigma factor σ^S and also functions to deliver σ^S to ClpXP for degradation (Becker *et al.*, 2000). In exponentially growing cultures, RssB is kept in an active form to quickly facilitate the turn over of σ^S. (Zhou & Gottesman, 1998). ClpXP and ClpAP can both degrade SsrA-tagged protein *in vitro*, but ClpXP is primarily responsible for the *in vivo* degradation of the majority of misfolded proteins or truncated products tagged with the SsrA peptide. ClpXP-catalyzed degradation of SsrA-tagged products is enhanced by an adaptor, the ribosome-associated protein SspB, which can specifically recognize SsrA-tagged proteins and deliver them to ClpXP rather than ClpAP (Levchenko *et al.*, 2000).

In *B. subtilis*, two-dimensional protein gel electrophoresis compared the protein pattern of wild-type, *clpP*, and *clpX* strains, which showed increased levels of GroEL, PpiB, PykA, SucD, YhfP, YqkF, YugJ and YvyD in both *clpP* and *clpX* mutant strains. Some of the above proteins likely are the substrates for ClpXP protease (Gerth *et al.*, 1998) or encoded by genes activated by Spx, a ClpXP substrate [such as YugJ (Kock *et al.*, 2004)].

Proteolysis is one of the most precise post-translational regulatory mechanisms for broad-range control of cellular processes, with multiple targets being recognized by proteases and molecular chaperones (Wickner *et al.*, 1999).

1.6.2 Spx as a ClpXP substrate

The *spx* gene was previously identified as the site of suppressor mutations of *clpP* and *clpX*. Higher levels of Spx are produced in *clpP* mutants than in wild-type cells grown in competence medium. This suggests that the Spx protein is a substrate for ClpP-containing proteases (Nakano *et al.*, 2001). *In vitro* proteolysis experiments using purified proteins demonstrated that Spx was degraded by ClpCP but only in the presence of one of the ClpC adapter proteins, MecA or YpbH (Nakano *et al.*, 2002b). When *spx* transcription is placed under the control by an IPTG-inducible promoter, the IPTG-induced Spx only accumulated when ClpX or ClpP were absent, suggesting that ClpX and ClpP are required for post-translational proteolytic control of Spx protein and not transcriptional control of the *spx* gene (Nakano *et al.*, 2001; Nakano *et al.*, 2002b). These results suggest that both ClpCP and ClpXP degrade Spx, but ClpXP activity was more important in controlling Spx concentration *in vivo*.

1.6.3 Physiological role of ClpP protease

The induction of genetic competence is prevented through the degradation of the ComK transcription factor by ClpCP (Nakano *et al.*, 2002a; Turgay *et al.*, 1998). ClpCP also plays an important role during spore development. During competence development, an important target of ClpXP during the initiation of competence is the transcription factor Spx (Nakano *et al.*, 2001). ClpXP is required for the efficient induction of a subset of stationary phase genes, including early sporulation-specific genes (Liu *et al.*, 1999; Msadek *et al.*, 1998; Nanamiya *et al.*, 2000), and ClpCP is required at a later stage of spore development, just after polar septation, to activate cell type-specific gene expression in the forespore (Pan *et al.*, 2001). It is likely that normal spore development requires the proteolysis of additional substrates by ClpXP, ClpCP and, perhaps, other ATP-dependent proteases, though the roles of individual proteases may be difficult to discern when two or more proteases recognize the same substrate.

1.6.4 Structure of AAA+ protease

In *B subtilis*, the Clp holoenzyme is composed of two contiguous heptametric rings containing 14 proteolytic ClpP subunits, which are flanked by a hexameric ring of regulatory Clp subunits of the Clp/Hsp100 chaperone family at one or both ends of the ClpP chamber. The proteolytic chamber formed by ClpP protects the catalytic site inside the chamber from cytoplasmic peptides (Wang *et al.*, 1997). The narrow channel in the chamber functions to degrade the denatured substrate and small peptides sent in from the ATPase complex. The regulatory ATPase associated with the proteolytic unit functions to recognize, unfold and tranlocate the substrate. The energy required for denaturing the target protein is usually four times higher than the energy required for translocation, and the more stable the substrate more higher energy is consumed during denaturation (Kenniston *et al.*, 2003). So six-fold ATPase will provide a constitutive energy source for destabilizing different target proteins. The overall structure and organization of the Clp proteases bears a resemblance to the 26S proteasome of eukaryotic cells (Kessel *et al.*, 1995).

The AAA+ ATP-dependent protease usually contains either one or two nucleotide binding domains (AAA-1, AAA-2) and functional domains include the P domain required for binding to ClpP (Kim *et al.*, 2001) and the N1 and N2 domains proposed to be involved in protein binding (Barnett *et al.*, 2005). In addition, a domain (UVR) resembling the interaction domain between the nucleotide excision repair proteins, UvrB and UvrC was identified in several ClpATPases such as ClpC and ClpE (Ingmer *et al.*, 1999).

1.6.5 C4-type Zinc-binding domain (ZBD) of ClpX and zinc metabolism

Protease subunits ClpE and ClpX contain a C4-type Zinc-binding domain (ZBD) which is required for subunit dimerization (Wojtyra *et al.*, 2003), the hexamer really being a “trimmer of dimers”. In *E. coli*, a zinc-deficient ClpX derivative is unable to bind ATP, to oligomerize, or to bind to ClpP (Banecki *et al.*, 2001). In *L. lactis* strain carrying a *clpE* gene with a mutated zinc finger motif showed decreased negative control of CtsR

(Varmanen *et al.*, 2003). In *B. subtilis* the ZBD of ClpE is essential for its basal level ATPase activity (Miethke *et al.*, 2006).

Zinc is an essential nutrient for all living organisms, though it is only the 27th most abundant metal in the earth's crust. Zinc serves as the catalytic cofactor for numerous enzymes and DNA-binding proteins and also provides a structural scaffold for metalloproteins (Vallee & Falchuk, 1993) .

Metalloregulatory proteins mediate transcriptional or translational control via sensing the intracellular concentration of a specific metal. The *B. subtilis* ferric uptake regulator (Fur) protein mediates the iron-dependent repression of at least 20 operons encoding approximately 40 genes. Its homologues in *B. subtilis* are PerR (regulator for response to peroxide stress through iron or manganese) and Zur (Zinc uptake repressor). Zinc-specific metalloregulation has been reported in yeast, mammals and bacteria.

The Fur homologue, Zur, in *B. subtilis* negatively controls two putative zinc homeostasis pathways in response to the micromolar levels of zinc (Gaballa & Helmann, 1998). Zinc starvation induces derepression of Zur regulon including the high affinity uptake pathway through the control of the *ycdHI-yceA* operon, which encodes an ABC transporter (Gaballa & Helmann, 1998), while the low affinity pathway specified by *yciABC* encodes a membrane protein belonging to a new metal transporter family (Gaballa *et al.*, 2002).

When the zinc level in the cell reaches the micromoler level, Zur represses the two uptake pathways and a third regulatory system involving a Zinc-binding P-type transporting ATPase ZosA [Zn(II) uptake under oxidative stress conditions] (Gaballa & Helmann, 2002). Deletion of all three zinc uptake system results in a cell that can only grow with addition of micromolar concentrations of Zn(II) (Gaballa & Helmann, 2002). Expression of *zosA* is activated by hydrogen peroxide and repressed by the metalloregulatory protein PerR rather than Zur. PerR binds to a PerR box at the *zosA* promoter. ZosA is important for resistant to H_2O_2 and thiol-specific oxidant diamide (Gaballa & Helmann, 1998; Gaballa & Helmann, 2002). Enhanced zinc uptake through ZosA upon peroxide stress might protect thiols from disulfide stress and oxidative stress. The study of the zinc-containing enzyme CDA (Cytidine deaminase) from *B. subtilis,* which is responsible for the hydrolytic deamination of cytidine to uridine and 2'-

deoxycytidine to 2'-deoxyuridine, indicates that zinc-reconstituted enzyme can regain activity in the presence of reductant (Mejlhede & Neuhard, 2000). Without zinc the zinc-coordinating cysteine residues are exposed to oxidation that leads to disulfide bond formation.

ZBD is also required for the activity of proteins that function in the disulfide stress response. For instance, Hsp33, the heat shock chaperon protein, contains the Cys4 type ZBD motif that coordinates one zinc atom (Jakob *et al.*, 2000). Recent studies indicate the requirement for the ZBD to sense peroxide stress and a linker region to sense the unfolding at its C-terminal redox switch domain. This exposes the N-terminal substrate-binding domain to achieve full unfolding activity in response to oxidative stress (Ilbert *et al.*, 2006; Ilbert *et al.*, 2007).

Zinc concentration also determines the switch between ZBD containing or Zn-free ribosomal protein paraglogs (Natori *et al.*, 2007). Zinc starvation causes derepression of *ytiA*, which encodes the ribosomal protein paralog that does not bind Zn. The ytiA gene is controlled by the repressor Zur and its product replaces the ZBD-containing paralog RpmE, which functions under normal condition (Akanuma *et al.*, 2006).

1.7 TRANSCRIPTIONAL REGULATOR SPX

As described above, the transcription initiation complex includes accessory proteins, which will aid in productive interaction between RNA polymerase and promoter DNA. These are transcriptional activators that function to recruit RNA polymerase to target promoters. Another kind of transcriptional regulator, known as a repressor, binds to DNA to block the RNA polymerase binding site or interacts with RNA polymerase to cause a conformational change, thereby preventing DNA and RNA polymerase interaction and blocking transcriptional initiation.

1.7.1 Negative transcriptional control exerted by Spx

αCTD is a common target for activator-RNA polymerase interaction that allows RNA polymerase to fully contact promoter DNA (Busby & Ebright, 1999; Igarashi *et al.*, 1991; Igarashi & Ishihama, 1991; Mencia *et al.*, 1998; Ross & Gourse, 2005). αCTD is a target for a form of negative control that is exerted by the Spx protein in *B. subtilis*

(Nakano *et al.*, 2003b). Spx prevents or disrupts activator-RNA polymerase interaction by binding to αCTD.

The *spx* gene was first identified as one of the suppressor loci of *clpP* and *clpX* mutations (Nakano *et al.*, 2001). The *clpP* and *clpX* mutants are defective in genetic competence, sporulation and growth in minimum media, which can be partially bypassed by suppresser mutations in *spx* or the αCTD-encoding part of *rpoA*. Spx protein concentration increases in *clpP* and *clpX* strains (Nakano *et al.*, 2001; Nakano *et al.*, 2002b). Mutation of the *clp* genes also blocks anaerobic growth by preventing the expression of genes within the *resDE* regulon (Nakano *et al.*, 1996; Sun *et al.*, 1996). ResD and ResE constitute a two-component signal transduction system required for activation of the *fnr* (anaerobic transcriptional regulator) gene, the *hmp* (flavohemoglobin) gene, the *nasDEF* (nitrite reductase) operon, and other genes required for aerobic and anaerobic respiration. *In vitro* run-off transcription reactions indicated protein Spx repressed *srf* and *hmp* transcription in the presence of *B. subtilis* RNA polymerase and their transcriptional activators ComA and ResDE (Nakano *et al.*, 2003b). Since ComA controls genes required for competence development and ResDE control genes for adaptation to oxygen limitation, the high levels of Spx in the *clpX* and *clpP* mutants are responsible for the defective competence and anaerobic growth phenotypes of *clpX* and *clpP* mutants of *B. subtilis*. The negative transcriptional effects of Spx are reduced when Spx mutant protein Spx^{G53R} also called $Spx^{Cxs\text{-}16}$ or RNA polymerase with an αCTD mutant ($RpoA^{Y263C}$ also called $RpoA^{Cxs\text{-}1}$) is present in the *in vitro* transcription reactions. Yeast-two hybrid experiment indicated that either Spx^{G53R} or $RpoA^{Cxs\text{-}1}$ abolishes the interaction between Spx and αCTD (Nakano *et al.*, 2003b; Zuber, 2004), indicating that the interaction between Spx and αCTD is necessary for its negative control of activator ComA-dependent *srf* and activator ResD-dependent transcription. Spx does not show DNA binding activity on its own, and αCTD has been shown to be its only target, thus far. According to this specific regulation mechanism Spx was called anti-alpha protein (Nakano *et al.*, 2003b; Newberry *et al.*, 2005).

1.7.2 Transcriptional activation by Spx

In wild-type cells under normal growth conditions, the concentration of Spx is kept at low levels. Spx activity *in vivo* had been observed only in a *clpX* or *clpP* mutant background (Nakano *et al.*, 2001; Nakano *et al.*, 2002b). However, the physiological role of Spx in the wild-type *B. subtilis* cell, was not known. To address this, Spx was over-expressed and rendered resistant to degradation by ClpXP by changing the Spx C-terminal proteolysis recognition sequence from LAN to LDD (Nakano *et al.*, 2003a). A microarray hybridization analysis was then undertaken to identify the genes whose expression is negatively or positively affected by a Spx-RNA polymerase interaction (Nakano *et al.*, 2003a). Expression of proteolysis-resistant form of Spx was controlled by an IPTG-inducible promoter. The genome transcriptional activities were compared in wild-type and *rpoA*$^{Cxs-1}$ mutant backgrounds. In total, 106 genes showed 3-fold or more induction and 176 genes showed 3-fold or more repression upon Spx-αCTD interaction. Two of the most highly induced genes were *trxA* (thioredoxin, 14.8-fold induction) and *trxB* (thioredoxin reductase 9.3-fold). Several other induced genes also encode products that are associated with thiol-redox homeostasis, such as *tpx* (probable thiol peroxidase, 4.2-fold), *msrA* (peptide methionine sulfoxide reductase, 4.0-fold), as well as genes *ycgT* (4.2-fold), *ydbP* (3.1-fold), and *ytpP* (3.2-fold), which encode thioredoxin-like proteins. All were induced in *spxDD rpoA*$^{+}$ cells but showed no or poor induction in *spxDD rpoA*$^{Cxs-1}$ cells. The discovery of this set of Spx regulon genes indicated that Spx might function in the cell's response to oxidative stress. This was supported by the finding that the *spx* null mutant and *rpoA*$^{Cxs-1}$ cells showed hypersensitivity to the thiol-specific oxidant diamide (Nakano *et al.*, 2003a). The expression of *trxA* and *trxB* genes in wild-type cells increased after diamide treatment, but not in the *spx* and *rpoA*$^{Cxs-1}$ mutants. Diamide-induced disulfide stress also increased Spx-dependent repression of *srfA* transcription, which was not observed in the *spx* null and *rpoA*$^{Cxs-1}$ mutants.

The microarray data and measurement of specific transcript levels indicated that Spx-dependent transcriptional activation and repression increased in cells undergoing disulfide stress. Spx-αCTD interaction is required for the cell to alter the pattern of gene expression in order to repair the damage caused by toxic oxidants.

1.7.3 Spx homologues

Spx is highly conserved among a large number of low G+C Gram-positive species including many pathogens such as *Staphylococcus*, *Listeria*, *Enterococcus*, and *Streptococcus* and resembles members of the arsenate reductase (ArsC) (Martin *et al.*, 2001) family of proteins.

In *Lactococcus lactis*, *trmA* encoding a protein which is now considered a *spx* homologue, is the site of mutations that alleviated temperature sensitivity cause by *recA* mutations and that suppressed *clpP* mutations by causing a general increase in proteolytic activity (Duwat *et al.*, 1999; Frees *et al.*, 2001). RecA and ClpXP are involved in self-cleavage of LexA-like protein HdiR, which is a negative transcriptional regulator that induces target gene expression in response to both heatshock and DNA damage (Savijoki *et al.*, 2003)

Another homologue of *B. subtilis spx* in *L. lactis* is *spxB*, which is activated by CesSR two-component regulatory system in response to cell-surface stress. SpxB was shown to interact with RpoA in yeast two hybrid assay and activates expression of *oatA*, that encodes peptidoglycan *O*-acetylase, which functions to increase the resistance to cell wall peptidoglycan hydrolysis in *L. lactis* (Veiga *et al.*, 2007) .

Spx in pathogenic *Staphylococcus aureus* is required for the response to a wide range of stress conditions including high and low temperature, high osmolarity, and hydrogen peroxide. It is also required for normal growth, partially due to its transcriptional activation of *trxB* under all growth condition. *trxB*, encoding thioredoxin reductase, is an essential gene for *S. aureus*. Spx also inhibits biofilm formation in *S. aureus* since transcription of *icaR*, which encodes a repressor of genes whose products are associated with biofilm formation, is increased in the absence of Spx. Thus, Spx is also a global effecter impacting stress tolerance and biofilm formation in *S. aureus* (Pamp *et al.*, 2006) .

In Chapter 2 experiments are described that provide insight into the mechanism of how Spx negatively controls the activator ComA-dependent transcription through the overlapping interaction surface of Spx/RNA polymerase and ComA/RNA polymerase. The proteolytic control of Spx affected by thiol-specific oxidant diamide will be

addressed in Chapter 3 and a putative post-transcriptional regulator of Spx will be characterized *in vivo* in Chapter 4.

CHAPTER 2 MUTATIONAL ANALYSIS OF THE *BACILLUS SUBTILIS* RNA POLYMERASE α C-TERMINAL DOMAIN SUPPORTS THE INTERFERENCE MODEL OF SPX-DEPENDENT REPRESSION*

2.1 INTRODUCTION

The *spx* gene of *Bacillus subtilis* was identified as the site of mutations that overcome the requirement for the protease ClpXP in the expression of genes that are transcriptionally activated by response regulator proteins (Nakano *et al.*, 2001; Nakano *et al.*, 2003b). The *srf* operon of *B. subtilis*, which contains genes encoding products that function in the control of competence development and in nonribosomal peptide synthesis (D'Souza *et al.*, 1994; Hamoen *et al.*, 1995; Nakano *et al.*, 1991; van Sinderen *et al.*, 1993), is activated by the ComPA two-component signal transduction system (Dubnau *et al.*, 1994; Dubnau & Lovett Jr., 2002). ComA is a response regulator that becomes phosphorylated by interaction with the histidine kinase ComP when the latter autophosphorylates in response to the peptide pheromone ComX (Grossman, 1995). This quorum-sensing system converts ComA to active ComA phosphate (ComA~P), which interacts as two dimers with the two ComA box elements residing upstream of the *srf* operon promoter (Nakano & Zuber, 1993; Roggiani & Dubnau, 1993). ComA-dependent transcriptional activation is one of the regulatory events in *B. subtilis* that are negatively affected by Spx (Nakano *et al.*, 2003b).

---

* Some part of this material has been published in this or similar form in *J. Bacteriol.* and is used here with permission of the American Society for Microbiology.

Zhang, **Y.**, **S. Nakano**, **S. Y. Choi**, **and P. Zuber.** 2006. Mutational analysis of the *Bacillus subtilis* RNA polymerase alpha C-terminal domain supports the interference model of Spx-dependent repression. *J. Bacteriol*. **188**:4300-4311.

Competence development, as well as several other transition state processes of *B. subtilis*, is severely impaired in strains bearing mutations in *clpX* or *clpP* (Liu *et al.*, 1999; Msadek *et al.*, 1998; Nakano *et al.*, 2001). Likewise, *srf* operon expression is diminished in *clpX* and *clpP* mutant cells (Nakano *et al.*, 2000; Nakano *et al.*, 2001). Some of the suppressor mutations resulting in restored *srf* expression in a *clpX* background mapped to the *rpoA* gene, which encodes the RNAP α subunit. Codon substitutions in the region encoding the C-terminal domain of α (αCTD) were uncovered through this suppressor analysis (Nakano *et al.*, 2000). One codon change conferring the *clpX* suppressor phenotype was Y263C, in the α1 helix of the αCTD. The other *clpX* suppressor locus is the *spx* gene (Nakano *et al.*, 2001), the product of which interacts with RNAP to affect transcription initiation (Nakano *et al.*, 2003b; Newberry *et al.*, 2005). Subsequent structural analysis confirmed that Spx interacts with the αCTD of RNAP and that the binding surface includes residue Y263 of the α subunit (Newberry *et al.*, 2005). This interaction is necessary for Spx-dependent repression of *srf* operon transcription (Nakano *et al.*, 2003b). Unlike other negative transcriptional regulators, however, Spx does not exhibit sequence-specific DNA binding activity (Nakano *et al.*, 2005).

Spx, while exerting negative control on activator-stimulated transcription, positively controls transcription of the thioredoxin (*trxA*) and thioredoxin reductase (*trxB*) genes as well as several genes that function in the oxidative stress response and in cysteine synthesis (Nakano *et al.*, 2003a; Nakano *et al.*, 2005). Positive control is observed after thiol-specific oxidative stress and requires disulfide formation at the highly conserved N-terminal CXXC motif. The detailed mechanism of Spx-dependent transcriptional activation is not known at this time.

The repression of the *srf* operon by Spx during oxidative stress is at least partly the result of a higher Spx protein concentration and interaction with the αCTD of RNAP (Nakano *et al.*, 2003a; Nakano *et al.*, 2003b). It is not known if oxidized Spx is required for repression. The mechanism of repression has been proposed to involve the interference of ComA interaction with RNAP by Spx (Nakano *et al.*, 2003b; Zuber, 2004). As with other prokaryotic transcriptional activators, ComA-dependent activation involves interaction of activated ComA with RNAP αCTD (this chapter). Alanine-scanning mutagenesis of RNAP αCTD and the identification of *rpoA* mutant alleles that

affect both Spx- and ComA-RNAP interaction are reported herein. The evidence presented supports the interference model of Spx-dependent repression.

2.2 RESULTS

2.2.1 Spx-RNAP interaction reduces ComA-assisted binding of RNAP to the *srf* promoter.

Previous studies provided evidence for a model of Spx-dependent transcriptional repression that involves the direct interference of interaction between the promoter-bound transcriptional activator and RNAP [reviewed in reference (Zuber, 2004)]. This was based on *in vitro* transcription studies using purified *srf* promoter DNA and ComA protein phosphorylated by treatment with acetyl phosphate. ComA~P had been shown to bind upstream of the *srf* promoter in two ComA boxes (Roggiani & Dubnau, 1993). However, electrophoretic mobility shift analysis (EMSA) suggested that ComA binding to *srf* promoter DNA requires its interaction with RNAP (Nakano *et al.*, 2003b). The binding of ComA~P to *srf* promoter DNA was reexamined, and the effect of Spx-RNAP interaction was investigated. DNase I footprinting showed that ComA~P is able to bind to ComA boxes 1 and 2 upstream of the *srf* -35 sequence (Fig. 2.1A) as previously shown (Roggiani & Dubnau, 1993). Higher concentrations resulted in protection near the -35 region (Fig. 2.1A, lane 3). RNAP alone protected sequences between -70 and -90 and between -10 and -30, but protection was extended upstream to the -35 region when ComA~P was included in the reaction (Fig. 2.1A, lanes 4 to 6). The ComA-assisted binding of RNAP to the -35 region is observed again in Fig. 2.1B. While higher concentrations of RNAP seemed to interact with DNA in the region between -10 to -30 in the absence of ComA (Fig. 2.1B, lanes 2 to 4), protection in the -30 to -40 region was observed when ComA~P was present (Fig. 2.1B, lanes 6 to 8). Figure 2.1B also shows that the addition of Spx protein significantly reduced RNAP binding and weakened ComA~P binding (lanes 10 to 12).

The *rpoA*$^{Cxs\text{-}1}$ and *spx*$^{Cxs\text{-}16}$ mutations block Spx-dependent inhibition of ComA-assisted RNAP binding to the *srf* promoter. A concentration of Spx (5 μM) that prevents binding of WT RNAP to the *srf* promoter (Fig. 2.1C, lanes 11 and 12) did not have a

significant negative effect on binding of RNAP bearing the mutant RpoA^{Cxs-1} subunit (Fig. 2.1C, lanes 7 to 9). The binding of WT RNAP to the *srf* promoter in the presence of ComA~P, while inhibited by WT Spx (Fig. 2.1D, lanes 5 to 7), was not affected by the inclusion of the mutant inactive Spx^{Cxs-16}, which was previously shown to confer reduced interaction between Spx and αCTD (Fig. 2.1D, lanes 8 to 10). Recently reported structural analysis has shown that the amino acid positions altered by the *rpoA*$^{Cxs-1}$ and *spx*$^{Cxs-16}$ mutations define part of the αCTD-Spx interaction interface (Newberry *et al.*, 2005).

The fact that ComA~P is able to interact with the *srf* promoter region and, in doing so, assists RNAP interaction contradicts previously reported EMSA results, which showed poor ComA interaction in the absence of RNAP (Nakano *et al.*, 2003b). Hence, we employed a third method to examine the function of ComA and Spx in RNAP-promoter interaction. The method, the Solid-phase promoter retention (SPPR) method, involves immobilization of biotinylated promoter DNA to a streptavidin-agarose bead support. Proteins are added to the bead-bound DNA, and the proteins retained after washing are examined by SDS-polyacrylamide electrophoresis and a colloidal Coomassie blue staining protocol (Candiano *et al.*, 2004). A blocking solution containing a mixture of BSA and Casamino Acids (amino acid solution) was used to prevent nonspecific binding of protein to the streptavidin-agarose beads (Fig. 2.2A). RNAP β, β' and α subunits are visible on the gels, but σ is obscured by the BSA band. RNAP binds poorly to the bead-bound *srf* promoter DNA (Fig. 2.2A, lane 3), and the addition of ComA~P to the mixture enhances RNAP retention (lanes 4 and 5). A ComA box 2 mutant version of *srf* promoter reduces the amount of ComA binding and reduces RNAP retention (Fig. 2.2A, lanes 6 and 7). The same outcome was observed with a -35 mutant form of the *srf* promoter (Fig. 2.2A, lanes 8 and 9), which has been shown to eliminate ComA-stimulated *srf* transcription (data not shown). This is consistent with the result that the protection observed in the -35 region of *srf* promoter DNA in footprinting reactions containing RNAP and ComA~P (Fig. 2.1) is due to RNAP binding. Figure 2B shows that equal amounts of DNA were applied to the streptavidin-agarose beads and could be recovered by phenol-chloroform extraction from the beads. Thus, the SPPR method provides an authentic picture of ComA/RNAP interaction at the *srf* promoter.

The addition of Spx to the SPPR reaction mixture containing ComA~P and RNAP reduces binding of RNAP to the bead-bound *srf* promoter DNA (Fig. 2.2C). When mutant *rpoA*$^{Cxs\text{-}1}$ RNAP was used in the reaction in place of WT RNAP, a reduction in Spx-dependent RNAP release was observed (Fig. 2.2C, lanes 6 to 8), a result consistent with the DNaseI footprinting data of Fig. 2.1 and previously published data (Nakano *et al.*, 2003a). In both footprinting and SPPR experiments, Spx substantially reduced RNAP binding to the *srf* promoter but also reduced ComA-DNA interaction. We conclude from these experiments that ComA is capable of interacting with *srf* promoter DNA, as was previously shown (Roggiani & Dubnau, 1993), but that this interaction is strengthened when RNAP is present, as was observed in the footprinting experiments of Fig. 2.1.

The hypothesis that ComA interacts with RNAP by binding to αCTD was supported by the observation that ComA can recruit purified αCTD protein to the bead-bound *srf* promoter DNA. The SPPR experiment of Fig. 2.2D shows that αCTD cannot interact with *srf* promoter DNA unless ComA~P is present. Spx addition causes the release of αCTD. This indicates that promoter-bound ComA~P can interact with αCTD and this interaction is sensitive to Spx.

2.2.2 Alanine-scanning mutagenesis of RNAP αCTD uncovers residues required for ComA-dependent activation of *srf* transcription.

We sought to employ *lacZ* fusion expression, *in vitro* transcription, footprinting, and SPPR analyses to study the effects of αCTD mutations on ComA and Spx function and to gain a better understanding of how Spx represses transcription. Our objective was to determine if ComA and Spx have overlapping binding surfaces on αCTD, indicating that Spx sterically hinders ComA-RNAP interaction.

The αCTD-coding region of the *B. subtilis rpoA* gene was subjected to alanine-scanning mutagenesis, and the resulting mutant alleles were introduced into the *rpoA* locus by a previously reported procedure (Nakano *et al.*, 2000). The expression of a *lacZ* fusion controlled by the *srf* promoter was examined in the αCTD mutants (Fig. 2.3). The activity of *srf-lacZ* was tested at the onset of stationary phase, while the mid-log expression of *rpsD-lacZ* was also monitored as a ComA- and Spx-independent control

fusion. The *rpsD* gene encodes ribosomal protein S4 (Grundy & Henkin, 1992), and its expression is maximal during the middle of exponential phase.

The C265, L266, K267, K287, and G307 residues are required for optimal ComA-dependent *srf-lacZ* expression (Fig. 2.3). The Y263C mutation, previously shown to confer reduced interaction between Spx and αCTD, has a negative effect on both *srf-lacZ* and *rpsD-lacZ* fusions.

Since the region around α1 of αCTD (Fig. 2.3, bracket) contains the binding interface between RNAP αCTD and Spx, as shown by crystal structure analysis (Newberry *et al.*, 2005), the effects of mutations altering residues C265 and K267 on ComA- and Spx-dependent control of the *srf* promoter were further examined.

2.2.3 The *rpoA*(C265A) mutation affects ComA-activated *srf* transcription and RNAP binding to the *srf* promoter.

The effect of the C265A mutation in *rpoA* on *srf* promoter utilization was examined using *in vitro* transcription and SPPR analysis. RNAP was purified from the *rpoA*(C265A) mutant cells and combined with ComA~P for time course transcription experiments. The reaction mixtures containing mutant RNAP and ComA~P showed a reduced rate of transcript accumulation (Fig. 2.4A and B), which was in keeping with the reduced expression of *srf-lacZ* in *rpoA*(C265A) mutant cells (Fig. 2.3). The mutant polymerase showed a level of *rpsD* transcript accumulation similar to that of wild-type RNAP. SPPR analysis shows that ComA-assisted binding of *rpoA*(C265A) RNAP to the *srf* promoter is defective (Fig. 2.4C, lanes 3 and 4), while no defect in binding of mutant RNAP to the *rpsD* promoter is observed (Fig. 2.4C, lanes 5 and 6). The mutation has no detectable effect on Spx repression (see below). The C265A substitution has no effect on ResD-dependent transcriptional activation *in vivo* and *in vitro* (H. Geng and M. M. Nakano, unpublished data), indicating that the mutation has a specific effect on ComA-RNAP interaction and does not confer a general defect on RNAP activity.

2.2.4 The *rpoA*(K267A) mutation affects ComA- and Spx-activated transcription and the Spx-dependent negative control.

Based on the recently published crystal structure of the αCTD-Spx complex, the K267 residue of αCTD contacts the conserved R47 of Spx (Newberry *et al.*, 2005) and might be important for stable RNAP-Spx interaction. A *B. subtilis* strain bearing the *rpoA* allele with a K267A codon substitution is hypersensitive to diamide-induced thiol-specific oxidative stress (Fig. 2.5A), which is the phenotype associated with defective RNAP-Spx interaction. The C265A mutation has no significant effect on diamide-resistance, and the Y263C mutation, as shown previously (Nakano *et al.*, 2003a), confers hypersensitivity to diamide due to reduced Spx-RNAP interaction and consequent defective oxidative stress response. *In vitro* transcription analysis of the mutant *rpoA*(K267A) RNAP in the presence of Spx showed that Spx-stimulated transcription from the *trxB* promoter is reduced compared to the reaction containing WT RNAP (Fig. 2.5B). The mutation also appears to have a modest effect on *rpsD-lacZ* expression (Fig. 2.3) and transcription from the *rpsD* promoter *in vitro* (Fig. 2.6A).

The reduced *in vitro* transcriptional activity and ComA-assisted promoter binding of the mutant RpoA(K267A) RNAP (Fig. 2.6B) is in keeping with the reduced *in vivo* activity observed in *srf-lacZ* strains bearing the *rpoA*(K267A) mutation (Fig. 2.6C). This low activity was not affected by Spx, as expression of the *spxLDD* allele (which encodes the protease-resistant form of Spx), while causing repression in *rpoA*$^+$ *srf-lacZ* cells, did not result in repression of *srf-lacZ* in *rpoA*(K267A) cells. No significant effect of SpxLDD expression or *rpoA*(K267A) mutation on the expression of *rpsD-lacZ* (Fig. 2.6C and D) was observed. The *rpoA*(C265A) mutation did not prevent Spx-dependent repression, in that reduced expression of *srf-lacZ* was observed when SpxLDD is produced (Fig. 2.6C and D), and ComA-dependent *srf* transcription *in vitro* was repressed when Spx protein was added to the transcription reaction mixture containing the mutant RpoA(C265A) form of RNAP (data not shown).

SPPR analysis shows that ComA-assisted RNAP binding to P*srf* is impaired by the mutant RpoA(K267A) subunit (Fig. 2.6E, compare lanes 3 and 4). However, interaction of RNAP with the *rpsD* promoter was not affected by the *rpoA*(K267A) mutation (Fig. 2.6E, lanes 7 to 10). Spx disrupted the ComA-RNAP complex at the *srf*

promoter, as shown in SPPR reactions (Fig. 2.6E, lanes 3 and 5). However, Spx had no significant effect on ComA-assisted promoter binding of RpoA(K267A) RNAP (Fig. 2.6E, lanes 4 and 6). The K267 amino acid position in the αCTD is important for ComA-dependent transcriptional activation, for Spx-dependent repression, and for Spx-dependent transcriptional activation.

The CXXC motif of Spx is not essential for repression of *srf* transcription. Transcriptional activation by *B. subtilis* Spx at the *trxA* and *trxB* promoters requires the oxidized form of Spx having an intrachain disulfide at the N-terminal CXXC motif (Nakano *et al.*, 2005). It was not known if the CXXC motif was also required for Spx-dependent repression. A protease-resistant form of Spx (Nakano *et al.*, 2003a) bearing a C10A substitution in the CXXC motif was produced from an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression system in a *B. subtilis* strain bearing a *srf-lacZ* fusion. Separate cultures expressing wild-type and C10A mutant forms of SpxLDD were analyzed by Western blotting, and equal amounts of SpxLDD protein were observed in each strain (data not shown). When the SpxLDD protein with the C10A substitution was expressed in IPTG-treated cells, *srf-lacZ* was repressed to nearly the same level as observed in cells expressing the parental *spxLDD* construct (Fig. 2.7A). An attempt was made to express a C13A mutant form of SpxLDD in *srf-lacZ* cells, but the product was unstable in *B. subtilis*, and only low levels of protein were detected (data not shown).

The activity of Spx(C10A) and Spx(C13A) *in vitro* was examined in transcription reactions and by DNase I footprinting (Fig. 2.7B and C). *In vitro* transcription reaction mixtures containing *srf* promoter DNA, ComA~P, RNAP, and Spx were assembled to examine Spx-dependent repression. The WT Spx repressed transcription from the *srf* promoter, while the negative control, $Spx^{Cxs\text{-}16}$, showed reduced repressing activity. Both Spx(C10A) and Spx(C13A) repressed transcription nearly to the level of WT Spx (Fig. 2.7B). Analysis of RNAP-ComA binding to the *srf* promoter in footprinting reactions showed that the mutant Spx(C10A) had a reduced ability to displace RNAP and ComA compared to WT Spx (Fig. 2.7C, compare lanes 5 and 6 with lanes 9 and 10). It was concluded that the CXXC motif, while enhancing repression, is not essential for the repressor activity of Spx.

2.3 DISCUSSION

Among the genes repressed by Spx, the ComA regulon genes, particularly those of the *srf* operon, were found to undergo the greatest reduction in transcript levels when Spx interacted with RNAP in cells over expressing Spx (Nakano *et al.*, 2003a). ComA activates transcription of the *srf* operon by interacting with two regions of dyad symmetry residing upstream from the *srf* promoter -35 sequence. Transcription requires these interactions as well as RNAP contact with the -35 region, which is assisted by ComA~P. Our data suggest that ComA interacts with RNAP αCTD in a region previously shown to contact Spx (Newberry *et al.*, 2005). Thus, Spx blocks productive interaction between ComA and RNAP at the *srf* promoter by occupying an overlapping site on αCTD.

The Y263C mutation of αCTD reduced *rpsD* (ribosomal S4) and ComA-dependent *srf* transcription to nearly the same extent (Fig. 2.3). This residue is also necessary for functional Spx-RNAP interaction and in response regulator ResD-stimulated transcription (Geng *et al.*, 2006), which induces anaerobic-specific gene transcription in response to oxygen limitation (Nakano *et al.*, 1996). As detailed previously, this residue is highly conserved in low-GC Gram-positive bacteria that also carry *spx*. These observations reinforce the view that the Y263 residue is an important feature of RNAP in Gram-positive organisms.

The footprinting and SPPR data indicate that ComA-RNAP interaction is necessary for ComA-assisted recruitment of RNAP to the *srf* promoter to form a stable promoter complex. ComA is capable of binding to the *srf* promoter without RNAP, as observed in previous studies (Roggiani & Dubnau, 1993), but RNAP-promoter binding appears to solidify ComA-*srf* promoter interaction by interaction with ComA. Spx interferes with ComA-RNAP interaction, since addition of Spx to the EMSA (Nakano *et al.*, 2003b), footprinting, and SPPR reaction mixtures weakens both RNAP-promoter and ComA-promoter complexes. Footprinting shows protection in the -35 region, particularly nucleotide -30, that is attributable to ComA-assisted RNAP binding. Despite little change in DNase I protection in the -10 region, the interactions observed in the footprinting result represent a productive complex, as shown by *in vitro* transcription data.

C265 and K267, along with Y263, reside in the α1 helix of αCTD (Fig. 2.8). The C and K residues correspond to residues C269 and K271 in the *E. coli* RNAP αCTD. The two residues are located C-terminal to the α1 residues that constitute part of the DNA-binding "265 determinant" of αCTD (Busby & Ebright, 1999; Gaal *et al.*, 1996; Savery *et al.*, 2002), which includes R265 (R261 in *B. subtilis*), V264 (V260), and N268 (N264). The conservation of the three residues in *B. subtilis* and the overall structural similarity between *E. coli* and *B. subtilis* αCTD (Newberry *et al.*, 2005) suggests that the *B. subtilis* αCTD also contains the analogous 265 determinant that binds to extended promoter DNA. The residues required for ComA and Spx interaction with RNAP lie adjacent to the 265 determinant sequences of *B. subtilis* αCTD. The residues N264, K294, and S295 in *B. subtilis* αCTD, which correspond to the 265 determinant residues of *E. coli* αCTD, are required for optimal *srf* and have a modest effect on *rpsD* expression. These confer a 40 to 70% reduction in *srf-lacZ* expression. These data suggest that upstream promoter binding by αCTD is necessary for ComA-activated *srf* transcription. Alanine substitutions of residues G292 and R261 in *B. subtilis* αCTD were not recovered in our screen after 20 attempts to obtain mutant *rpoA* recombinants, raising the possibility that these substitutions were lethal.

Crystal structure analysis of Spx reveals the two-domain structure of the ArsC homolog (Newberry *et al.*, 2005), a central domain that interacts with RNAP α and the redox domain formed by the N- and C-terminal sequences of Spx and containing the CXXC motif. Two peptide coils connect the central domain with the redox domain. The central domain contacts αCTD at the α1 region and involves the participation of helices α2 and α5 of the Spx central domain. The fact that the CXXC motif is necessary for positive transcriptional control, yet is some distance from the αCTD binding surface of Spx, suggests that Spx may contact other components of RNAP holoenzyme.

σ^{70} region 4.2 is important for interaction with the promoter -35 element (Lonetto *et al.*, 1992). The region 4 of *E. coli* σ^{70} and *B. subtilis* σ^{A} also functions in the interaction with DNA-bound transcriptional activators. The transcription factor AsiA of phage T4 contacts σ^{70} of *E. coli* RNAP holoenzyme and the flap domain of the β subunit. In doing so, the distance between regions 4 and 2 of σ^{70} is altered, and σ^{70} region 4 is now in a position to contact MotA, which is bound to phage T4-specific promoters (Gregory *et al.*,

2004; Pande *et al.*, 2002; Simeonov *et al.*, 2003). It is possible that the oxidized form of Spx also contacts σ^A, the homologue of σ^{70}, in *B. subtilis* and, perhaps, β as part of the mechanism of positive transcription control.

Our evidence indicated that *sigA* region 4.2 mutants L366A affected ComA-dependent *srf* transcription and K356A (not K356E), R358A and R362A were defective in Spx-dependent repression of *srf-lacZ* (Fig. 2.9A). K356A and K356E affected Spx-dependent induction of *trxA-lacZ* (Fig. 2.9B). Spx did not repress *srf* transcription in the presence of the region 4 mutant proteins SigA(R362A) RNAP or SigA(K356A) RNAP *in vitro* (Fig. 2.9C). These results suggest that R362 and K356 of σ^A region 4.2 are required for productive Spx interaction with RNAP.

SigA(L366A) RNAP shows reduced transcriptional activity from the *srfA*, *rpsD*, *trxA* and *trxB* promoters *in vitro*. RNAP purified from *sigA*(WT), *sigA*(R362A) *sigA*(K356A) and *sigA*(K356E) contain similar levels of α, β, β′, σ^A, δ, ω subunits, but SigA(L366A) holoenzyme preparations contained little or no σ^A (Fig. 2.10A). SigA(L366A) reconstituted RNAP could not activate *rpsD* and *trxA* transcription *in vitro* (Fig. 2.10B and C), indicating that σ^A L366 has a global effect on RNAP-catalyzed transcription. Recent studies indicated that the corresponding Leu residue of *E. coli* σ^{70} L607 when substituted by Pro weakens the interaction between σ^{70} region 4 and the β-flap domain (Nickels *et al.*, 2005). So *sigA*(L366) is required for σ^A association with RNA polymerase core enzyme to form the functional holoenzyme probably through sigma and β-flap interaction.

rpoE encoding the delta subunit of RNA polymerase functions to enhance promoter recognition and core enzyme recycling but inhibit the open complex formation (Juang & Helmann, 1994). We observed that an *rpoE* insertion mutant was sensitive to high concentration diamide. However, the *rpoE* insertion mutation did not affect transcription of *srfA*, *rapA* and *rpsD* genes and Spx-dependent transcriptional control of *srfA*, *rapA* and *trxA* promoter *in vivo*.

Our results show that the redox disulfide center of Spx, while enhancing the repressor activity, is not essential for negative control. This finding highlights the importance of control mechanisms affecting Spx concentration, which would seem to determine in large part when and under what conditions Spx negative control is exerted.

An increase in Spx concentration is observed upon oxidative stress (Nakano *et al.*, 2003a), which is the result of increased *spx* gene transcription (Leelakriangsak & Zuber, 2007) and enhanced Spx stability (Nakano *et al.*, 2003a). Oxidative stress induced the reduction of proteolytic control of Spx will be further addressed in Chapter 3.

2.4 MATERIALS AND METHODS

2.4.1 Bacterial strains and plasmids

Bacillus subtilis strains used in the study are listed in Table 2.1. *B. subtilis* strains constructed with alanine-scanning αCTD alleles are listed in Table 2.2 in the supplemental material. Oligonucleotides used in the study, including those used for alanine-scanning mutagenesis of *rpoA*, are listed in Table 2.3 in the supplemental material. To express mutant *spx*^{C10A} from the isopropyl-β-thiogalactopyranoside (IPTG)-inducible Phyperspank (Pspank-hy) promoter (Britton *et al.*, 2002), plasmid pZY14 was constructed. Plasmid pSN56 (Nakano *et al.*, 2003a) was digested with *Bcl*I and *Sal*I to obtain the 201-bp fragment containing the 3' half of the *spxLDD* allele, which encodes the ClpXP-resistant form of Spx. The plasmid pSN95 (Nakano *et al.*, 2005) was digested with *Hin*dIII and *Bcl*I to obtain a 281-bp fragment containing the N-terminal portion of the *spx*^{C10A} allele. Ligation of the two fragments with pUC18, which was digested with *Sal*I and *Hin*dIII, was followed by transformation of *Escherichia coli* DH5α competent cells with the ligation mix. This resulted in construction of plasmid pZY11, which encodes the mutant SpxLDD(C10A). Plasmid pZY11 was digested with *Sal*I and *Hin*dIII to obtain the 482-bp SpxLDD(C10A)-encoding fragment for ligation with pDR111, which was digested with *Sal*I and *Hin*dIII, to yield plasmid pZY14 [P*spank-hy-spxLDD*(C10A)]. Plasmid pZY14 was used to transform *B. subtilis* strain LAB545 (*srfA-lacZ*, contains pMMN92) (Nakano & Zuber, 1993) integrated into SPβ*c2del2*::Tn*917*::pSK10Δ6 prophage (Zuber & Losick, 1987) to obtain ORB6307. SpxLDD-expressing strains having mutations in *rpoA* were obtained by transforming ORB5259 [*rpoA*(C265A)] and ORB5262 [*rpoA*(K267A)] with chromosomal DNA from ORB4342 [*amyE*::pSN56 (P*spank-hy-spxLDD*)] to yield ORB6127 and ORB6128. The *srf-lacZ* fusion was introduced into the resulting strains and into ORB4342

(*amyE*::pSN56 *rpoA*$^{+}$) by transduction with the SPβ phage lysate (Zuber & Losick, 1987) carrying *srf-lacZ* (pMMN92) (Nakano & Zuber, 1993) with selection for chloramphenicol-resistance (5 μg/ml) to yield ORB6127, ORB6128, and ORB6129. Construction of a *srf* -35 promoter mutant plasmid (-34, -35 TG to CC) was carried out by site-directed mutagenesis (Nakano *et al.*, 2005). Upstream and downstream fragments were synthesized via PCR by using the mutagenic oligonucleotides oYZ02-6 and oYZ02-3a along with the upstream primer oYZ02-3(-347) and downstream primer oYZ02-4 (+65). The two resulting PCR fragments were used as templates for PCR with primers oYZ02-3 and oYZ02-4. The PCR fragment digested with *Bam*HI and *Hin*dIII was inserted into pUC18 to obtain plasmid pZY6. The plasmid pMMN101, containing the mutant *srf* promoter fragment bearing a ComA box 2 mutation, was previously described (Nakano & Zuber, 1993).

2.4.2 Alanine-scanning mutagenesis of the *rpoA* CTD region

The *rpoA-rplQ* region of the *B. subtilis* chromosome was amplified by PCR using the primers oMN99-91 and oMN001-106 (Nakano *et al.*, 2000). The fragment was inserted into *Hin*dIII- and *Xba*I-cleaved pAG58-ble-1 (Youngman *et al.*, 1989) to yield pSN108. Alanine-scanning mutagenesis was conducted using mutagenic primers and the PCR amplification method of site-directed mutagenesis. Two primers (forward and reverse) specifying a single mutation were used to perform inverse PCR on whole pSN108 plasmid DNA. The mutation also introduced an additional restriction site in the mutated DNA insert. The PCR product was extracted with phenol-chloroform-isoamyl alcohol and precipitated with ethanol using yeast RNA as the carrier. The DNA was cleaved with *Dpn*I to eliminate template DNA, and the restriction reaction was used to directly transform competent cells of *E. coli* strain DH5α. Introduction of the mutation into the *rpoA-rplQ* fragment was confirmed by nucleotide sequencing (Oregon National Primate Research Center, Core Facility, Beaverton). The primers used for alanine-scanning mutagenesis are listed in Table 2.3 in the supplemental material. The pSN108 derivative bearing the alanine codon substitution was used to transform competent cells of strain JH642. The plasmid integrated into the *rpoA* locus of the chromosome by a Campbell recombination mechanism. The selection for elimination of the plasmid vector

DNA by loop-out recombination, thus leaving the alanine codon substitution in the *rpoA* gene, was accomplished according to a previous published procedure (Nakano *et al.*, 2000). The presence of the mutation was confirmed by PCR of the *rpoA* CTD region followed by cleavage with the restriction enzyme that recognizes the mutated sequence.

2.4.3 Diamide sensitivity

Wild-type *B. subtilis* strain JH642, ORB3621(*rpoA*$^{Cxs\text{-}1}$), ORB5259 [*rpoA*(C265A)], and ORB5262 [*rpoA*(K267A)] were grown in Difco sporulation medium (DSM) at 37°C with shaking until mid-log phase (optical density at 600 nm = 0.5). Viable-cell numbers were measured by plating 5 μl of cells from a dilution series onto DSM agar medium with or without 0.1 mM diamide. Cells were also spotted onto DSM plates without drug in the same way as above.

2.4.4 Protein purification

RNAP containing a His_{10}-tagged RpoC (β') subunit was purified from *B. subtilis* MH5636 (wild-type [WT]), ORB4123 (*rpoA*$^{Cxs\text{-}1}$), ORB5501 [*rpoA*(C265A)], or ORB6116 [*rpoA*(K267A)] by using a procedure described previously (Qi & Hulett, 1998). Intein-tagged ComA was purified using a procedure described previously (Nakano *et al.*, 2003b). The self-cleavable affinity tag system IMPACT (New England Biolabs) was used to purify ComA from *E. coli* strain BL21(DE3)(pLysS). The ComA proteins obtained have a Pro-Gly extension at the C termini and were further purified by elution with a 100-to-600 mM KCl gradient from a High Q column (Bio-Rad). Intein-tagged Spx was purified by using a procedure described previously (Nakano *et al.*, 2002b). His_6-tagged wild-type, Cxs-16, C10A, and C13A Spx proteins were purified using a previously published procedure (Nakano *et al.*, 2005).

2.4.5 *In vitro* transcription reactions

Linear DNA fragments for templates of promoters P*rpsD* (from -115 to about +71), P*srfA* (-347 to about +104) and P*trxB* (-220 to about +88) for *in vitro* transcription were generated by PCR. The oligonucleotides used in PCR to generate promoter fragments are listed in Table 2.5 in the supplemental material. The transcription reaction

mixtures (20 µl) contained 40 mM Tris HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol (DTT) (unless otherwise indicated), 10 units RNasin (Promega), 50 nM P*srf* or 20 nM P*trxB* or 50 nM P*rpsD* template, 0.05 µM RNAP, and 1.6 µM ComA phosphorylated by treatment with acetyl phosphate as previously described (Nakano *et al.*, 2003b). The mixtures were incubated at 37°C for 10 min with or without Spx before the addition of 40 µM ATP, CTP, and GTP, 10 µM UTP, and 5 µCi [α-^{32}P]UTP. After incubation (times are indicated in figure legends and text), the reactions were stopped by addition of 10 µl stop buffer (1 M ammonium acetate, 0.1 mg/ml yeast RNA, 0.03 M EDTA) and then precipitated with 75 µl ethanol at -80°C. Electrophoresis was performed on 6% urea gel as described previously (Liu & Zuber, 2000).

2.4.6 Assay of β-galactosidase activity.

β-galactosidase activity was determined as previously described (Nakano *et al.*, 1988) and is presented as Miller units (Miller, 1972).

2.4.7 DNase I footprinting experiment

A radioactively end-labeled fragment of the *srf* promoter (from -138 to +65) was made by PCR amplification using primers o-MN02-195 and o-YZ02-4 and JH642 chromosomal DNA as a template. To end-label the template or coding strand, one member of each primer set was treated with T4 polynucleotide kinase and [γ-^{32}P]ATP. The PCR products were separated on a nondenaturing polyacrylamide gel and purified with Elutip-d columns (Schleicher and Schuell). Dideoxy sequencing ladders were obtained using a Thermo Sequenase cycle sequencing kit (USB) with the primers used for the footprinting reactions. DNase I footprinting experiments were performed in 20 µl reaction buffer containing 10 mM Tris HCl (pH 7.9), 30 mM KCl, 10 mM $MgCl_2$, and 0.5 mM β-mercaptoethanol. Proteins were incubated with labeled probe (50,000 cpm) at 37°C for 20 min. The reaction mixtures were treated with 3 µl of 0.02 mg/ml DNase I (diluted in 5 mM $MgCl_2$, 5 mM $CaCl_2$) at room temperature for 15 s (without proteins) or 30 s (with proteins). The reactions were then stopped with 10 µl stop buffer (6.25 mM EDTA [pH 8.0], 0.125% sodium dodecyl sulfate (SDS), 0.375 M sodium acetate, 62.5

μg/ml yeast RNA). After phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation, pellets were dissolved in loading dye and subjected to 6% polyacrylamide-8 M urea gel electrophoresis as previously described (Nakano *et al.*, 2005).

2.4.8 Solid-phase promoter retention (SPPR) experiments

Solid-phase promoter retention (SPPR) experiments used streptavidin-attached agarose beads that bind to biotinylated DNA fragments along with any interacting proteins. Biotinylated DNA fragments were synthesized by PCR with biotinylated 5' upstream oligonucleotides and underivatized downstream oligonucleotides (see Table 2.4 in the supplemental material). Streptavidin agarose beads were equilibrated with binding buffer (10 mM Tris-HCl [pH 8.0], 100 mM KCl, 10 mM $MgCl_2$ and 0.5 mM β-mercaptoethanol). The beads were preincubated with the biotinylated DNA fragment for 30 min in binding buffer containing 1% Casamino Acids and 0.1 mg/ml bovine serum albumin (BSA) to shield the nonspecific binding sites on the agarose beads. After the unbound DNA fragment was washed out, protein mixtures were added to the beads in binding buffer containing 0.05 mg/ml yeast RNA, 0.08 mg/ml pUC18 plasmid, and 0.1 mg/ml BSA and then incubated for 1 h at room temperature with gentle shaking. After the unbound protein was washed out by suspension in and centrifugation from binding buffer, the beads were heated at 95°C in SDS loading dye to release the proteins from the agarose beads. SDS-polyacrylamide gel electrophoresis was performed to examine the proteins that were immobilized on the biotinylated DNA-streptavidin agarose complex. The 12% SDS-polyacrylamide gel was stained by colloidal Coomassie G (Candiano *et al.*, 2004), and the images were taken with a UV Transilluminator with a visible-spectrum conversion filter.

2.5 ACKNOWLEDGMENTS

We thank H. Geng for assisting in construction of αCTD alanine-scanning mutant library and M. M. Nakano for valuable discussion and critical reading of the manuscript.

Research was supported by grant GM45898 (to P.Z.) from the National Institutes of Health and a grant from the Medical Research Foundation of Oregon (to P.Z.). This

work was also supported by a Korea Research Foundation grant (KRF-2004-013-F00001) to S.Y.C.

Table 2.1 *Bacillus subtilis* strains

S*tra*in	Genoty*pe*	Reference
LAB545	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	(Nakano & Zuber, 1993)
MH5636	*trpC2 pheA1* His10 *rpoC*	(Qi & Hulett, 1998)
OKB167	*trpC2 pheA1 comPA*::Erm *srfB*::Tn*917*	(Nakano & Zuber, 1989)
ORB3621	*trpC2 pheA1 rpoA*(Y263C)*	(Nakano *et al.*, 2000)
ORB4123	*trpC2 pheA1* His10 *rpoC rpoA*(Y263C)*	(Nakano *et al.*, 2005)
ORB4342	*trpC2 pheA1 amyE*::pSN56	(Nakano *et al.*, 2003a)
ORB4343	*trpC2 pheA1 rpoA*(Y263C)* *amyE*::pSN56	(Nakano *et al.*, 2003a)
ORB5501	*trpC2 pheA1 rpoA*(C265A) His10 *rpoC*	This study
ORB5259	*trpC2 pheA1 rpoA*(C265A)	This study
ORB5262	*trpC2 pheA1 rpoA*(K267A)	This study
ORB5327	*trpC2 pheA1 rpoA*(K267A) SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB5422	*trpC2 pheA1 rpoA*(C265A) SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB5553	*trpC2 pheA1 rpoA*(Y263C)* SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB5661	*trpC2 pheA1 comPA*::Erm SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB6116	*trpC2 pheA1 rpoA*(K267A) *His*10 *rpoC*	This study
ORB6127	*trpC2 pheA1 rpoA*(C265A) *amyE*::pSN56 (pDR111-*spxLDD*)	This study
ORB6128	*trpC2 pheA1 rpoA*(K267A) *amyE*::pSN56 (pDR111-*spxLDD*)	This study
ORB6129	*trpC2 pheA1 amyE*::pSN56 SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB6130	*trpC2 pheA1 rpoA*(Y263C)* *amyE*::pSN56 SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB6131	*trpC2 pheA1 rpoA*(C265A) *amyE*::pSN56 SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB6132	*trpC2 pheA1 rpoA*(K267A) *amyE*::pSN56 SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB6137	*trpC2 pheA1 amyE*::pSN56 (pDR111-*spxLDD*) SPβ*c2del2*::Tn*917*::pTMH112(*rpsD-lacZ*)	This study
ORB6138	*trpC2 pheA1 rpoA*(Y263C)* *amyE*::pSN56 (pDR111-*spxLDD*) SPβ*c2del2*::Tn*917*::pTMH112(*rpsD-lacZ*)	This study
ORB6139	*trpC2 pheA1 rpoA*(C265A) *amyE*::pSN56 (pDR111-*spxLDD*)	This study

	SPβ*c2del2*::Tn*917*::pTMH112(*rpsD-lacZ*)	
ORB6140	*trpC2 pheA1 rpoA*(K267A) *amyE*::pSN56 (pDR111-*spxLDD*) SPβ*c2del2*::Tn*917*::pTMH112(*rpsD-lacZ*)	This study
ORB6303	*trpC2 pheA1 amyE*::pDR111	This study
ORB6304	*trpC2 pheA1 amyE*::pZY14(*spxLDD*(C10A))	This study
ORB6305	*trpC2 pheA1 amyE*::pDR111 SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study
ORB6307	*trpC2 pheA1 amyE*::pZY14(*spxLDD*(C10A)) SPβ*c2del2*::Tn*917*::pMMN92(*srfA-lacZ*)	This study

* *rpoA*(Y263C) is as same as *rpoA*$^{Cxs\text{-}1}$

Table 2.2 Plasmids encoding alanine-scanning mutant αCTD polypeptides.

All plasmids are derivatives of pAG58-phleo (Youngman *et al.*, 1989).

pSN123	alanine scanning *rpoA-rplQ*/pAG58	R268A
pSN124	alanine scanning *rpoA-rplQ*/pAG58	G270A
pSN125	alanine scanning *rpoA-rplQ*/pAG58	V274A
pSN126	alanine scanning *rpoA-rplQ*/pAG58	L277A
pSN127	alanine scanning *rpoA-rplQ*/pAG58	N279A
pSN128	alanine scanning *rpoA-rplQ*/pAG58	M286A
pSN129	alanine scanning *rpoA-rplQ*/pAG58	T281A
pSN130	alanine scanning *rpoA-rplQ*/pAG58	E282A
pSN131	alanine scanning *rpoA-rplQ*/pAG58	K287A
pSN132	alanine scanning *rpoA-rplQ*/pAG58	E276A
pSN133	alanine scanning *rpoA-rplQ*/pAG58	N290A
pSN134	alanine scanning *rpoA-rplQ*/pAG58	D284A
pSN135	alanine scanning *rpoA-rplQ*/pAG58	R293A
pSN136	alanine scanning *rpoA-rplQ*/pAG58	E298A
pSN137	alanine scanning *rpoA-rplQ*/pAG58	S295A
pSN138	alanine scanning *rpoA-rplQ*/pAG58	R261A
pSN139	alanine scanning *rpoA-rplQ*/pAG58	K267A
pSN140	alanine scanning *rpoA-rplQ*/pAG58	K300A
pSN141	alanine scanning *rpoA-rplQ*/pAG58	E283A
pSN142	alanine scanning *rpoA-rplQ*/pAG58	V299A
pSN143	alanine scanning *rpoA-rplQ*/pAG58	L303A
pSN144	alanine scanning *rpoA-rplQ*/pAG58	E304A
pSN145	alanine scanning *rpoA-rplQ*/pAG58	E306A
pSN146	alanine scanning *rpoA-rplQ*/pAG58	L308A
pSN147	alanine scanning *rpoA-rplQ*/pAG58	L296A
pSN148	alanine scanning *rpoA-rplQ*/pAG58	V288A
pSN149	alanine scanning *rpoA-rplQ*/pAG58	K294A
pSN150	alanine scanning *rpoA-rplQ*/pAG58	G307A
pSN151	alanine scanning *rpoA-rplQ*/pAG58	G309A
pSN152	alanine scanning *rpoA-rplQ*/pAG58	K302A
pSN153	alanine scanning *rpoA-rplQ*/pAG58	D313A

pSN154	alanine scanning *rpoA-rplQ*/pAG58	T273A
pSN155	alanine scanning *rpoA-rplQ*/pAG58	K280A
pSN156	alanine scanning *rpoA-rplQ*/pAG58	M285A
pSN157	alanine scanning *rpoA-rplQ*/pAG58	N272A
pSN158	alanine scanning *rpoA-rplQ*/pAG58	Q275A
pSN159	alanine scanning *rpoA-rplQ*/pAG58	R289A
pSN160	alanine scanning *rpoA-rplQ*/pAG58	L291A
pSN161	alanine scanning *rpoA-rplQ*/pAG58	K312A
pSN162	alanine scanning *rpoA-rplQ*/pAG58	D314A
pSN163	alanine scanning *rpoA-rplQ*/pAG58	L310A
pSN164	alanine scanning *rpoA-rplQ*/pAG58	E297A
pSN165	alanine scanning *rpoA-rplQ*/pAG58	R311A
pSN166	alanine scanning *rpoA-rplQ*/pAG58	I271A
pSN167	alanine scanning *rpoA-rplQ*/pAG58	G292A
pSN168	alanine scanning *rpoA-rplQ*/pAG58	E305A

Table 2.3 Oligonucleotides used in alanine-scanning mutagenesis of *rpoA* DNA encoding αCTD.

M251A-F	GAAAGTTCTTGAAGCTACAATTGAAGAAT
M251A-R	ATTCTTCAATTGTAGCTTCAAGAACTTTC
I253A-F	TCTTGAAATGACAGCTGAAGAATTGGATC
I253A-R	GATCCAATTCTTCAGCTGTCATTTCAAGA
E255A-F	ATGACAATTGAAGCTTTGGATCTTTCTG
E255A-R	CAGAAAGATCCAAAGCTTCAATTGTCAT
L256A-F	CTTGAAATGACAAGCTAAGAATTGGATCT
L256A-R	AGATCCAATTCTTAGCTTGTCATTTCAAG
L256A-F2	GACAATTGAAGAAGCTGATCTTTCTGTTC
L256A-R2	GAACAGAAAGATCAGCTTCTTCAATTGTC
L258A-F	GAAGAATTGGATGCTTCTGTTCGTTCTT
L258A-R	AAGAACGAACAGAAGCATCCAATTCTTC
S259A-F	GAATTGGATCTTGCGGTTCGTTCTTAC
S259A-R	GTAAGAACGAACCGCAAGATCCAATTC
R261A-F	GATCTTTCTGTTGCGTCTTACAACTGC
R261A-R	GCAGTTGTAAGACGCAACAGAAAGATC

S262A-F	CTTTCTGTTCGTGCGTACAACTGCTTAA
S262A-R	TTAAGCAGTTGTACGCACGAACAGAAAG
N264A-F	GTTCGTTCTTACGCGTGCTTAAAGCGTG
N264A-R	CACGCTTTAAGCACGCGTAAGAACGAAC
C265A-F	CGTTCTTACAACGCTTTAAAGCGTGCG
C265A-R	CGCACGCTTTAAAGCGTTGTAAGAACG
L266A-F	GTTCTTACAACTGCGCTAAGCGTGCGGGTAT
L266A-R	ATACCCGCACGCTTAGCGCAGTTGTAAGAAC
K267A-F	CTTACAACTGCTTAGCACGTGCGGGTATTAAC
K267A-R	GTTAATACCCGCACGTGCTAAGCAGTTGTAAG
R268A-F	CAACTGCTTAAAGGCCGCGGGTATTAACAC
R268A-R	GTGTTAATACCCGCGGCCTTTAAGCAGTTG
G270A-F	CTTAAAGCGTGCGGCCATTAACACGGTTC
G270A-R	GAACCGTGTTAATGGCCGCACGCTTTAAG
I271A-F	CTTAAAGCGTGCGGGTGCCAACACGGTTCAAGAG
I271A-R	CTCTTGAACCGTGTTGGCACCCGCACGCTTTAAG
N272A-F	GCGTGCGGGTATTGCCACGGTTCAAGAGC
N272A-R	GCTCTTGAACCGTGGCAATACCCGCACGC
T273A-F	GTGCGGGTATTAACGCGGTTCAAGAGCTTG
T273A-R	CAAGCTCTTGAACCGCGTTAATACCCGCAC
V274A-F	GGGTATTAACACGGCCCAAGAGCTTGCG
V274A-R	CGCAAGCTCTTGGGCCGTGTTAATACCC
Q275A-F	GTATTAACACGGTTGCTGAGCTTGCGAAC
Q275A-R	GTTCGCAAGCTCAGCAACCGTGTTAATAC
E276A-F	GTATTAACACGGTTCAAGCGCTTGCGAACAAGAC
E276A-R	GTCTTGTTCGCAAGCGCTTGAACCGTGTTAATAC
L277A-F	CACGGTTCAAGAGGCCGCGAACAAGACGG
L277A-R	CCGTCTTGTTCGCGGCCTCTTGAACCGTG
N279A-F	GTTCAAGAGCTTGCGGCCAAGACGGAAGAAG
N279A-R	CTTCTTCCGTCTTGGCCGCAAGCTCTTGAAC
K280A-F	GAGCTTGCGAACGCGACGGAAGAAGATATG
K280A-R	CATATCTTCTTCCGTCGCGTTCGCAAGCTC
T281A-F	GCTTGCGAACAAGGCCGAAGAAGATATGATG
T281A-R	CATCATATCTTCTTCGGCCTTGTTCGCAAGC
E282A-F	CTTGCGAACAAGACGGCCGAAGATATGATGAAAG

E282A-R	CTTTCATCATATCTTCGGCCGTCTTGTTCGCAAG
E283A-F	GAACAAGACGGAAGCTGATATGATGAAAG
E283A-R	CTTTCATCATATCAGCTTCCGTCTTGTTC
D284A-F	CAAGACGGAAGAAGCTATGATGAAAGTTC
D284A-R	GAACTTTCATCATAGCTTCTTCCGTCTTG
M285A-F	GACGGAAGAAGATGCCATGAAAGTTCGAAATC
M285A-R	GATTTCGAACTTTCATGGCATCTTCTTCCGTC
M286A-F	GGAAGAAGATATGGCCAAAGTTCGAAATC
M286A-R	GATTTCGAACTTTGGCCATATCTTCTTCC
K287A-F	GAAGAAGATATGATGGCCGTTCGAAATCTAG
K287A-R	CTAGATTTCGAACGGCCATCATATCTTCTTC
V288A-F	GAAGATATGATGAAAGCGCGAAATCTAGGAC
V288A-R	GTCCTAGATTTCGCGCTTTCATCATATCTTC
R289A-F	GATATGATGAAAGTTGCGAATCTAGGACGCA
R289A-R	TGCGTCCTAGATTCGCAACTTTCATCATATC
N290A-F	GATGAAAGTTCGAGCGCTAGGACGCAAATC
N290A-R	GATTTGCGTCCTAGCGCTCGAACTTTCATC
L291A-F	GAAAGTTCGAAATGCCGGACGCAAATCAC
L291A-R	GTGATTTGCGTCCGGCATTTCGAACTTTC
G292A-F	GAAAGTTCGAAATCTAGCGCGCAAATCACTTG
G292A-R	CAAGTGATTTGCGCGCTAGATTTCGAACTTTC
R293A-F	GTTCGAAATCTAGGAGCTAAATCACTTGAAG
R293A-R	CTTCAAGTGATTTAGCTCCTAGATTTCGAAC
K294A-F	GAAATCTAGGACGCGCATCACTTGAAGAAGTG
K294A-R	CACTTCTTCAAGTGATGCGCGTCCTAGATTTC
S295A-F	TCTAGGACGCAAAGCGCTTGAAGAAGTGA
S295A-R	TCACTTCTTCAAGCGCTTTGCGTCCTAGA
L296A-F	CTAGGACGCAAATCAGCTGAAGAAGTGAAAG
L296A-R	CTTTCACTTCTTCAGCTGATTTGCGTCCTAG
E297A-F	GACGCAAATCACTTGCGGAAGTGAAAGCGAAAC
E297A-R	GTTTCGCTTTCACTTCCGCAAGTGATTTGCGTC
E298A-F	GCAAATCACTTGAAGCTGTGAAAGCGAAAC
E298A-R	GTTTCGCTTTCACAGCTTCAAGTGATTTGC
V299A-F	CAAATCACTTGAAGAAGCTAAAGCGAAACTAG
V299A-R	CTAGTTTCGCTTTAGCTTCTTCAAGTGATTTG

K300A-F	CACTTGAAGAAGTGGCCGCGAAACTAGAAG
K300A-R	CTTCTAGTTTCGCGGCCACTTCTTCAAGTG
K302A-F	GAAGAAGTGAAAGCGGCGCTAGAAGAACTTGG
K302A-R	CCAAGTTCTTCTAGCGCCGCTTTCACTTCTTC
L303A-F	GAAGTGAAAGCGAAAGCTGAAGAACTTGGAC
L303A-R	GTCCAAGTTCTTCAGCTTTCGCTTTCACTTC
E304A-F	GAAAGCGAAACTAGCTGAACTTGGACTCGG
E304A-R	CCGAGTCCAAGTTCAGCTAGTTTCGCTTTC
E305A-F	GCGAAACTAGAAGCGCTTGGACTCGGAC
E305A-R	GTCCGAGTCCAAGCGCTTCTAGTTTCGC
L306A-F	GAAACTAGAAGAAGCTGGACTCGGACTTC
L306A-R	GAAGTCCGAGTCCAGCTTCTTCTAGTTTC
G307A-F	CTAGAAGAACTTGCGCTCGGACTTCGC
G307A-R	GCGAAGTCCGAGCGCAAGTTCTTCTAG
L308A-F	GAAGAACTTGGAGCTGGACTTCGCAAAG
L308A-R	CTTTGCGAAGTCCAGCTCCAAGTTCTTC
G309A-F	GAACTTGGACTCGCGCTTCGCAAAGACG
G309A-R	CGTCTTTGCGAAGCGCGAGTCCAAGTTC
L310A-F	GAACTTGGACTCGGAGCGCGCAAAGACGATTG
L310A-R	CAATCGTCTTTGCGCGCTCCGAGTCCAAGTTC
R311A-F	GGACTCGGACTTGCGAAAGACGATTGAC
R311A-R	GTCAATCGTCTTTCGCAAGTCCGAGTCC
K312A-F	GACTCGGACTTCGCGCAGACGATTGACTAG
K312A-R	CTAGTCAATCGTCTGCGCGAAGTCCGAGTC
D313A-F	CGGACTTCGCAAAGCTGATTGACTAGTTTC
D313A-R	GAAACTAGTCAATCAGCTTTGCGAAGTCCG
D314A-F	CTTCGCAAAGACGCGTGACTAGTTTCCC
D314A-R	GGGAAACTAGTCACGCGTCTTTGCGAAG

Table 2.4 Oligonucleotides for SPPR analysis

Promoter	Oligo	Sequence	Position	Fragment Length
P*rpsD*	oYZ05-1	BIO-TCGAGCATATGATAATGAAAGGCGGA	Fw −166 ~ −141	220
	oYZ04-2	CGGGATCCAAATGAAAAC	Rv +37 ~ +54	
P*srfA*	oYZ1-01	BIO-GAGTGGGGGAAAGGCTATATGGAATT	Fw −349 ~ −324	414
	oYZ02-4	CCCCACCCTAATAAGAAACCAATTTTGGC	Rv +37 ~ +65	

Table 2.5 Oligonucleotides for synthesis of *in vitro* transcription templates and for DNase I footprinting substrates.

Name	O*ligo*	Sequence	Position	Template Length	Transcript Length
rpsD	oSN03-86	CATGTTTTTATCACCTAAAAGTTTACCAC	Fw −115 ~ −88	186	71
	oSN03-87	CGATACACCTTATTGATAAGGAACAAATG	Rv +42 ~ +71		
srfA	oYZ1-01	GTGGGGGAAAGGCTATATGGAATT	Fw −347 ~ −324	451	104
	oYZ2-01	CATTGCGGCGTTTAACATAAGCGGATAAAG	Rv +75 ~ +104		
trxB	oSN03-72	GACAATTACATCTCATGGCGTATC	Fw −220 ~ −198	308	88
	oSN03-61	CTTCTGACACACTATTGACTCCTTAAACC	Rv +60 ~ +88		

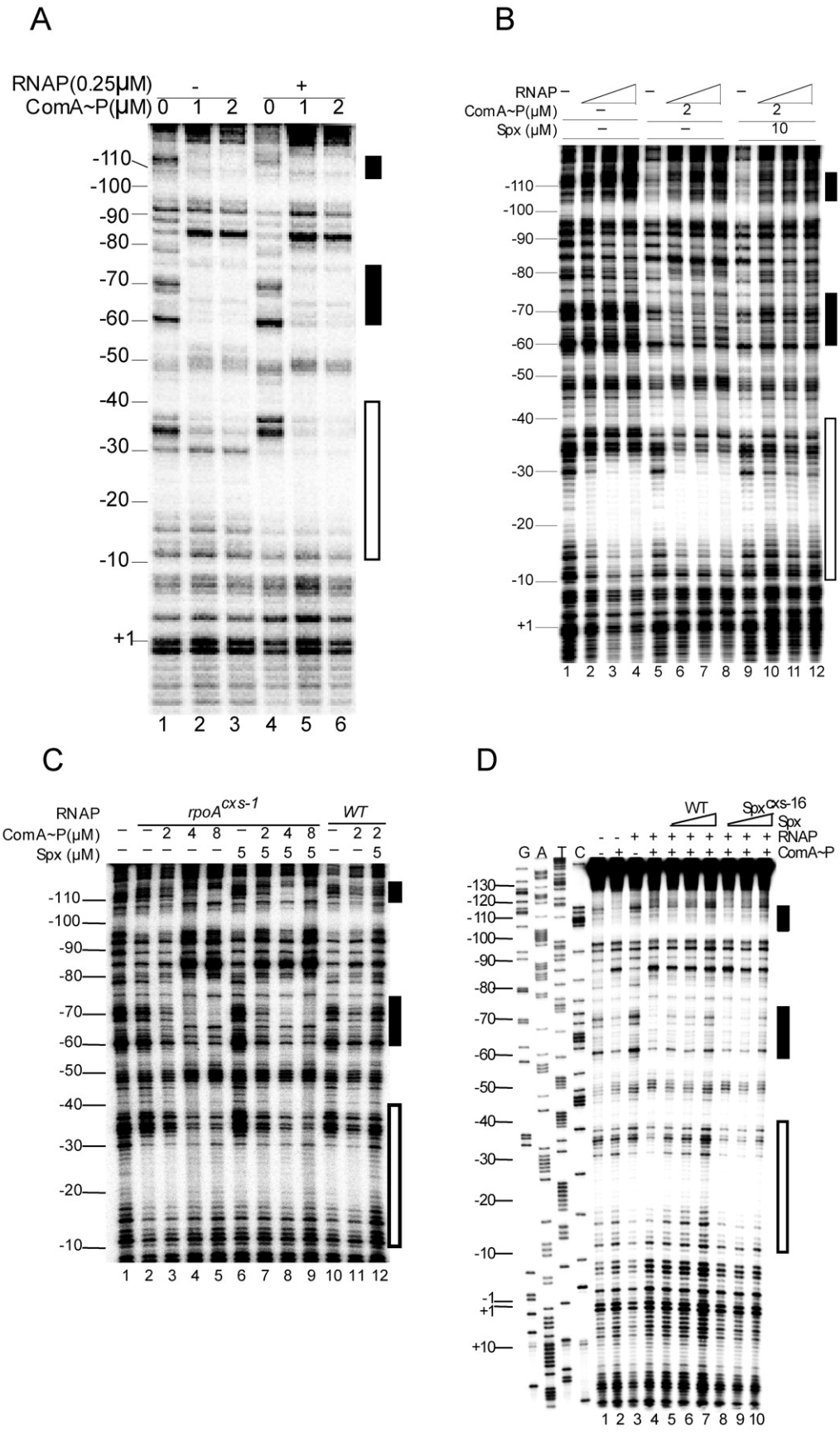

A
RNAP(0.25μM) - +
ComA~P(μM) 0 1 2 0 1 2
-110
-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
+1
1 2 3 4 5 6
B
RNAP – – –
ComA~P(μM) – 2 2
Spx (μM) – – 10
-110
-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
+1
1 2 3 4 5 6 7 8 9 10 11 12
C
RNAP rpoA^cxs-1 WT
ComA~P(μM) – – 2 4 8 – 2 4 8 – 2 2
Spx (μM) – – – – – 5 5 5 5 – – 5
-110
-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
1 2 3 4 5 6 7 8 9 10 11 12
D
WT Spx^cxs-16
Spx
RNAP - - + + + + + + + +
ComA~P - + - + + + + + + +
G A T C
-130
-120
-110
-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
-1
+1
+10
1 2 3 4 5 6 7 8 9 10

Figure 2.1 Effect of Spx on binding of ComA~P and RNAP to the *srf* promoter region.

(A) RNAP and ComA~P (Nakano *et al.*, 2003b) were added to DNase I footprinting reaction mixtures containing the *srf* promoter fragment synthesized by PCR and end labeled on the noncoding strand. Concentrations of RNAP and ComA are indicated. Reaction conditions are described in Materials and Methods. The two black rectangles indicate the locations of the ComA binding elements, box 1 (upper) and box 2 (lower). The white rectangle marks the site of RNAP-promoter interaction.

(B) RNAP and ComA~P were combined with the end-labeled *srf* promoter DNA as described for panel A, but a gradient of RNAP concentration was tested in the footprinting reaction. RNAP concentrations (from left to right and marked by the white ramped triangle) are 0.25, 0.5, and 1 μM. Spx is included in the rightmost reactions at the indicated concentration. ComA box 2 is indicated by the black rectangle (box 1 is obscured at the top of the gel image).

(C) Footprinting reactions containing either WT or *rpoA*$^{Cxs\text{-}1}$ RNAP with and without ComA~P and Spx. White and black rectangles indicate RNAP and ComA binding sites as in panel A. Protein reaction components were applied in the concentrations indicated.

(D) WT RNAP and ComA~P were added to footprinting reaction mixtures in the absence or presence of Spx or the mutant Spx$^{Cxs\text{-}16}$ protein. RNAP, 0.1 μM; ComA, 2 μM; Spx, 5, 10, and 20 μM; Spx$^{Cxs\text{-}16}$, 5, 10, and 20 μM.

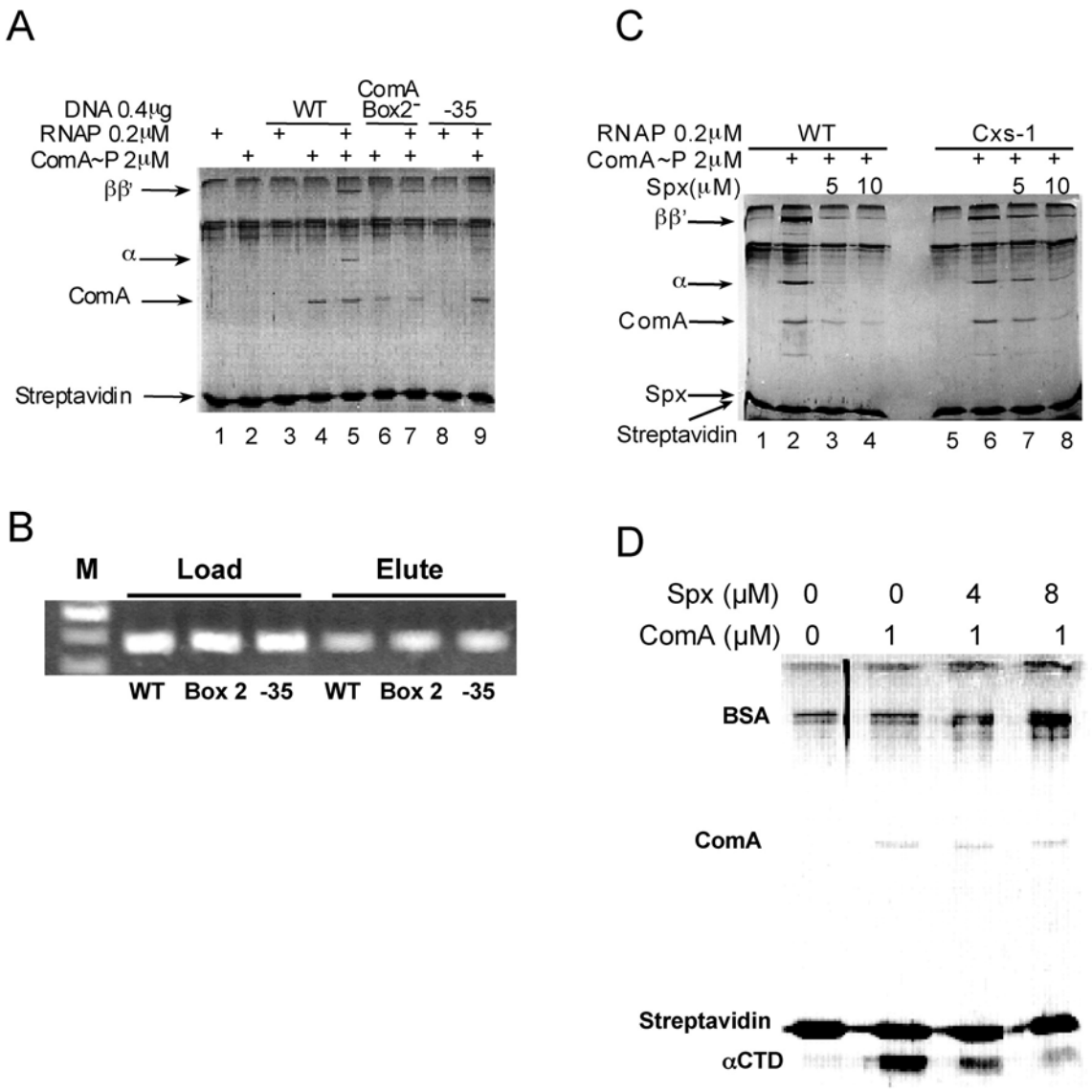


Figure 2.2 Binding of ComA~P and RNAP to the *srf* promoter as observed using SPPR analysis.

(A) Biotinylated *srf* or mutant *srf* promoter DNA bound to streptavidin beads was combined with RNAP and/or ComA phosphorylated by acetyl phosphate (ComA~P). Bound protein was analyzed by SDS-polyacrylamide gel electrophoresis as outlined in Materials and Methods. WT, box 2 mutant, and -35 mutant *srf* promoter DNA was used in the indicated reactions. Protein concentrations: RNAP, 0.2 μM; ComA~P, 2 μM; Spx, 10 μM.

(B) Ethidium bromide-stained DNA on a 1% agarose gel. Biotinylated DNA fragments of WT, box 2 mutant, and -35 mutant DNA were applied to the

streptavidin beads and extracted with phenol-chloroform from streptavidin agarose beads.

(C) Effect of Spx on RNAP and ComA~P binding to *srf* promoter DNA as determined by SPPR analysis. RNAP of WT and *rpoA*$^{Cxs\text{-}1}$ (Cxs-1) strains was used in the reactions. ComA~P and Spx were added in the concentrations indicated.

(D) SPPR reactions with purified αCTD and ComA~P, untreated or treated with Spx. Amounts of proteins in reactions are indicated. The SPPR method is described in Materials and Methods.

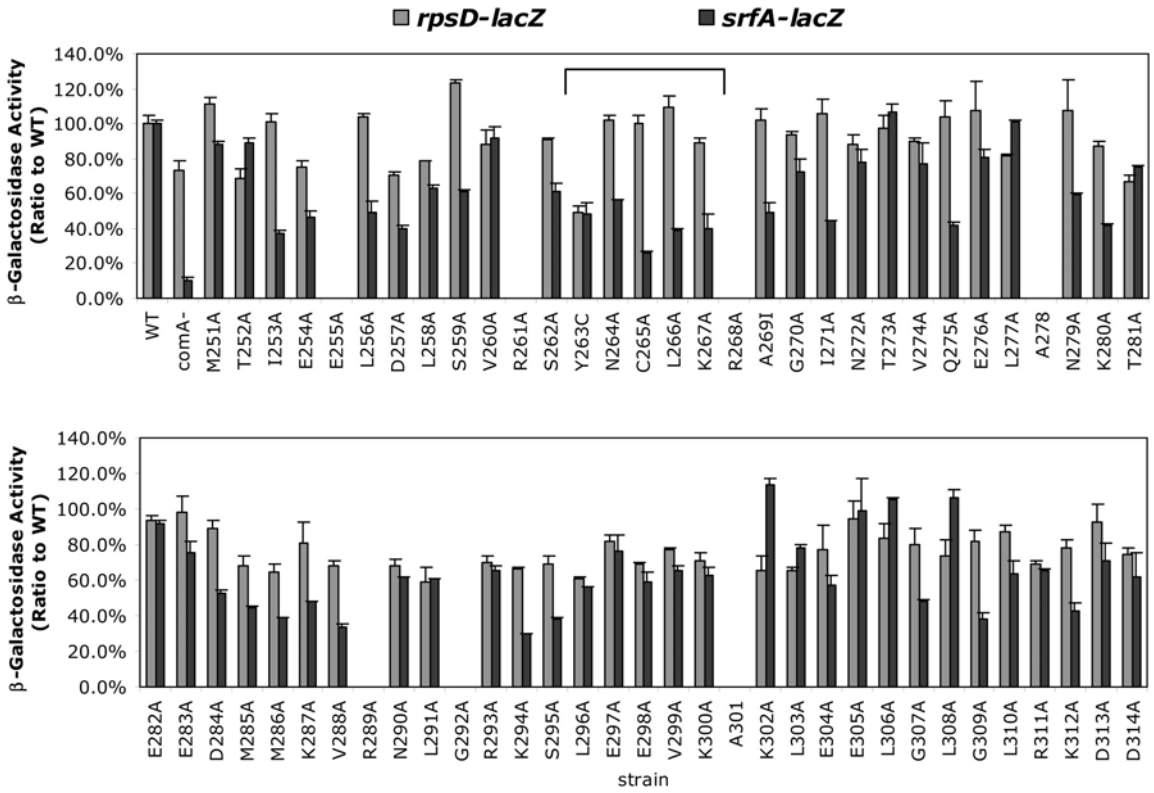


Figure 2.3 Measurement of *lacZ* fusion activity in *B. subtilis* strains bearing alanine codon substitutions in the αCTD-coding region of the *rpoA* gene.

β-galactosidase activity was measured in culture samples collected at the beginning of stationary phase for *srf-lacZ*-bearing cells and mid-log phase for *rpsD-lacZ* cells. Activity is expressed as a percentage of the activity measured in *rpoA*$^{+}$ cells. The bracket shows the region around α1 of αCTD.

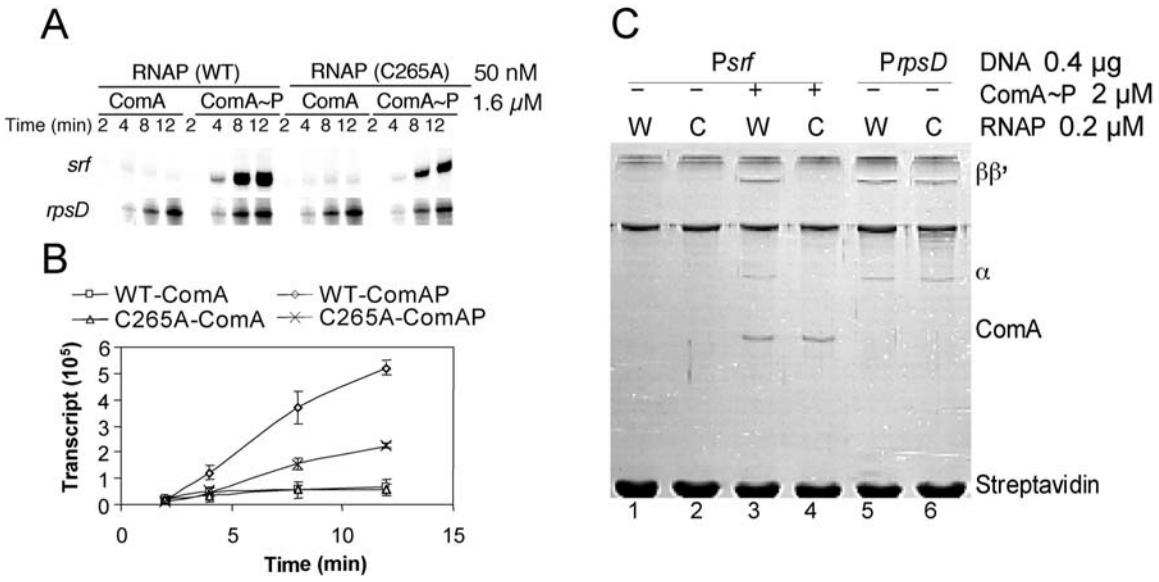


Figure 2.4 Effect of *rpoA*(C265A) mutation on *srf* transcription and on ComA and RNAP binding to *srf* promoter DNA.

(A) Time course *in vitro* runoff transcription experiment using *srf* promoter DNA as the template and untreated ComA (ComA) or ComA treated with acetyl phosphate (ComA~P), plus RNAP or *rpoA*(C265A) RNAP.

(B) Plot of band intensities derived from three repeats of the experiment shown in panel A against time of incubation.

(C) Binding of WT and mutant *rpoA*(C265A) RNAP with ComA~P to the *srf* promoter as determined by SPPR analysis. Reactions containing wild-type RNAP (W) and mutant *rpoA*(C265A) RNAP (C) are indicated.

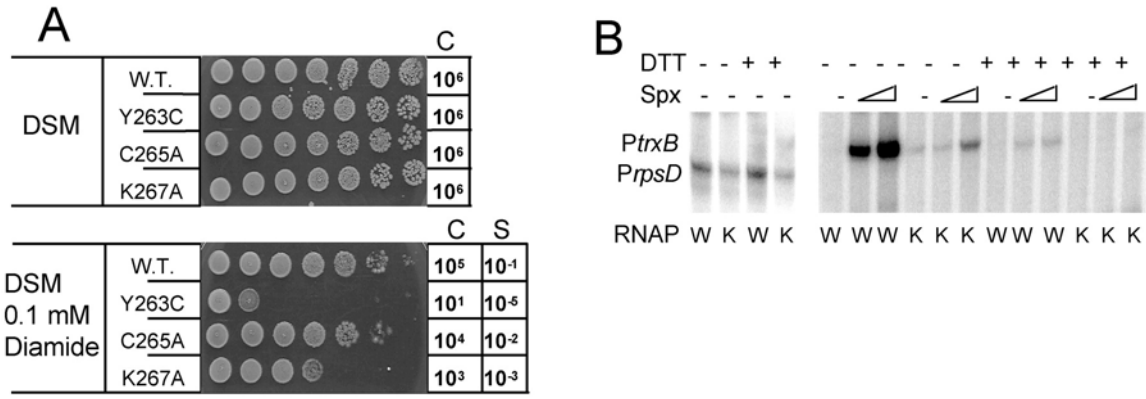


Figure 2.5 *In vivo* and *in vitro* phenotypes of *rpoA* mutants.

(A) Sensitivity of *rpoA*$^{Cxs-1}$, *rpoA*(C265A), and *rpoA*(K267A) mutants to the thiol-specific oxidant diamide. Cultures of WT cells and those of each mutant grown in DSM to mid-log phase were serially diluted to 10^{-6}, and 5 µl of each dilution was spotted onto DSM agar and DSM agar containing diamide. C, control indicating the final dilution spotted that showed growth; S, sensitivity, shown as the approximate fraction of total cells surviving exposure to diamide [(C + diamide)/C - diamide)].

(B) Transcription from the *trxB* promoter catalyzed by WT RNAP (W) and mutant *rpoA*(K267A) RNAP (K) in the absence (-) and presence (+) of Spx (0.4 µM and 0.8 µM). Where used, DTT was added to a final concentration of 5 mM. The control transcription reaction mixture contained *rpsD* gene promoter DNA.

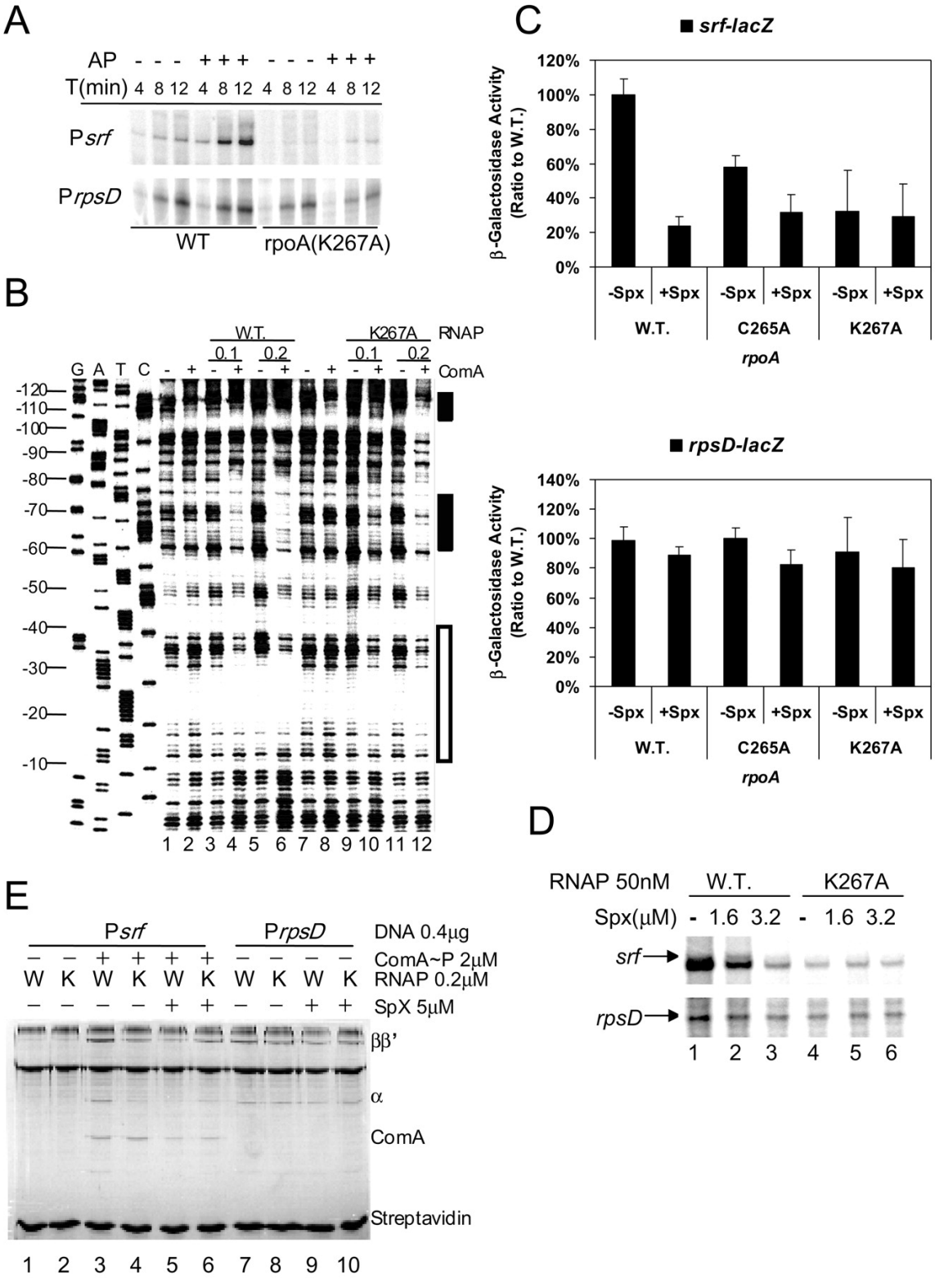

A
AP - - - + + + - - - + + +
T(min) 4 8 12 4 8 12 4 8 12 4 8 12
Psrf
PrpsD
WT
rpoA(K267A)
B
W.T.
K267A
RNAP
0.1
0.2
0.1
0.2
G A T C
ComA
-120
-110
-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
1 2 3 4 5 6 7 8 9 10 11 12
C
srf-lacZ
β-Galactosidase Activity (Ratio to W.T.)
120%
100%
80%
60%
40%
20%
0%
-Spx
+Spx
W.T.
C265A
K267A
rpoA
rpsD-lacZ
140%
D
RNAP 50nM
W.T.
K267A
Spx(μM) - 1.6 3.2 - 1.6 3.2
srf
rpsD
1 2 3 4 5 6
E
Psrf
PrpsD
DNA 0.4μg
ComA~P 2μM
W K W K W K W K W K
RNAP 0.2μM
SpX 5μM
ββ'
α
ComA
Streptavidin
1 2 3 4 5 6 7 8 9 10

Figure 2.6 Effect of *rpoA*(K267A) mutation on ComA-dependent *srf* transcription and Spx-dependent repression.

(A) Time course *in vitro* transcription experiment showing the accumulation of *srf* transcript in reaction mixtures containing untreated (-) or acetyl phosphate (1.6 mM)-treated (+) ComA, and either wild-type RNAP (WT) or *rpoA*(K267A) RNAP (50 nM).

(B) Denaturing gel analysis of DNase I footprint reactions containing P*srf* DNA with (+) and without (-) ComA~P in the presence of wild-type RNAP (WT) or RpoA(K267A) RNAP. RNAP was used at 0.1 μM and 0.2 μM as indicated.

(C) Effect of SpxLDD production on levels of β-galactosidase activity in *srf-lacZ* (ORB6129, ORB6131, and ORB6132) and *rpsD-lacZ* (ORB6137, ORB6139 and ORB6140) cells bearing the wild-type *rpoA* allele or the *rpoA*(C265A) or *rpoA*(K267A) mutant allele. *srf-lacZ* fusion-bearing cells were collected from cultures at the end of exponential growth, while *rpsD-lacZ* cells were collected from mid-log-phase cultures.

(D) Effect of Spx (1.6 μM) on transcription of *srf* in reaction mixtures containing ComA (1.6 μM), acetyl phosphate (1.6 mM), and either wild-type (WT) or *rpoA*(K267A) RNAP (50 nM). Control transcripts from reactions containing *rpsD* promoter DNA are shown at the bottom.

(E) Effect of *rpoA* mutations on ComA-dependent RNAP binding to the *srf* and *rpsD* promoter and on Spx-dependent RNAP release from promoter DNA. SPPR reaction mixtures contained ComA (+) and either wild-type (W) or *rpoA*(K267A) (K) RNAP in the presence (+) or absence of Spx.

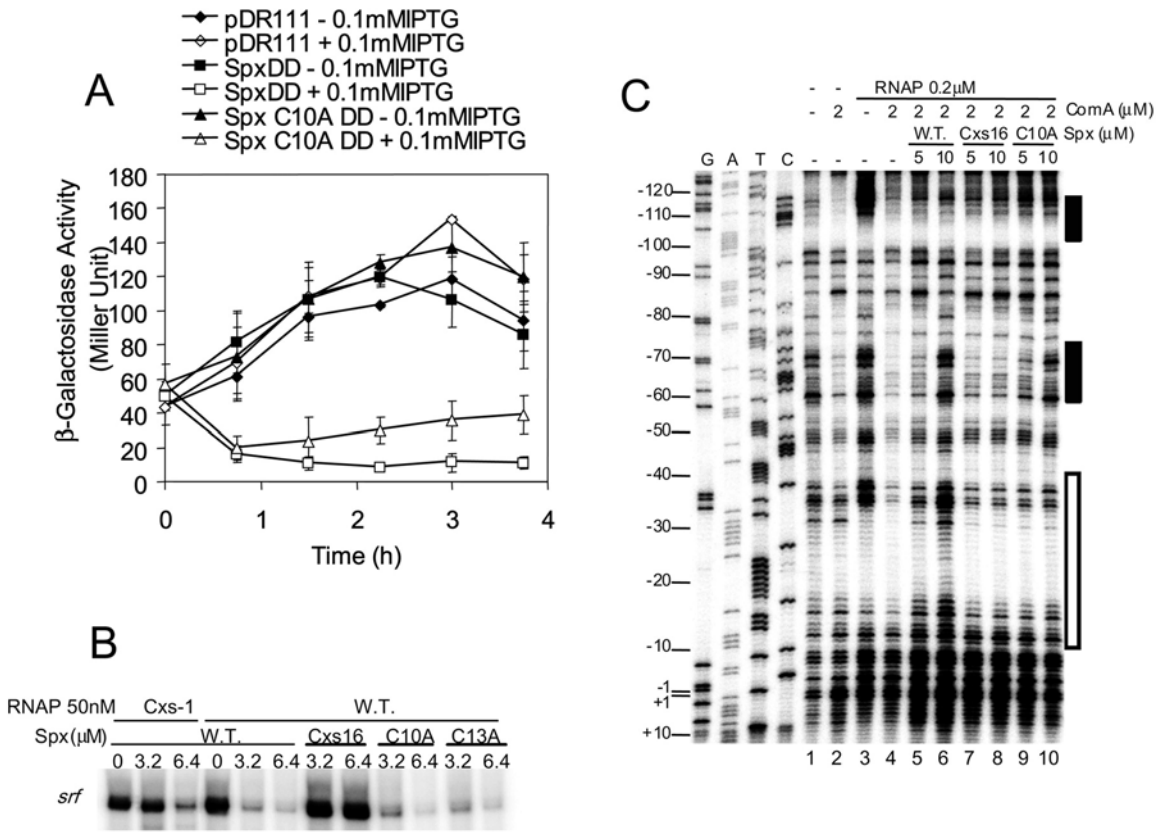


Figure 2.7 Effect of amino acid substitutions in the CXXC motif of Spx on Spx-dependent repression of *srf* transcription.

(A) Measurement of β-galactosidase activity in a time course experiment of cultures of *srf-lacZ* bearing cells expressing either SpxLDD or SpxLDD(C10A). *spxLDD* and mutant *spxLDD*(C10A) alleles were expressed from an IPTG-inducible construct derived from pDR111. Vector control cultures are indicated (pDR111), as are *spxLDD* and mutant *spxLDD*(C10A) cultures. Data are from two experiments.

(B) *In vitro* transcription data from reactions with WT or $rpoA^{Cxs-1}$ RNAP, ComA~P (1.6 μM), and increasing concentrations of either Spx, Spx^{Cxs-16}, Spx(C10A), or Spx(C13A).

(C) Image of denaturing polyacrylamide gel of DNase I footprinting reactions containing the end-labeled *srf* promoter DNA, RNAP, ComA~P, and either Spx, Spx^{Cxs-16}, or Spx(C10A), at the concentrations indicated.

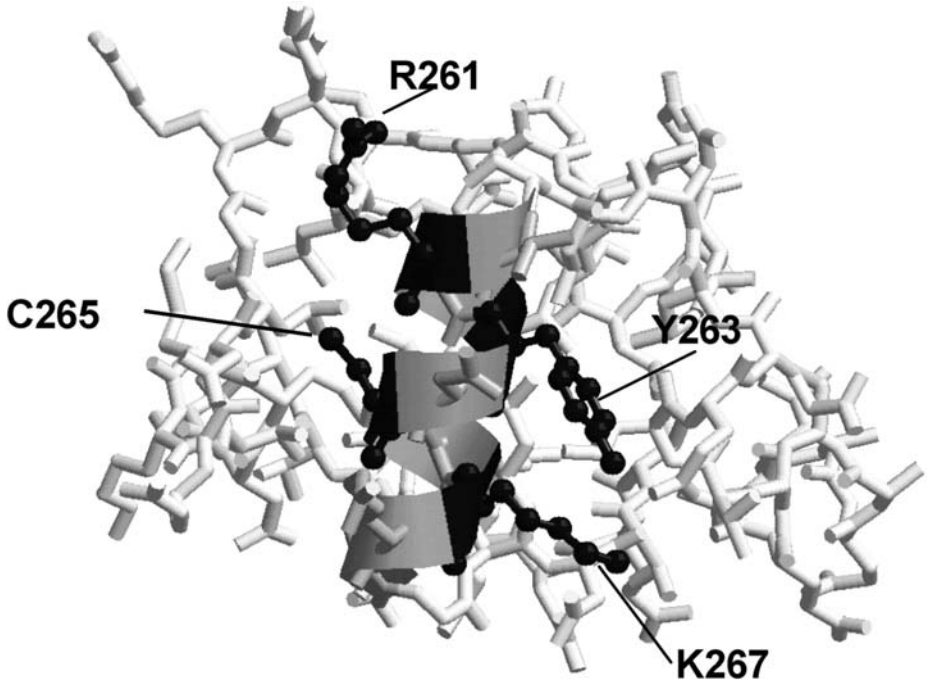


Figure 2.8 Structure of *B. subtilis* RNA polymerase αCTD (Newberry *et al.*, 2005).

Stick structures denote peptide backbone and amino acid side chains. The ribbon indicates the α1 helix. The side chains of residues R261, Y263, C265, and K267 are presented as black ball-and-stick structures.

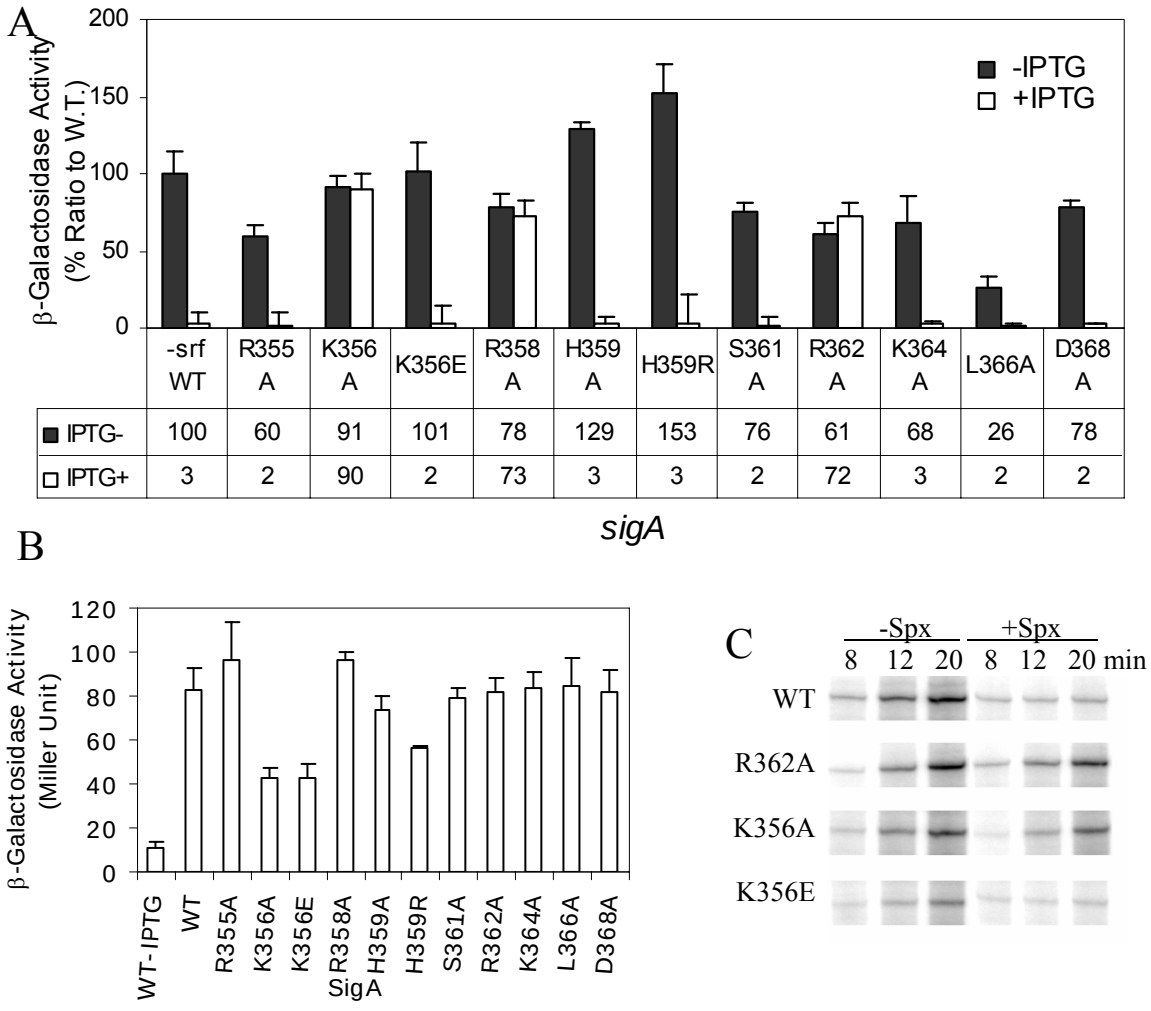


Figure 2.9 Effect of *sigA* region 4.2 on Spx-dependent transcriptional control of *srfA* and *trxB*.

(A) β-galactosidase activity was measured in DSM culture samples collected at the beginning of stationary phase for *srf-lacZ*, Phyperspank-*spxDD* bearing cells. When O.D. 600 reached 0.5, culture was separated, and half was continue grow with or without 0.5 mM IPTG. Activity was expressed as a percentage of the activity measured in *sigA*(WT) cells without IPTG.

(B) β-galactosidase activity of *trxB-lacZ* was measured in DSM culture samples collected at the mid of exponential phase in Phyperspank-*spxDD* bearing cells. Cells started grow from O.D. 600=0.02 with or without 0.5 mM IPTG. Activity

was expressed as a percentage of the activity measured in *sigA*(WT) cells with IPTG. All other *sigA* mutant cell exhibited similar basal level β-galactosidase activity of *trxB-lacZ* in the absence of IPTG.

(C) *In vitro* transcriptions from the *srfA* promoters catalyzed by WT RNAP (WT) and mutant *sigA*(R362A), *sigA*(K356A) and *sigA*(K356E) RNAP. Reactions contained 50nM *srfA* template, 50nM RNAP and 2 μM ComA~P (ComA mixed with Acetyl-phosphate at the ratio 1:1000 for 15min in 37°C before added to the reaction) in the reaction buffer with or without 2μM *Spx* and with 5 mM DTT. Reactions were initiated by adding nucleotides mix with radioactive labeled UTP. Reactions were stopped at 8, 12, 20 min by adding stop buffer to the reactions.

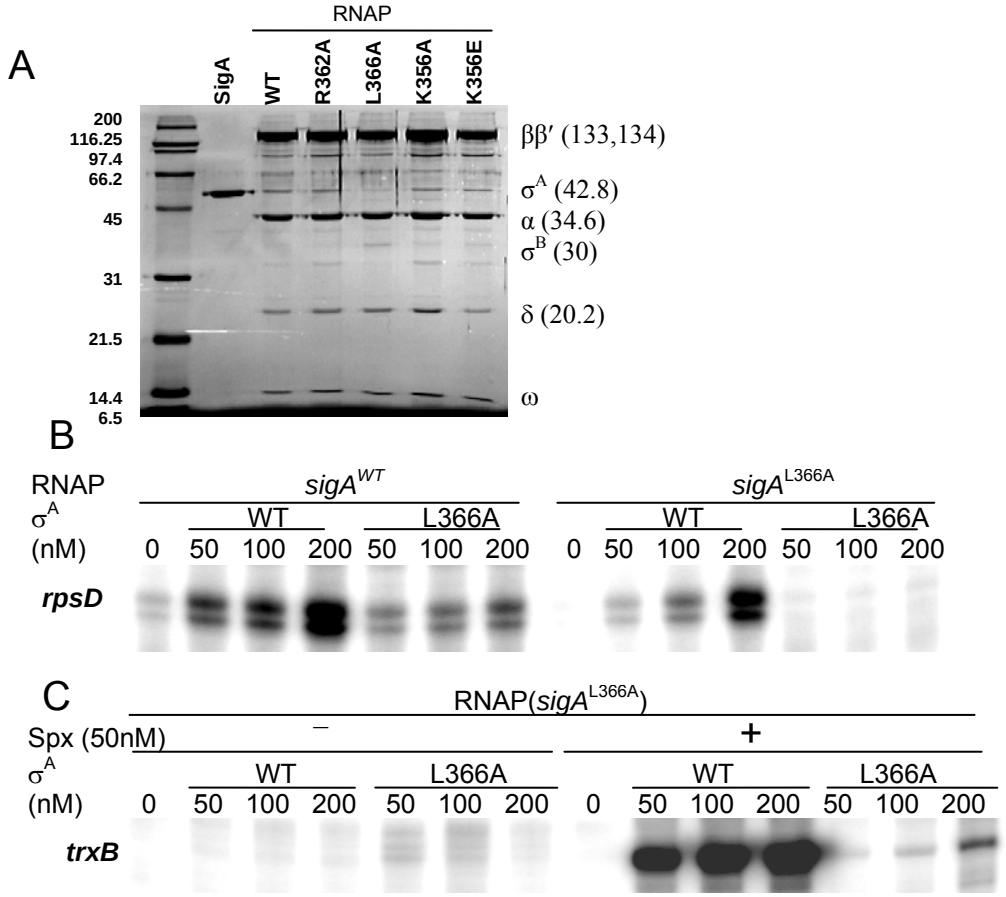


Figure 2.10 *In vitro* transcription with WT or *sigA*(L366A) σ^A reconstituted RNAP.

(A) SDS-PAGE of *sigA* mutant containing RNAP. 10 pmols of each RNAP were separated by 12% SDS-polyacrylamide gel electrophoresis, followed by staining with Coomassie blue. The subunits of RNAP, β (133.4kd), β′ (133.9kd), σ^A (42.8kd), α (34.6), σ^B (30kd), δ (20.2kd) and ω were at the right side of the gel. Purified σ^A protein was used as a control to indicate the position of the σ^A subunit.

(B) *In vitro* transcription from the *rpsD* promoters catalyzed by reconstituted RNAP with WT or *sigA*(L366A) σ^A subunit. Reactions contained 25 nM *rpsD* template, 50 nM RNAP and different concentrations (0, 50, 100, 200 nM) of WT or *sigA*(L366A) σ^A protein in the presence of 5 mM DTT. After 15 min 37°C preincubation reactions were initiated with addition of nucleotides with radioactive labeled UTP. Reactions were stopped after 15 min by adding stop buffer to the reactions.

(C) Transcriptions from the *trxB* promoters catalyzed by reconstituted RNAP with WT or *sigA*(L366A) σ^A subunit. Reactions contained 10 nM *trxB* template, 50 nM *sigA*(L366A) RNAP (reconstituted with 0, 50, 100, 200 nM of WT or *sigA*(L366A) σ^A subunit) with and without 50 nM *Spx* in the reaction buffer without DTT. After 15 min 37°C preincubation, reactions were initiated with addition of nucleotides with radioactive labeled UTP. Reactions were stopped after 15 min by adding stop buffer to the reactions.

CHAPTER 3 REQUIREMENT OF THE ZINC-BINDING DOMAIN (ZBD) OF CLPX FOR SPX PROTEOLYSIS IN *BACILLUS SUBTILIS*: EFFECTS OF DISULFIDE STRESS ON CLPXP ACTIVITY.[†]

3.1 INTRODUCTION

The ATP-dependent protease, ClpXP, plays an important role in protein quality control during developmental processes and in the cell's response to harmful physical and chemical agents (Frees *et al.*, 2007; Gottesman, 1996; Gottesman, 1999). ClpX is one of several AAA+, Clp/Hsp100 family members in prokaryotes that function as molecular chaperones or as the substrate-binding, ATPase subunits of multicomponent Clp proteases. ClpX functions as an "unfoldase" that can either disassemble stable macromolecular complexes or denature substrates for delivery to the proteolytic chamber formed by its Clp protease partner subunit, ClpP (Sauer *et al.*, 2004). ClpX can recognize one or more substrate amino acid sequences, which serve as tethering or degradation tags that interact with the ATPase protease subunit (Baker & Sauer, 2006; Flynn *et al.*, 2003). ClpX, as well as other Clp protease ATPase subunits, sometimes require an adaptor to offer substrates to the ATP-dependent unfoldase (Becker *et al.*, 2000; Dougan *et al.*, 2002; Levchenko *et al.*, 2000; Neher *et al.*, 2003; Turgay *et al.*, 1997). The affinity range for recognition tags and the partnering with adaptor proteins provides broad flexibility for substrate interaction that allows the Clp proteases to respond appropriately to the regulatory signals generated by changing environmental conditions and metabolic states

[†] Some part of this material has been published in this or similar form in *J. Bacteriol.* and is used here with permission of the American Society for Microbiology.

Zhang, Y., and P. Zuber 7 September 2007. Requirement of the Zinc-binding domain of ClpX for Spx proteolysis in *Bacillus subtilis* and Effects of disulfide stress on ClpXP Activity. *J. Bacteriol.* doi:10.1128/JB.00745-07

that require turnover of specific protein substrates (Sauer *et al.*, 2004). ClpXP can target specific proteins for degradation, including the SsrA-tagged products of interrupted translation (Gottesman *et al.*, 1998), the stationary phase RNA polymerase sigma subunit of *E. coli*, σ^S (Becker *et al.*, 1999; Zhou & Gottesman, 1998), proteins whose production is induced by the SOS response (Neher *et al.*, 2006), and proteins that function in phage development (Burton & Baker, 2003; Gonciarz-Swiatek *et al.*, 1999; Jones *et al.*, 1998).

In the spore-forming bacterium, *Bacillus subtilis*, there are several AAA+ unfoldases that function as ATPase subunits of Clp proteases; such as ClpX, ClpC, and ClpE (Derre *et al.*, 1999a; Kruger *et al.*, 1994; Msadek *et al.*, 1994; Msadek *et al.*, 1998). ClpX is necessary for many of the late growth processes for which the bacterium is known, such as sporulation and competence development, and is also necessary for optimal growth in minimal medium and resistance to elevated temperature (Frees *et al.*, 2007; Msadek *et al.*, 1998; Nakano *et al.*, 2000; Nakano *et al.*, 2001). Aside from SsrA-tagged proteins (Wiegert & Schumann, 2001), only a few specific protein substrates of ClpXP have been identified in *B. subtilis*. Recently, the Sda peptide, which controls sporulation in response to replication stress in *B. subtilis* (Rowland *et al.*, 2004; Sagara *et al.*, 1998), was found to be a substrate for ClpXP (Ruvolo *et al.*, 2006). ClpXP has also been implicated in activation of the SigW regulon in *B. subtilis* by its requirement for complete degradation of the anti-sigma protein RsiW (Zellmeier *et al.*, 2006), as part of the cell's envelope stress response.

The transcriptional regulator, Spx (Zuber, 2004), is another ClpXP substrate that is under tight proteolytic control in cells of cultures undergoing unperturbed, exponential growth. The product of *spx* is a transcriptional regulator that functions in the disulfide stress response in *B. subtilis* by interacting with RNA polymerase to repress a variety of cellular process while activating the transcription of genes whose products function in alleviating the damage caused by thiol oxidation (Nakano *et al.*, 2003a; Nakano *et al.*, 2003b; Zuber, 2004). Spx has also been implicated as a regulatory factor for virulence-related functions in *Staphylococcus aureus* (Pamp *et al.*, 2006) and *Listeria monocytogenes* (Chatterjee *et al.*, 2006). Expression of *spx* is controlled at several levels. Transcription from the *spx* P3 promoter is under negative control by two oxidant-sensitive repressors, PerR and YodB (Leelakriangsak *et al.*, 2007; Leelakriangsak &

Zuber, 2007), and is also controlled transcriptionally from other promoters of the *yjbC spx* dicistronic operon (Antelmann *et al.*, 2000; Leelakriangsak & Zuber, 2007; Thackray & Moir, 2003). The activity of the Spx protein is under redox control by a thiol/disulfide switch involving its N-terminal CXXC motif that controls productive RNA polymerase interaction (Nakano *et al.*, 2005). Spx is also the substrate for ClpXP proteolysis (Nakano *et al.*, 2003a), and in a *clpX* or *clpP* mutant, Spx protein accumulates to high concentration, which is largely responsible for the severe detrimental effects conferred by a *clpX* or *clpP* mutation (Gerth *et al.*, 1998; Msadek *et al.*, 1998; Nakano *et al.*, 2000; Nakano *et al.*, 2001). Spx is also a substrate for MecA/ClpCP *in vitro* (Nakano *et al.*, 2002a; Nakano *et al.*, 2002b), but mutations in *mecA* or *clpC* do not significantly affect Spx protein levels. In the case of Spx, a recognition tag residing at the extreme C-terminus of the Spx protein is required for ClpXP-dependent proteolysis (Nakano *et al.*, 2003a).

Previously reported evidence suggested that higher Spx concentrations in cells undergoing disulfide stress might result from down-regulation of ClpXP-catalyzed Spx turnover (Nakano *et al.*, 2003a). That ClpXP might be under redox control is suggested by the presence of an essential Zinc-binding domain (ZBD) of the Cys4 variety (Banecki *et al.*, 2001). The N-terminal ZBD functions in dimerization, substrate recognition, and in adaptor binding (Park *et al.*, 2007; Thibault *et al.*, 2006b; Wojtyra *et al.*, 2003). It is also thought to function in directing the substrate to the proteolytic cavity formed by the heptameric rings of ClpP, through an ATP-dependent ClpX conformational change. The Cys4 clusters, like those coordinating the Zn atom of ClpX, are sensitive to oxidizing agents, exposure to which results in release of Zn or a change in the conformation of the ZBD (Jenkins *et al.*, 2006).

In this chapter, we show that ClpXP *in vitro* is hypersensitive to the thiol-specific oxidant, diamide, while MecA/ClpCP shows little diamide sensitivity. Diamide treatment causes a greater than 50% loss in Zn content and causes ClpX protein to aggregate, while little diamide-induced aggregation is observed in the case of ClpC. Mutations that change two of the Zn-coordinating Cys residues to Ser reduce Spx proteolysis *in vitro* and confer high Spx concentration and activity *in vivo*. A model is proposed that the N-terminal

ZBD of ClpX is required for Spx proteolysis and is the site of oxidant-induced protease inactivation.

3.2 RESULTS

3.2.1 Spx protein concentration is higher in diamide treated cells.

The Spx protein of *B. subtilis* is a substrate for the ATP-dependent protease ClpXP *in vivo* and *in vitro*, and it accumulates to high concentration in *clpX* and *clpP* mutant cells (Nakano *et al.*, 2001; Nakano *et al.*, 2003a; Nakano *et al.*, 2003b). Spx concentration also increases in response to disulfide stress brought about by treatment of cells with the thiol-specific oxidant, diamide (Nakano *et al.*, 2003a). Fig. 3.1A shows the result of a western blot experiment in which Spx protein levels were examined in cells of a culture treated with diamide and in cells of an untreated culture. As observed previously (Nakano *et al.*, 2003a), Spx concentration is higher in the diamide-treated cells versus untreated. Spx concentration is also high in an *clpX* mutant (Fig. 3.1B), suggesting that the increase in Spx concentration observed in diamide-treated cells could be due to a reduction in ClpXP activity, thus creating a condition resembling the phenotype of a *clpX* null mutant.

The stability of Spx protein in diamide-treated cells was examined to determine if the elevated concentration of Spx during oxidative stress was attributable to post-translational control. *B. subtilis* cells of JH642 were grown in TSS medium until mid-log phase. One culture was treated with diamide and the other was left untreated. The two cultures were split and one was treated with chloramphenicol. Protein extracts were obtained by lysozyme treatment in protoplast buffer and were applied to an SDS-polyacrylamide gel for electrophoresis. Western blot analysis was performed using anti-Spx antiserum. In the culture that was not treated with diamide, the level of Spx protein remained constant in chloramphenicol-untreated cells, but the protein disappeared after chloramphenicol treatment (Fig. 3.2), indicating low Spx stability. However, in the diamide-treated culture, subsequent treatment with chloramphenicol did not result in a significant reduction in Spx concentration (Fig. 3.2). These results suggest that there is a down-regulation of Spx proteolysis during oxidative stress, possibly directed at ClpXP.

3.2.2 Diamide treatment causes increase in SsrA-tagged protein concentration.

The *in vivo* activity of ClpXP in diamide-treated *B. subtilis* cells was next examined using an artificial ClpXP substrate, HrcA-SsrA constructed as previously reported (Wiegert & Schumann, 2001). The *hrcA-ssrA* allele is transcribed from a constitutive promoter (promoter of the *dnaK* gene without the CIRCE operator elements (Wiegert & Schumann, 2001), and integrated in the *lacA* gene. Removal of the CIRCE elements eliminates transcriptional and post-transcriptional control of the transcript synthesized from the *dnaK* promoter (Homuth *et al.*, 1999; Schulz & Schumann, 1996). Cells of wild-type (JH642) and *lacA*::*hrcA-ssrA* cells were grown in DSM to mid-log, then split into two cultures, one treated with diamide and the other untreated. After 30 min. of incubation, a sample was taken for western analysis. In JH642, HrcA protein increases in concentration after diamide treatment (Fig. 3.3), which is expected because the *hrcA* gene transcription is induced 8-fold by diamide treatment (Leichert *et al.*, 2003). HrcA-SsrA (HrcA-AA) protein is undetectable in a *clpX*$^+$ background, but is observed to increase in concentration after diamide treatment. A protease-resistant form of the HrcA-SsrA (HrcA-DD), bearing two aspartyl residues at the extreme C-terminus instead of the alanine residues normally found in the SsrA peptide, is observed to be present in both diamide-treated and untreated cells. Likewise, in *clpX* mutant cells, the HrcA-SsrA tagged product is observed in both untreated and diamide-treated cells. The increased levels of the product encoded by the *hrcA-ssrA* allele, expressed from the constitutive promoter, after diamide treatment, strongly suggests that ClpXP activity is reduced within *B. subtilis* cells which are exposed to a toxic oxidant.

3.2.3 ClpXP activity *in vitro* is reduced in the presence of oxidant.

Spx is degraded by ClpXP *in vitro* (Fig. 3.4A) (Nakano *et al.*, 2003a) but ClpXP-catalyzed proteolysis of Spx is reduced in the presence of diamide or hydrogen peroxide (Fig. 3.4A). While ClpXP-catalyzed proteolysis reduces Spx concentration 90%, there was little reduction in Spx protein levels in reactions containing diamide, and there was less than 20% reduction of Spx in reactions treated with H_2O_2 (Fig. 3.4B). This effect of

diamide on ClpXP-catalyzed proteolysis is not substrate-specific since an SsrA-tagged derivative of green fluorescent protein (His6-Gfp-SsrA), a substrate for ClpXP *in vitro* (Fig. 3.5), is degraded at a reduced rate when the reaction contains diamide. Hydrogen peroxide addition also reduces ClpXP-catalyzed proteolysis of His6-Gfp-SsrA (Fig. 3.5).

The ATP-dependent protease ClpCP/MecA can utilize Spx as a substrate *in vitro* (Nakano *et al.*, 2002a; Nakano *et al.*, 2002b), but mutations in *clpC* or *mecA* have little effect on the *in vivo* concentration of Spx. Fig. 3.6 shows that Spx concentration is sharply reduced in proteolytic reactions containing ClpCP/MecA. Addition of diamide at concentrations that inhibit proteolysis of Spx by ClpXP has little effect on ClpCP/MecA-catalyzed proteolysis, showing only an initial reduction in the reaction when the oxidant is present. While ClpC is structurally similar to ClpX, being a member of the HSP100/Clp family of proteins, ClpC does not possess a ZBD.

The addition of diamide to a reaction containing Spx will likely create a disulfide linkage between Cys10 and Cys13 at the N-terminal redox disulfide center of Spx (Nakano *et al.*, 2005). It was possible that the formation of the disulfide bond could render Spx resistant to ClpXP-catalyzed proteolysis. The Spx(C10A) mutant protein (Nakano *et al.*, 2005) was tested to see if it could be degraded in the presence or absence of diamide. Fig. 3.7 shows that Spx(C10A) was degraded by ClpXP but not when the reactions contained diamide, showing that Spx(C10A) exhibited the same resistance to proteolysis as wild-type Spx. This result along with the results of Fig. 3.5 indicated that diamide was affecting protease activity rather than changing the structure of the substrate, thereby rendering Spx protease-resistant.

3.2.4 Amino acid substitutions in the ZBD of ClpX reduce Spx proteolysis by ClpXP.

The ZBD of ClpX is a likely target for direct oxidant-dependent inactivation of ClpXP protease. To determine if the ZBD is required for Spx proteolysis, amino acid substitutions in the Cys4, Zinc-binding cluster were created by *in vitro* PCR mutagenesis and the products of the resulting alleles were tested for activity *in vitro* and *in vivo*. Cysteine to serine substitutions were created at position 16 and 35 of the ClpX N-terminal ZBD (Fig. 3.8A).

The alleles encoding the mutant ClpX proteins were introduced into the *thrC* locus of the *clpX* null mutant, bearing either a *trxB-lacZ* fusion or a *srfA-lacZ* fusion, to determine if they could complement *clpX* with respect to Spx activity. The *trxB* gene (encoding thioredoxin reductase) is positively controlled by Spx (Nakano *et al.*, 2003a; Nakano *et al.*, 2005) and is expressed at a high level in a *clpX* mutant due to the accumulation of Spx protein. The introduction of a wild-type allele to an ectopic location (the *thrC* locus) resulted in reduced *trxB-lacZ* expression relative to that of the *clpX* mutant (Fig. 3.8B). The introduction of either the C16S or the C35S alleles of *clpX* into the *thrC* locus of the *clpX trxB-lacZ* strain resulted in high levels of expression (Fig. 3.8B), similar to that observed in the *clpX* mutant, indicating a failure of either ZBD mutant allele to complement *clpX*.

The *srf* operon is repressed in a *clpX* mutant due to the accumulation of Spx, which blocks ComA-dependent activation of the *srf* operon (Nakano *et al.*, 2003a; Nakano *et al.*, 2003b; Zhang *et al.*, 2006). Expression of the *srf-lacZ* fusion is repressed in a *clpX* mutant (Fig. 3.8C) but the *clpX* null mutation can be complemented by the ectopically expressed, wild-type copy of the *clpX* gene, as shown by the increase in *srf-lacZ* expression (Fig. 3.8C). The introduction of either the C16S or C35S alleles of *clpX* into an ectopic position (the *thrC* locus) within the *clpX* mutant genome fails to increase *srf-lacZ* expression, indicative of a defect in Spx proteolysis.

The western blot in Fig. 3.8D shows that Spx protein levels are low in wild-type cells and in cells of the $clpX^{+}$/*clpX* merodiploid strain, but are high in the *clpX* mutant and in cells of the $clpX^{C16S}$/*clpX* and $clpX^{C35S}$/*clpX* strains, confirming that the ZBD mutants of ClpX are unable to participate in proteolytic turnover of Spx. ClpX protein was detected in the wild-type and mutant merodiploid strains, although somewhat lower levels were produced in the C to S mutant-producing cells (Fig. 3.8D).

Proteolysis reactions containing either wild-type ClpXP or protease bearing C to S mutant versions of ClpX showed that the mutant enzymes were defective in utilizing Spx protein as substrate *in vitro* (Fig. 3.9A and C). The addition of diamide to the reaction reduced proteolysis of Spx in reactions containing wild-type ClpXP enzyme, but little effect of diamide treatment was detected in the reactions of ZBD mutant ClpXP, the activity of which was already compromised (Fig. 3.9B and C).

3.2.5 Diamide treatment results in aggregation of ClpX protein.

The Zn content of wild-type and mutant proteins was examined by pyridyl azo resorcinol (PAR) staining of ClpX protein resolved on polyacrylamide gels (data not shown). The wild-type ClpX protein showed reduced Zn content after diamide treatment as judged from the band intensity after PAR staining. No detectable PAR staining was observed in lanes containing mutant $ClpX^{C35S}$ protein. Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) analysis of untreated and diamide-treated ClpX showed a 45% reduction in Zn content after diamide treatment. The mutant $ClpX^{C35S}$ and $ClpX^{C16S}$ proteins had only 33% of the Zn content found in wild-type ClpX as determined by ICP-OES. The modest reduction in Zn content after diamide treatment suggested that Zn release upon Cys oxidation provides a partial explanation for the reduction in ClpXP activity after treatment with the thiol-specific oxidant.

The ClpX protein was again examined in proteolysis reactions containing the Spx substrate and treated with varying concentrations of diamide (Fig. 3.10). A concentration of 8 μM diamide results in a 50% reduction in Spx proteolysis after 10 min (Fig. 3.10A). ClpX protein treated with diamide at the concentrations indicated caused a reduction in the intensity of the ClpX band in non-reducing SDS polyacrylamide gels, with the appearance of slower migrating bands (Fig. 3.10B, asterisk). Western analysis using anti-ClpX antibody shows that ClpX protein aggregates upon diamide treatment, as shown by the appearance of anti-ClpX-reacting proteins migrating slowly on the non-reducing SDS-polyacrylamide gel (Fig. 3.10B, lower panel).

The effects of the oxidants on ClpX protein were examined, this time treating *B. subtilis* log phase cells with the same concentrations of diamide and H_2O_2 as was used in the proteolysis reactions. Western analysis of the protein extracts was performed using either anti-ClpX or anti-ClpC antisera. Treatment with increasing concentrations of diamide resulted in a reduction in the intensity of the ClpX band and the appearance of higher molecular weight material reacting with anti ClpX antibody (Fig. 3.10C, upper panel). Some decrease in the ClpX band intensity is observed with H_2O_2, but with less higher molecular weight material observed. ClpC protein is affected only slightly by treatment with diamide and H_2O_2 (Fig. 3.10C, lower panel), with some higher molecular

weight material reacting with anti-ClpC antibody at higher concentrations of diamide. The data suggest that ClpX protein is induced to aggregate *in vivo* after treatment with oxidants. This and the reduction in Zn content observed *in vitro*, could contribute to reduced ClpX activity.

3.3 DISCUSSION

In previously reported work, we had shown that Spx protein concentration increases 30 min. after diamide treatment of *B. subtilis* cells (Nakano *et al.*, 2003a). This can be explained in part by the increase in transcription of the *spx* gene resulting from inactivation of the YodB and PerR (Lee & Helmann, 2006) repressors upon oxidative stress (Leelakriangsak *et al.*, 2007; Leelakriangsak & Zuber, 2007). However, replacement of the promoter region of *spx* with an IPTG-inducible promoter did not render Spx production constitutive, as shown by a dramatic increase in Spx protein concentration after diamide treatment [Fig. 3.2, (Nakano *et al.*, 2003a)]. A post-translational mechanism of Spx control involving the ClpXP protease was proposed (Zuber, 2004).

No adaptor protein for recognition of Spx for ClpXP-catalyzed proteolysis has been reported. However, recently a mutation in the gene *yjbH* of *B. subtilis* results in an increase in Spx concentration without affect *spx* transcript levels is reported (Larsson *et al.*, 2007), suggestive of a role in proteolytic control of Spx. We note that the rate of proteolysis as catalyzed by ClpXP *in vitro* is much less than that of ClpCP/MecA, while ClpXP appears to be a primary determinant of Spx stability *in vivo*. Hence, it is reasonable to propose that ClpXP might require a cofactor/adaptor for degradation of Spx and perhaps other substrates in *B. subtilis*. YjbH or a factor under its control could serve as an adaptor for Spx degradation by ClpXP.

The ZBD of ClpX has been implicated in substrate and adaptor interaction as well as ClpX multimerization (Park *et al.*, 2007; Thibault *et al.*, 2006b; Wojtyra *et al.*, 2003). Recent studies of *E. coli* ClpX indicate that the N-terminal section of ClpX, which includes the ZBD, undergoes dramatic, ATP-dependent changes in its position within the ClpXP complex (Thibault *et al.*, 2006a). These studies suggest a model in which the N-terminal domain of ClpX functions in the introduction of bound substrate into the

proteolytic cavity formed by the ClpP heptameric rings. The ZBD contains a Cys-4 type Zinc-binding motif that is sensitive to thiol-reactive compounds (Banecki *et al.*, 2001). Zn release has been reported to affect ATP-binding, interaction with ClpP, and oligomerization, which are also observed when the four Cys residues that coordinate Zn are changed to Ser (Banecki *et al.*, 2001).

In the work reported here, treatment with the thiol-specific oxidant, diamide, resulted in a severe reduction in ClpXP-catalyzed Spx degradation *in vitro*, and accumulation of Spx as well as an SsrA-tagged protein *in vivo*. The concentrations of diamide used did not affect the activity of ClpCP/MecA, which can also utilize Spx as a substrate. Hydrogen peroxide inhibited ClpXP activity *in vitro*, but little effect was observed in Spx concentration *in vivo*, perhaps because of the multiple mechanisms possessed by the cell for removing H_2O_2 (Dowds, 1994; Engelmann & Hecker, 1996; Gaballa & Helmann, 2002). The diamide effect was not due to enhanced resistance of oxidized Spx to proteolysis as a C10A mutant, defective in disulfide bond formation at the redox disulfide center of Spx, also shows reduced proteolysis by ClpXP in the presence of diamide. Furthermore, His6-GFP-SsrA, another substrate of ClpXP, is not degraded in ClpXP reactions containing diamide. A similar loss of proteolytic activity is observed when either of two Cys residues of the Cys-4 Zinc-binding motif is changed to Ser. The substitution of either one of the two Cys residues results in a significant loss of Zn as shown by Zn-specific staining of SDS-PAGE gels with PAR (data not shown) and by ICP-OES (33% Zn content in mutant protein compared to wild-type). Diamide has two discernable effects on wild-type ClpX protein. First, it causes an approximately 45% reduction in Zn content as determined by ICP-OES. Secondly, diamide treatment leads to the formation of higher molecular weight forms of ClpX, suggestive of aggregate formation. Aggregates of ClpX protein are observed along with a disappearance of monomeric ClpX after treatment with increasing concentrations of diamide (Fig. 3.10B), while less higher molecular weight forms of ClpC are observed on gels of diamide-treated ClpC protein preparations. Western blot analysis of *B. subtilis* soluble protein extracts shows a similar result after diamide treatment (Fig. 3.10C). The results suggest that ClpX, unlike ClpC, undergoes structural changes upon exposure to thiol-reactive compounds that correlate with reduced activity. ClpX bears seven cysteine residues, five

of which reside in the ZBD. ClpC and MecA proteins contain a single Cys residue each, which likely does not participate significantly in proteolytic activity or is not accessible to thiol-reactive azo-bearing compounds such as diamide.

Exposure of Cys4 ZBDs, such as that of GATA-1, to thioester-forming electrophiles results in efficient displacement of Zinc (Jenkins *et al.*, 2006). This is not the case for some Cys2-HisCys or Cys2-His2 ZBDs, which show resistance and retain the Zn atom after treatment with electrophile. Resistance is thought to be due in part to the substitution of a thiolate for a coordinating histidine and to secondary interactions involving residues surrounding the metal-binding site. Studies of the vulnerability of Zinc-binding domains to thiol-reactive agents showed that Cys4 ZBDs, such as the one occupying the N-terminal domain of ClpX, might be particularly sensitive to oxidation (Jenkins *et al.*, 2006). As mentioned above, the treatment of ClpXP with diamide results in a modest reduction (45%) in Zn content. However, reaction of ZBDs with electrophiles need not result in Zn release in order to alter protein activity. The Ada protein undergoes a methylation to create a charge-neutral thioether at a Cys4 Zn-coordinating Cys residue without Zn release from the Ada N-terminal domain (He *et al.*, 2005). This changes the sequence specificity of Ada's DNA-binding activity. While reaction of ClpX with diamide leads to some loss of Zn, aggregation that is likely the result of thiol oxidation and disulfide formation is also observed. The ZBDs of the ClpX hexamer have been reported to interact to form three dimers (Wojtyra *et al.*, 2003), with Zn-coordinating Cys residues of adjacent monomers in position to possibly react covalently. Oxidation of the Cys residues of the ZBD might lead to both intra- and inter-chain disulfide crosslinks that contribute to the formation of the higher molecular weight species observed on non-reducing gels, as shown in Fig. 3.10. The higher molecular weight forms might still contain coordinated Zn despite reduced ligand coverage due to disulfide formation.

A mobile substrate- and adaptor-binding N-terminal domain is characteristic of the AAA+ component of Clp proteases and chaperones (Guo *et al.*, 2002; Hinnerwisch *et al.*, 2005; Kirstein *et al.*, 2006; Thibault *et al.*, 2006a; Thibault *et al.*, 2006b). That the N-terminal domain may also possess a sensory function can be proposed based on the data reported herein. Among the questions one could address is if the changes to ClpX brought about by exposure to thiol-reactive electrophiles are reversible, as is the case

with other redox- controlled, Zinc-binding proteins (Ilbert *et al.*, 2006). Disruption of the ZBD could affect substrate and/or the putative substrate-adaptor (YjbH) interaction with the protease.

3.4 MATERIALS AND METHODS

3.4.1 Bacterial strains and growth conditions

Bacillus subtilis strains used in this study are derivatives of JH642 and are listed in Table 3.1. *B. subtilis* cells were cultured in a shaking water bath at 37°C in Difco Sporulation medium (DSM) (Schaeffer *et al.*, 1965) for β-galactosidase assays or TSS minimal medium (Rosenkrantz *et al.*, 1985) for diamide treatment experiments and genotype verification. Diamide was purchased from SIGMA.

For complementation experiments ectopically expressed *clpX* alleles were constructed as follows. Primers oGL03-7 and oGL03-8 (Table 3.2) were used to amplify the *clpX* gene from *B. subtilis* strain JH642 chromosomal DNA. The PCR fragment (from -786 to +1856, about 2642 bp, including 1260 bp of the coding region of *clpX* as well as 786 bp of upstream sequence and 596 bp of downstream sequence) was digested with *Kpn*I and *Bam*HI, then ligated with pUC18 that had been digested with the same enzymes, to generate pZY23. The *clpX* sequence in plasmid pZY23 was verified by DNA sequencing. Plasmid pZY23 was cleaved with *Kpn*I before treatment with T4 DNA polymerase (New England BioLabs) to create a blunt end, and then further digested with *Bam*HI to release the *clpX* fragment. To generate pZY30, the fragment was ligated with pDG795 (Guerout-Fleury *et al.*, 1996) that was digested with *Eco*RI before treatment with T4 DNA polymerase (New England BioLabs) to create blunt ends, and then further digested with *Bam*HI. Plasmid pZY30 was introduced by transformation, with selection for erythromycin/lincomycin and screening for threonine auxotrophy, into *B. subtilis* strain JH642, where the *clpX* fragment was integrated into the *thrC* locus. The resulting strain was designated ORB6624 (*thrC*::*clpX*$^{+}$). Chromosomal DNA of LAB2876 (*clpX*::Spc) was used to transform ORB6624 to generate ORB6628 (*clpX*::Spc, *thrC*::*clpX*$^{+}$). Mutant *clpX*^{C16S} and *clpX*^{C35S} alleles were constructed by PCR-based site-directed mutagenesis. The first round of PCR was performed by using pZY23 (pUC18-

clpX^{+}) plasmid DNA as template with primers oGL03-2 (oGL03-4) and oGL03-8 for the upstream fragment of *clpX*^{C16S} (*clpX*^{C35S}), and primer oGL03-1 (oGL03-3) and oGL03-7 for the downstream fragment of *clpX*^{C16S} (*clpX*^{C35S}). Two PCR fragments, purified on low-melting agarose gels, were mixed and used as templates for the second round PCR with primers oGL03-7 and oGL03-8 to generate the full-length fragment (–786 ~ +1856) bearing the desired mutant allele. The same procedure were used to create pZY24 (pUC18 with *clpX*^{C16S}), pZY25 (pUC18 with *clpX*^{C35S} allele), pZY31 (pDG795 with *clpX*^{C16S} allele) and pZY32 (pDG795 with *clpX*^{C35S} allele). The *clpX*^{C16S} and *clpX*^{C35S} sequences in plasmid pZY24 and pZY25 were verified by DNA sequencing. The plasmids were introduced by transformation into JH642 to create the *thrC*::pZY31- and *thrC*::pZY32-bearing strains. These strains were then transformed with DNA from the *clpX*::Spc null mutant, strain LAB2876, yielding the mutant complementation strains ORB6650 (*clpX*::Spc, *thrC*::pZY31) and ORB6651 (*clpX*::Spc, *thrC*::pZY32).

The plasmids used for mutant ClpX protein production and purification were constructed by PCR with primers oZY06-1 and oMN02-200 (Table 3.2) using either pZY24 or pZY25 as template. The PCR fragment was digested with *Nde*I and *Sap*I, then ligated with pTYB1 that was digested with the same restriction enzymes to create pZY29 (pTYB1 carrying the *clpX*^{C16S} allele) and pZY27 (pTYB1 with *clpX*^{C35S}). The mutant *clpX* allele sequences in plasmid pZY27 and pZY29 were verified by DNA sequencing.

The promoter region of *trxB* was amplified by PCR with primer oSN03-48 and oSN03-49 from JH642 chromosomal DNA. The resulting PCR fragment was digested with *Bam*HI and *Eco*RI, and then ligated with plasmid pTKlac that was digested with the same restriction enzymes, to generate pSN67. Plasmid pSN67 (*trxB-lacZ* fusion) was used to transform cells of strain ZB307A (Zuber & Losick, 1987) with selection of chloramphenicol-resistance. An SPβ- transducing lysate was produced by heat induction, and was then used to transduce cells of strain ZB278 (Zuber & Losick, 1987). Phage generated from this strain was used to transfer the *trxB-lacZ* fusion into a wild-type background by transduction into JH642 with selection for chloramphenicol-resistance to generate strain ORB6701.

3.4.2 Production and purification of proteins

For production of proteins used in this study, the IMPACT system (New England BioLabs), which utilizes the inducible self-cleaving intein tag, was used. Intein-tagged ClpX, ClpP, MecA and ClpC were purified using a previously reported procedure (Nakano *et al.*, 2002a; Nakano *et al.*, 2002b; Nakano *et al.*, 2003a; Nakano *et al.*, 2003b). His6-tagged wild-type, Spx(C10A) and His6-GFP-SsrA proteins were purified using a previously published procedure (Nakano *et al.*, 2005).

3.4.3 Transformation and transduction

Preparation of competent cells of *B. subtilis* and DNA-mediated transformation were carried out as described previously (Dubnau & Davidoff-Abelson, 1971; Hoch *et al.*, 1967; Niaudet & Ehrlich, 1979). Specialized transduction using SPβ phage constructs was carried out as described previously (Zuber & Losick, 1987).

3.4.4 Spx protein stability

Total protein extracts were prepared from cultures of wild-type *B. subtilis* JH642 grown in TSS liquid media. When OD600 reached 0.5, the culture was split, and one subculture was treated with 1 mM (final concentration) diamide, while the other was left untreated. After 10min, each subculture was split again and to one, 0.1 mg/ml (final concentration) chloramphenicol was added. Samples (3 ml) were taken at the indicated time points and centrifuged. Cells were then treated with 1 mg/ml lysozyme in protoplast buffer (20 mM potassium phosphate pH 7.5; 15 mM $MgCl_2$; 20% sucrose) for 30 min and centrifuged. The protoplasts were then suspended in lysis buffer (30 mM Tris-HCl, 1 mM EDTA, pH 8.0). Total protein (30 μg) from each sample was applied to a 15% SDS-polyacrylamide gel and electrophoresis was performed. The protein levels of Spx were examined by western blot analysis using anti-Spx antiserum (Nakano *et al.*, 2001) followed by incubating with the secondary antibody conjugated to alkaline phosphatase.

3.4.5 Assay of β-galactosidase activity

Cells were grown in DSM medium until OD600 ≈ 0.4-0.5. The cells were incubated further for 3 h, during which time samples were collected every 30 min and prepared for β-galactosidase assays. β-galactosidase activity was determined as previously described (Nakano *et al.*, 1988) and is presented as Miller units (Miller, 1972).

3.4.6 Western blot analysis

The total protein extracts were prepared from cells of *B. subtilis* cultures grown in DSM. Samples (1 ml) were taken at the indicated time points and centrifuged. Cells were then treated with protoplast buffer (20 mM K-phosphate pH 7.5; 15 mM $MgCl_2$; 20% sucrose; 1 mg/ml lysozyme) for 30 min and centrifuged. The protoplasts were then suspended in lysis buffer (30 mM Tris-HCl, 1 mM EDTA, pH 8.0). Total protein (30 μg) from each sample was applied to an 8% (for ClpX and ClpC) or 12% (for HrcA) or 15% (for Spx) SDS-polyacrylamide gel and electrophoresis was performed. The protein levels of Spx, ClpX, and ClpC were examined by western blot analysis using anti-Spx, anti-ClpX, anti-ClpC (Nakano *et al.*, 2001)or anti-HrcA (Wiegert & Schumann, 2001) antiserum followed by incubating with the secondary antibody conjugated to alkaline phosphatase.

3.4.7 *In vitro* ClpXP-catalyzed proteolysis reaction

In vitro proteolysis reactions were assembled under conditions as described previously (Nakano *et al.*, 2003a) with some modifications. The reactions were carried out in 50 mM HEPES/KOH (pH 7.6), 50 mM KCl, 10 mM Mg acetate, 5 mM DTT (unless diamide or H_2O_2 was added as indicated), 5 mM ATP, 5 mM creatine phosphate, 0.05 U/μl creatine kinase (Sigma) and Spx (6 μM) or GFP-SsrA (3 μM). Reactions were incubated at 37°C in the presence of ClpP (12 μM) and ClpX (6 μM) or ClpC (2.5 μM), ClpP (4 μM) and MecA (2.5 μM) in a 50 μl reaction mixture. At time intervals, 10 μl sample from each reaction was collected and treated with 2 μl stop buffer (SDS loading dye with 0.1 M DTT). The proteins were then resolved on a 12% SDS/PAGE followed by staining with Coomassie blue. Levels of Spx were defined as the ratio of Spx band

intensity/ClpP band intensity, since ClpP concentrations in all reactions were equal. The Spx/ClpP ratio in a reaction containing no ClpX was given the value 100%.

3.5 ACKNOWLEDGEMENTS

The authors wish to thank Amanda Barry and Ninian Blackburn for performing ICP-OES, Michiko Nakano for critical reading of the manuscript and helpful discussions, and Shunji Nakano for preparation of ClpP, MecA, and ClpC protein. The authors also wish to thank Dr. W. Schumann for the gifts of *hrcA-ssrA* expression *B. subtilis* strains and the anti-HrcA antibody. Research reported herein was supported by grant GM45898 from the National Institutes of Health, USA, and by a grant from the Research Foundation of Oregon.

Table 3.1 *Bacillus subtilis* strains and plasmids

Strain	Relevant genotype or properties	Source and/or reference
LAB 2876	*trpC2 pheA clpX*::Spc	(Liu *et al.*, 1999)
ORB6624	*trpC2 pheA thrC*::pZY30 (pDG795-$clpX^{WT}$)	This study
ORB6648	*trpC2 pheA thrC*::pZY31(pDG795-$clpX^{C16S}$)	This study
ORB6649	*trpC2 pheA thrC*::pZY32(pDG795-$clpX^{C35S}$)	This study
ORB6628	*trpC2 pheA thrC*::pZY30 (pDG795- $clpX^{+}$), *clpX*::Spc	This study
ORB6650	*trpC2 pheA thrC*::pZY31(pDG795- $clpX^{C16S}$), *clpX*::Spc	This study
ORB6651	*trpC2 pheA thrC*::pZY32(pDG795- $clpX^{C35S}$), *clpX*::Spc	This study
LAB 545	*trpC2 pheA* SPβ*c2del2*::Tn*917*:: pMMN92(*srfA-lacZ*)	(Nakano & Zuber, 1993)
ORB6763	*trpC2 pheA* SPβ*c2del2*::Tn*917*:: pMMN92(*srfA-lacZ*), *clpX*::Spc	This study
ORB6681	*trpC2 pheA* SPβ*c2del2*::Tn*917*:: pMMN92(*srfA-lacZ*), *thrC*::pZY30(pDG795-$clpX^{WT}$), *clpX*::Spc	This study
ORB6682	*trpC2 pheA* SPβ*c2del2*::Tn*917*:: pMMN92(*srfA-lacZ*), *thrC*::pZY32(pDG795-$clpX^{C16S}$), *clpX*::Spc	This study
ORB6683	*trpC2 pheA* SPβ*c2del2*::Tn*917*:: pMMN92(*srfA-lacZ*), *thrC*::pZY32(pDG795-$clpX^{C35S}$), *clpX*::Spc	This study
ORB6701	*trpC2 pheA* SPβ*c2del2*::Tn*917*:: pSN67(*trxB-lacZ*)	This study
ORB6702	*trpC2 pheA* SPβ*c2del2*::Tn*917*:: pSN67(*trxB-lacZ*), *clpX*::Spc	This study
ORB6703	*trpC2 pheA* SPβ*c2del2*::Tn*917*:: pSN67(*trxB-lacZ*), *thrC*::pZY30(pDG795-$clpX^{WT}$), *clpX*::Spc	This study
ORB6704	*trpC2 pheA* SPβ*c2del2*::Tn*917*:: pSN67(*trxB-lacZ*),	This study

	thrC::pZY32(pDG795-*clpX*^{C16S}), *clpX*::Spc	
ORB6705	*trpC2 pheA* SPβ*c2del2*::Tn*917*:: pSN67(*trxB-lacZ*), *thrC*::pZY32(pDG795-*clpX*^{C35S}), *clpX*::Spc	This study
ORB4381	*trpC2 pheA1 lacA*::*hrcA-ssrA*(*AA*)	This study
ORB4382	*trpC2 pheA1 lacA*::*hrcA-ssrA*(*DD*)	This study
ORB4384	*trpC2 pheA1 lacA*::*hrcA-ssrA*(*AA*) *clpX*::Spc	This study
Plasmids		
pLysS	Plasmid to produce T7 lysozyme	Stratagene
pPROEX-1	Plasmid for construction of His-6-tagged fusion	Life Technologies, Rockville, MD
pTKlac	Plasmid for construction of *lacZ* transcriptional fusion	(Kenney & Moran Jr, 1991)
pDG795	Plasmid for construction of transcriptional fusion into *thrC* locus	(Guerout-Fleury *et al.*, 1996)
pTYB1 ~ 4	Cloning vector for IMPACT T7 system	New England BioLabs
pSN17	pPROEX-1 with *spx*	(Nakano *et al.*, 2002b)
pMMN470	pTYB4 with *spx*	(Nakano *et al.*, 2001)
pSN3	pTYB1 with *mecA*	(Nakano *et al.*, 2002b)
pClpC	pTYB2 with *clpC*	(Nakano *et al.*, 2002b)
pClpP	pTYB1 with *clpP*	(Nakano *et al.*, 2002b)
pGFP-ssrA	pPROEX-1 with GFP-*ssrA*	
pMMN92	*pTKlacZ with srfA-lacZ*	(Nakano & Zuber, 1993)
pSN67	*pTKlacZ with trxB-lacZ*	This study
pZY23	pUC18 with *clpX*$^{+}$(–786 ~ +1856)	This study

pZY24	pUC18 with *clpX*^{C16S} (–786 ~ +1856)	This study
pZY25	pUC18 with *clpX*^{C35S} (–786 ~ +1856)	This study
pZY30	pUC18 with *clpX*$^{+}$(–786 ~ +1856)	This study
pZY31	pDG795 with *clpX*^{C16S} (–786 ~ +1856)	This study
pZY32	pDG795 with *clpX*^{C35S} (–786 ~ +1856)	This study
pZY29	pTYB1 with *clpX*^{C16S}	This study
pZY27	pTYB1 with *clpX*^{C35S}	This study
pMMN509	pTYB1 with *clpX*$^{+}$	(Nakano *et al.*, 2003b)

Table 3.2 Oligonucleotides

Oligo	Oligo Sequence	Position†	Note††
oGL03-1	tgctcgttctctggaaaaacacaa	Fw *clpX* +36 ~ +59	$clpX^{C16S}$
oGL03-2	ttgtgtttttccagagaacgagca	Rv *clpX* +59 ~ +36	$clpX^{C16S}$
oGL03-3	ggtgtatatatatctgacgaatgtatc	Fw *clpX* +90 ~ +116	$clpX^{C35S}$
oGL03-4	acattcgtcagatatatatacacc	Rv *clpX* +113 ~ +90	$clpX^{C35S}$
oGL03-7	atgagcggatccgcaattcctctttca	Rv *clpX* +1856 ~ +1830	*Bam*HI
oGL03-8	cgcaaaaggtaccgatgaagaagtggaaac	Fw *clpX* –786 ~ –757	*Kpn*I
oGL03-11	gctacatctttgactgaagctggata	Fw *clpX* +423 ~ +448	Sequence
oZY06-1	gggaattcatatgtttaaatttaacgaggaaaaaggac	Fw *clpX* +1 ~ +28	*Nde*I
oMN02-200	taataagctcttccgcatgcagatgttttatc	Rv *clpX* +1260 ~ +1236	*Sap*I
oSN03-48	gaattcagcgttggttcaagcattgtaggac	Fw *trxB* –510 ~ –484	*Eco*RI
oSN03-49	gcggatcctctttcaatcattaatgtcg	Rv *trxB* +112 ~ +92	*Bam*HI

† Fw = forward primer, Rv = reverse primer

††mutation or restriction site created by the primer, oGL03-11 was used as a sequencing primer.

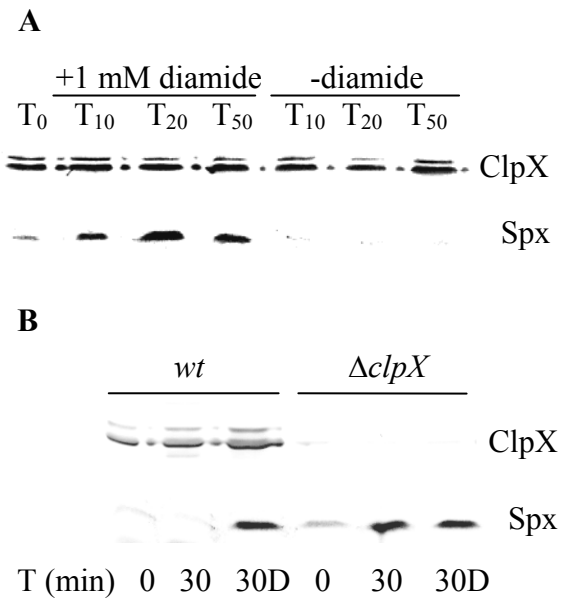


Figure 3.1 Effect of diamide on the protein level of ClpX and Spx in wild-type and *clpX* cells.

Cells were grown in DSM media until mid-exponential phase, then were treated with 1 mM diamide or left untreated. Samples were taken at indicated time point after treatments. Cells were lysed with protoplast buffer and were suspended in lysis buffer (See Materials and Methods). Thirty μg protein from each sample was applied to an SDS polyacrylamide gel for electrophoresis. The protein levels of Spx or ClpX were examined by western blot analysis using rabbit anti-Spx or anti-ClpX antiserum.

(A) Western blot analysis of ClpX and Spx levels. The wild-type strain JH642, which was grown in DSM media until mid-exponential phase, was treated with or without 1 mM diamide. Samples were taken at 0, 10, 20, 50 min after treatments.

(B) Western blot analysis of ClpX and Spx levels in wild-type and *clpX* strains. Strain JH642 (wild-type) and ORB2876 (*clpX*). Samples were collected 0 and 30 min after diamide treatment. “d” stands for 1 mM diamide treatment.

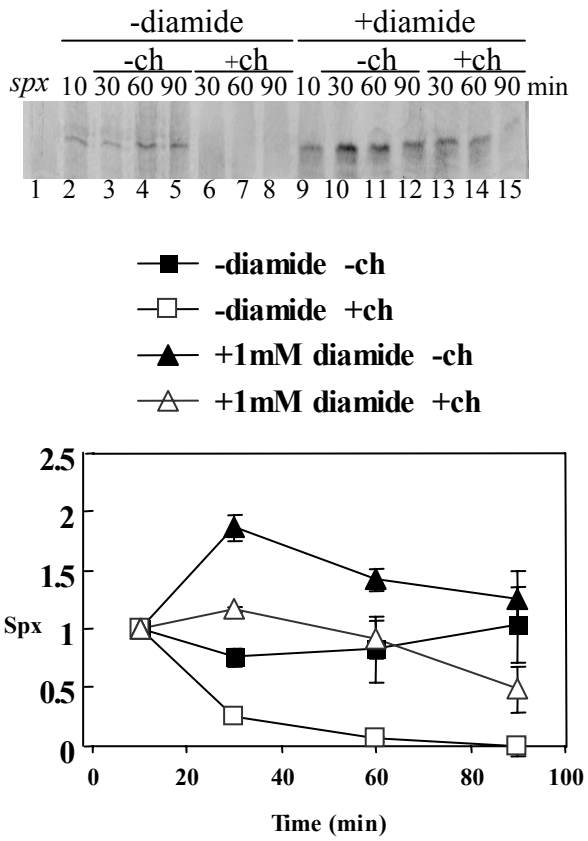


Figure 3.2 Western blot analysis of Spx protein stability in cells of cultures treated with diamide and chloramphenicol.

The wild-type strain JH642, which was grown in TSS media until OD600=0.3, was treated with or without 1 mM diamide, and after 10 min, the culture was split into two sub-cultures of equal volume. 0.1 mg/ml chloramphenicol was added one of the sub-cultures. Samples were taken at 10, 30, 60 90 min after diamide treatment. Cells were harvested by centrifugation and lysed by the protoplast method. The protein extracts were applied to an SDS-polyacrylamide gel for electrophoresis and then blotted for western analysis using anti-Spx antiserum. Sample at the lane 1 from ORB3834 (*spx*::*neo*) strain was taken at OD600=0.5 from TSS media. The lower panel is a plot of Spx band intensity versus time. The Spx amount at 10 min without diamide treatment is denoted as 1. Standard deviations on the plot are obtained from three independent experiments. “ch” represents 0.1 mg/ml chloramphenicol.

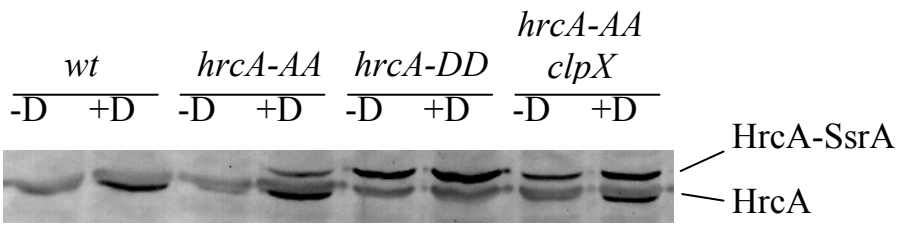


Figure 3.3 Western blot analysis of HrcA protein stability in cells of cultures treated with diamide.

Strains JH642 (*wild-type*), ORB4381 (*lacA*::*hrcA-ssrA* [HrcA-AA]), ORB4382 (*lacA*::*hrcA-ssrADD* [HrcA-DD]), ORB4383 (*lacA*::*hrcA-ssrA* [HrcA-AA], *clpX*::Spc), were grown in DSM media at 37°C with shaking. Samples were taken when OD600=0.5. and each culture was treated with 1 mM diamide. Samples were taken 30 min after diamide treatment. Cells were lysed with protoplast buffer and were suspended in lysis buffer (See Materials and Methods). Thirty μg protein from each sample was applied to an SDS polyacrylamide gel for electrophoresis. The protein levels of HrcA were examined by western blot analysis using anti-HrcA antibody.

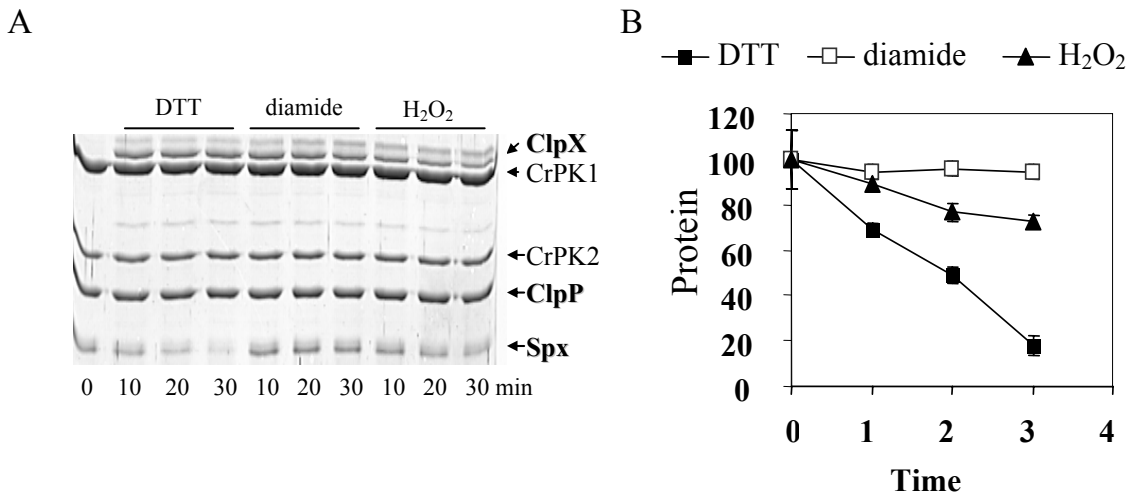


Figure 3.4 Effect of diamide and H_2O_2 on ClpXP-catalyzed proteolysis of Spx *in vitro*.

(A) Spx (6 μM), ClpX (6 μM) and ClpP (12 μM) were incubated at 37°C in the presence of ATP and an ATP-generating system (creatine kinase) with 5 mM DTT, diamide or H_2O_2 in a proteolysis reaction buffer containing 50 mM HEPES/KOH (pH 7.6), 50 mM KCl, 10 mM Mg acetate as described in Materials and Methods. 10 μl Samples were taken at 0, 10, 20, and 30 min, and the reactions were stopped by mixing with 2 μl SDS-loading dye containing 0.1 M DTT. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by staining with Coomassie blue. CrPK, creatine phosphate kinase 0.05 U/μl, 5 mM ATP and 5 mM creatine phosphate were used as an ATP-regenerating system.

(B) Plot of Spx band intensities derived from three repeats of the experiment against time of reaction. The intensities of ClpP protein in each reaction were used as internal controls. The Spx/ClpP ratio in the reaction without ClpX was referred as 100%.

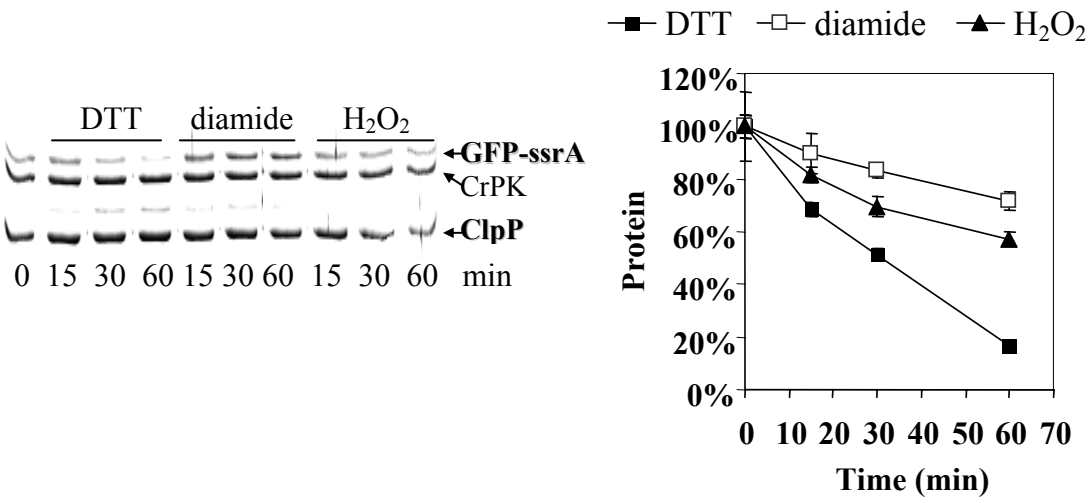


Figure 3.5 Effect of diamide and H_2O_2 on ClpXP-catalyzed proteolysis of GFP-SsrA *in vitro*

GFP-SsrA (6 μM), ClpX (6 μM) and ClpP (12 μM) were incubated at 37°C in the presence of ATP and an ATP-regenerating system with 5 mM DTT, diamide or H_2O_2 as described in Material and Methods. 10 μl Samples were taken at 0, 15, 30, and 60 min time points. Plot of GFP-SsrA/ClpP band intensity ratios against time of reaction was derived from triplicate experiments. The intensities of ClpP protein in each reaction were used as internal control. The GFP-SsrA/ClpP ratio in the reaction without ClpX was referred as 100%.

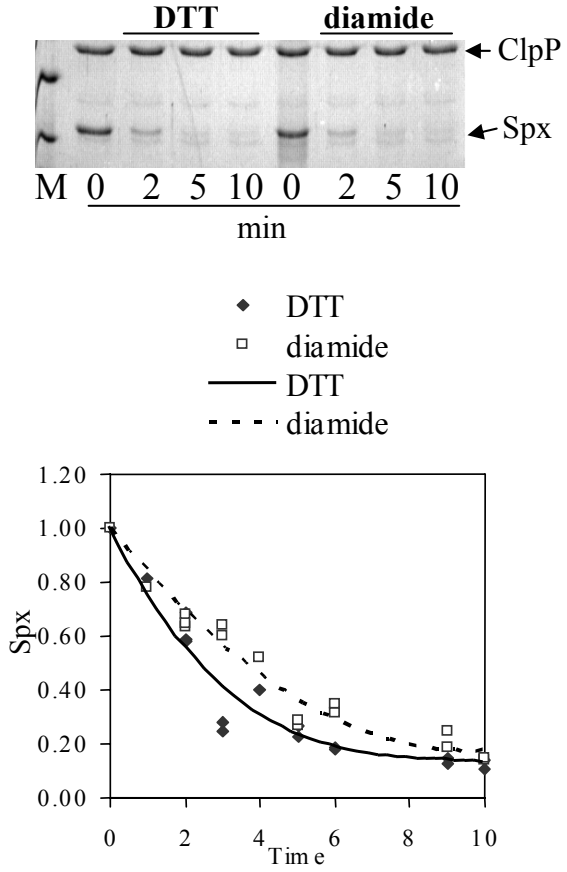


Figure 3.6 Effect of diamide and H_2O_2 on ClpCP proteolysis of Spx *in vitro*

Spx (8 μM), ClpC (2.5 μM), ClpP (4 μM), and MecA (2.5 μM) were incubated at 37°C in the presence of ATP and an ATP-generating system with 5 mM DTT, diamide or H_2O_2 as described in Materials and Methods. Samples (10 μl) were taken at 0, 2, 5, 10 min time intervals. The lower panel is a plot of Spx/ClpP band intensity ratios versus time were derived from triplicate experiments. The intensities of ClpP protein in each reaction were used as an internal control. The Spx/ClpP ratio in the reaction without ClpX was referred as 100%.

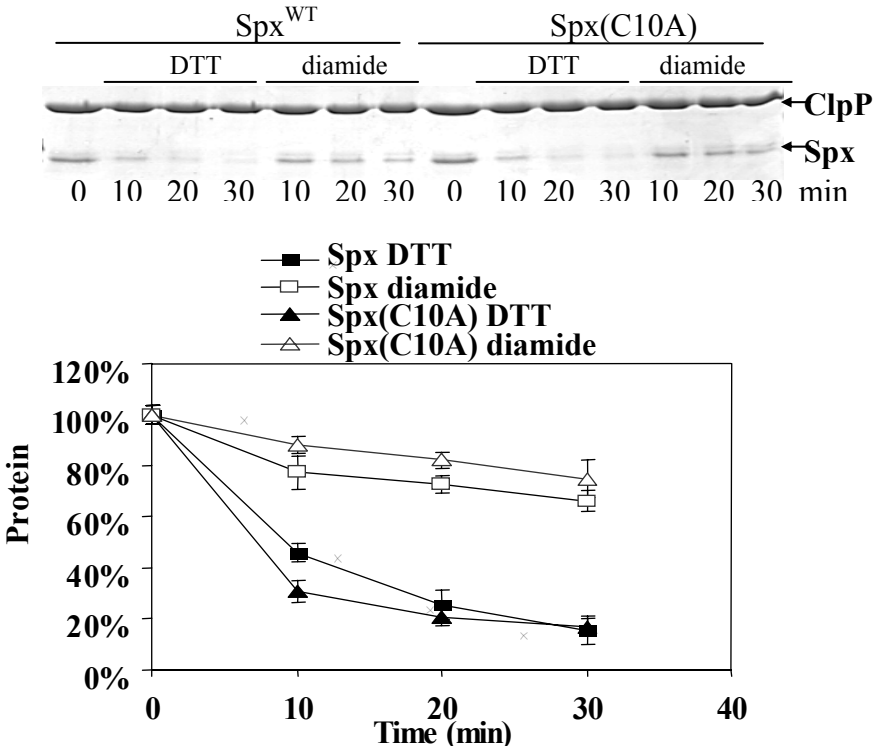


Figure 3.7 Effect of diamide on ClpXP proteolysis of wild-type Spx and C10A Spx *in vitro*

Wild-type Spx or C10A Spx (6 μM), 6 μM ClpX and 12 μM ClpP were incubated at 37°C in the presence of ATP and an ATP-generating system with 5 mM DTT or diamide in a proteolysis reaction buffer described in Materials and Methods. Samples (10 μl) were taken at 0, 10, 20, and 30 min time points. Plot of Spx/ClpP band intensity ratios against time of reaction was derived from triplicate experiments. The intensities of ClpP protein in each reaction were used as an internal control. The Spx/ClpP ratio in the reaction without ClpX was referred as 100%.

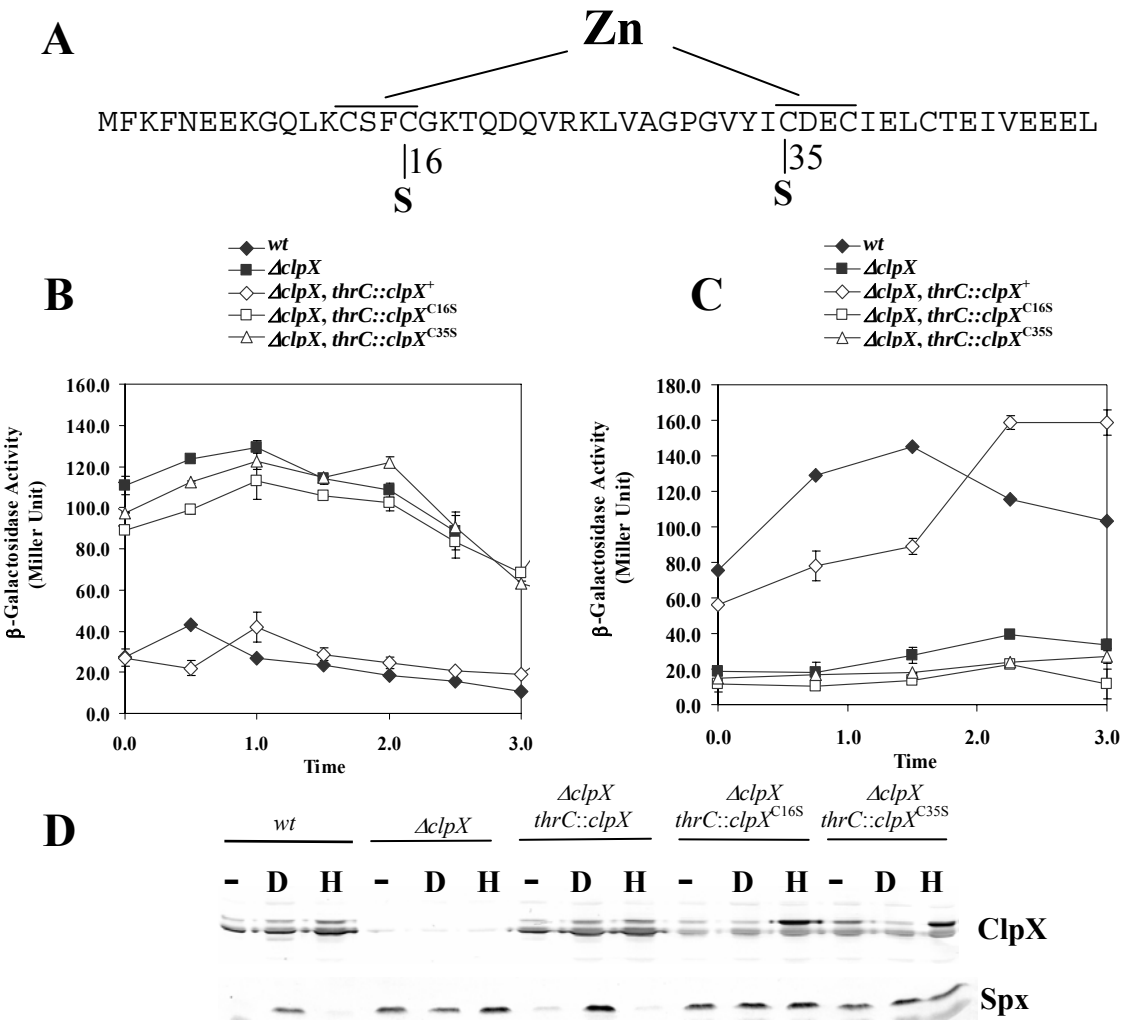


Figure 3.8. Effect of ZBD mutations of *clpX* on Spx-dependent regulation of *srf* and *trxB* transcription

(A) Diagram of C4-type Zinc-binding domain sequence showing the CXXC and CXXCXXXC motifs. Also shown are the Cys16 and Cys35 positions that were changed to Ser.

(B) Measurement of β-galactosidase activity in a time course experiment of cultures of *trxB-lacZ* bearing cells in either wild-type (♦), Δ*clpX* (■), Δ*clpX*, *thrC*::*clpX*$^{+}$ (◊), Δ*clpX*, *thrC*::*clpX*^{C16S} (□), or Δ*clpX*, *thrC*::*clpX*^{C35S} (Δ) background. Data were from three independent experiments.

(C) Measurement of β-galactosidase activity in a time course experiment of cultures of *srf-lacZ* bearing cells in either wild-type (♦), Δ*clpX* (■), Δ*clpX*, *thrC*::*clpX*$^{+}$ (◊),

Δ*clpX*, *thrC*::*clpX*^{C16S} (□), or Δ*clpX*, *thrC*::*clpX*^{C35S} (Δ) backgrounds. Data shown were from three independent experiments.

(D) Examination of ClpX and Spx protein levels in JH642 (wild-type), LAB2876 (Δ*clpX*), ORB6624 (Δ*clpX*, *thrC*::*clpX*$^{+}$), ORB6648 (Δ*clpX*, *thrC*::*clpX*^{C16S}), or ORB6649 (Δ*clpX*, *thrC*::*clpX*^{C35S}) by western blot analysis. Cultures were grown in DSM media until mid-exponential phase were treated with or without 1 mM diamide or H_2O_2. Samples were collected after 30 min and were suspended in protoplast buffer followed by resuspension in lysis buffer (Materials and Methods). Protein (30 μg) from each sample was applied to SDS polyacrylamide gel for electrophoresis. The levels of Spx or ClpX protein were determined by western blot analysis using rabbit anti-Spx or anti-ClpX antiserum.

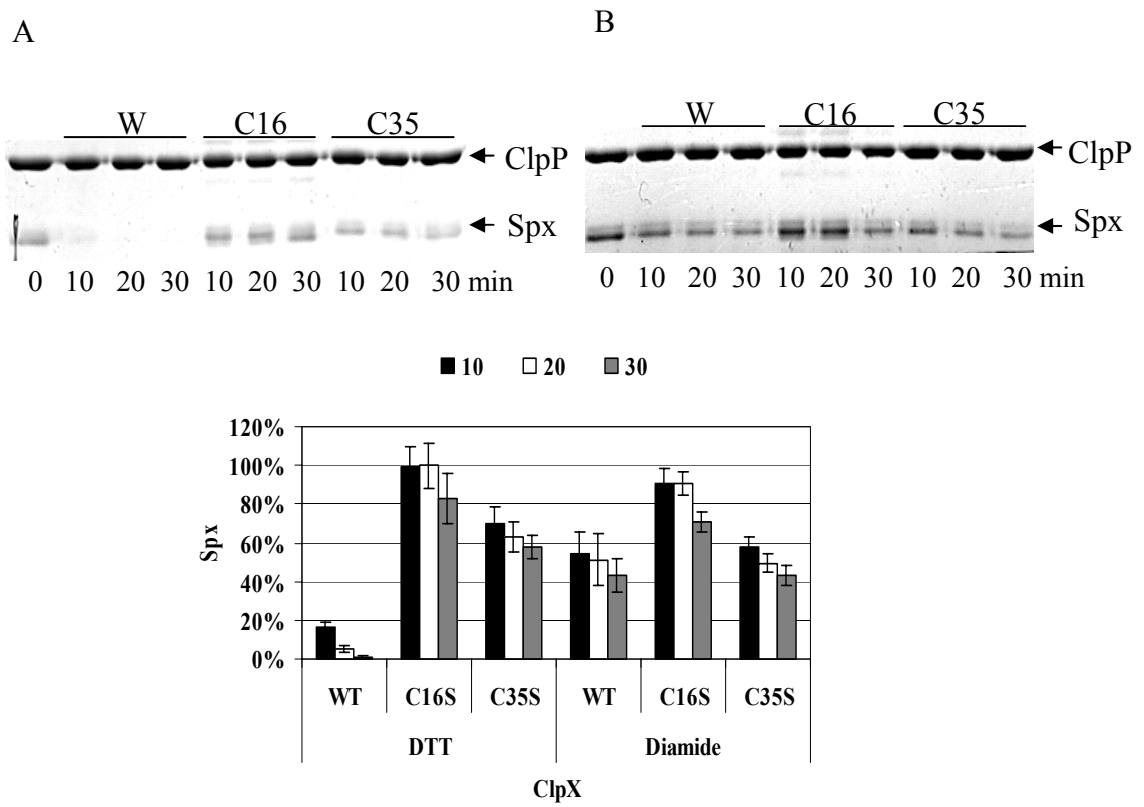


Figure 3.9. Effect of ZBD mutations of *clpX* on ClpXP-catalyzed proteolysis of Spx *in vitro*

A) And **B**) Spx (6 μM), ClpX (6 μM, wild-type, C16S, or C35S) and ClpP (12 μM) were incubated at 37°C in the presence of ATP 5 mM DTT (Figure 9A) or 5 mM diamide (Figure 9B) in proteolysis reaction buffer as described in Materials and Methods. Samples (10 μl) were collected at 0, 10, 20 30 min and the reactions were stopped by mixing with 2 μl SDS-Loading dye containing 0.1 M DTT. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by staining with Coomassie blue. CrPK, creatine phosphate kinase 0.05 U/μl, 5 mM ATP and 5 mM creatine phosphate were used as an ATP-regenerating system. **C**) Plot of Spx band intensities against time derived from triplicate experiments. Values of Spx levels determined as in Figs. 4-7.

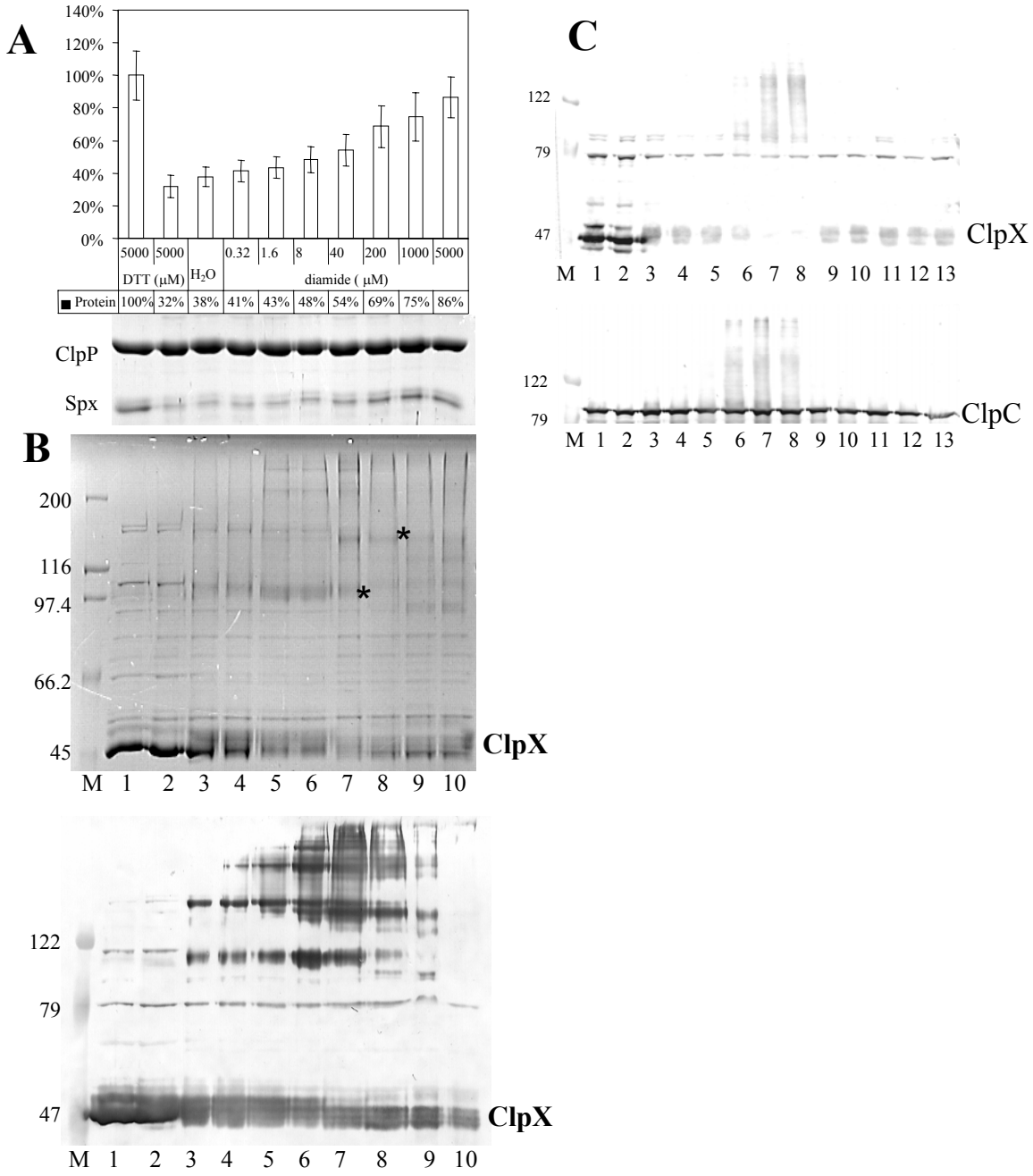


Figure 3.10. Diamide dose-dependent inhibition of ClpXP proteolysis of Spx *in vitro*

(A) Spx (6 μM), ClpX (6 μM) and ClpP (12 μM) were incubated at 37°C in the presence of ATP and an ATP-generating system with 5 mM DTT or varying concentrations of diamide in 10 μl proteolysis reaction buffer as described in Materials and Methods. 2 μl SDS-Loading dye containing 0.1 M DTT was mixed

with reaction after 10 min. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by staining with Coomassie blue. Plot of Spx band intensities against time of reaction was derived from triplicate experiments and values determined as in Figs. 3.4-3.7.

(B) ClpX protein (10 μl 6 μM) treated with DTT or varying concentration of diamide (lane 1 and 2: 5 mM DTT, lane 3: H_2O, lane 4 to 10: 0.32, 1.6, 8, 40, 200, 1000, 5000 μM diamide) was applied to non-reducing SDS polyacrylamide gels. The 5 mM DTT treated sample (lane 1) was mixed with SDS loading dye containing 0.1 M DTT. Samples in lane 2 to 10 were mixed with SDS loading dye without DTT. Samples were heated at 90°C for 2 min before loading. The 8% SDS poly-acrylamide gel was stained with Coomassie blue. Lower panel: The ClpX protein of Figure 10B up panel was detected by Western-blot analysis of the SDS polyacrylamide gel with anti-ClpX antiserum.

(C) Western blot analysis of ClpX and ClpC in wild-type cells treated with oxidants. Cells of strain JH642 (wild-type) were grown in DSM medium until mid-exponential phase, then treated with varying concentration of DTT, diamide, or H_2O_2 (lane 1 and 2: 5 mM DTT; lane 3: H_2O; lane 4 to 8: 0.5, 5, 50, 500, 5000 μM diamide; lane 9 to 13: 0.5, 5, 50, 500, 5000 μM H_2O_2). Cells were lysed with protoplast buffer, followed by lysis buffer as described in Materials and Methods. Protein (30 μg) was applied to non-reducing SDS polyacrylamide gels. The sample in lane 1 was treated with 5 mM DTT and mixed with SDS loading dye containing 0.1 M DTT. All the samples applied to lanes 2 to 13 were mixed with SDS loading dye without DTT. Each sample was heated at 90°C for 2 min before loading. The ClpX and ClpC protein on an 8% SDS polyacrylamide gel was detected by Western-blot analysis with rabbit anti-ClpX or anti-ClpC antiserum.

CHAPTER 4 YJBH AFFECTS THE CONCENTRATION OF SPX IN *BACILLUS SUBTILIS*

4.1 INTRODUCTION

In the spore-forming bacterium, *Bacillus subtilis*, the protein Spx is the global transcriptional regulator that exerts both positive and negative control on multiple genes during thiol-specific oxidative stress (Nakano *et al.*, 2003a). Such stress can be caused by treatment with diamide [diazenedicarboxylic acid bis(*N*,*N*-dimethylamide)] that can oxidize intracellular cysteines, causing inappropriate disulfide bond formation. Hence, thiol-specific oxidative stress is also called disulfide stress. Spx activity and concentration are elevated by disulfide stress, which leads to Spx-dependent activation of *trxA* (encoding thioredoxin) and *trxB* (thioredoxin reductase), whose products function in alleviating disulfide stress (Nakano *et al.*, 2003a). Diamide-induced Spx-dependent activation of *trxA* and *trxB* is not observed in the *spx* null or *rpoA*^{Y263C} mutant strains, indicating that the activation by Spx at the promoters of *trxA* and *trxB* requires interaction between Spx and the C-terminal domain of the RNA polymerase α subunit (RNAP αCTD) (Nakano *et al.*, 2005). During disulfide stress, Spx also inhibits transcription of the *srf* operon, which encodes an essential competence regulatory gene (D'Souza *et al.*, 1994; Hamoen *et al.*, 1995). Spx blocks the interaction between activator ComA and RNAP αCTD at the *srfA* promoter which required for initiation of *srf* transcription (Nakano *et al.*, 2003a; Nakano *et al.*, 2003b; Zhang *et al.*, 2006).

The *spx* gene was first identified as one of suppressor loci of *clpP* and *clpX* mutations (Nakano *et al.*, 2001). ClpX belongs to the AAA+ family (for ATPases associated with a variety of cellular activities) Clp/Hsp100 family of proteins and forms a hexametric, ring-shaped complex. ClpX is the ATPase subunit of the multicomponent ClpXP protease, where it functions as an unfoldase and translocase that recognizes certain stable protein substrates or denature protein. ClpX unfolds substrate proteins and

then translocates them to the proteolytic chamber composed of 14 ClpP subunits (Sauer *et al.*, 2004). In wild-type cells under normal growth conditions Spx is present at nearly undetectable levels because of its degradation by ClpXP (Nakano *et al.*, 2003b). The *clpP* and *clpX* mutants are defective in genetic competence, sporulation and growth in minimal media, defects that can be partially bypassed by suppresser mutations in *spx* or the αCTD-encoding part of *rpoA*. Because of the deleterious effects of high Spx concentrations, ClpXP-catalyzed Spx protein turnover during normal growth conditions is an important cellular process.

ClpX and ClpP orthologs are found in bacteria, mitochondria, and chloroplasts (Adam *et al.*, 2001). ClpXP protease is important for the quality and quantity control of protein during the cell's responses to stress and as part of developmental programs. Intensive studies have focused on *E coli* ClpX structure, its mechanism of action and the nature of its protein substrates. Through proteomic studies using a proteolytically deficient mutant ClpP to trap substrates, five distinct degradation signals recognized by ClpX were identified, including three sequence motifs at the N termini of natural substrates and two sequence motifs found at the C termini (Flynn *et al.*, 2003). The C-terminal residues of MuA transposase, the N-terminal residues of the lambda phage O protein are known recognition signals for ClpXP degradation (Gonciarz-Swiatek *et al.*, 1999; Levchenko *et al.*, 1997). In *Bacillus subtilis* the C-terminal residues LAN of Spx are required for its degradation by ClpXP (Nakano *et al.*, 2002b; Nakano *et al.*, 2003a; Nakano *et al.*, 2003b). This sequence shows similarity to the SsrA-tag (AANDENYALAA) protein which is another substrate recognition motif for ClpXP in both *E coli* and *B. subtilis* (Nakano *et al.*, 2002b; Nakano *et al.*, 2003a; Nakano *et al.*, 2003b). Except for the direct sequence recognition by ClpX, some substrates require an additional adaptor protein to tether substrates to the ATP-dependent unfoldase. The response regulator RssB in *E coli* can act like an anti-σ factor by recognizing the stationary phase sigma factor σ^S and delivering to ClpXP for degradation (Becker *et al.*, 2000). In exponentially growing cultures the RssB is kept in an active form to quickly facilitate the turn over of σ^S. (Zhou & Gottesman, 1998). *In vitro* ClpXP and ClpAP can both degrade SsrA-tagged proteins, but *in vivo* ClpXP is responsible for the degradation of the majority misfolded proteins or truncated products tagged with the SsrA peptide.

ClpXP-catalyzed degradation of SsrA-tagged products is enhanced by an adaptor the ribosome-associated protein SspB which can specifically recognize SsrA-tagged proteins and deliver them to ClpXP rather than ClpAP (Levchenko *et al.*, 2000).

Clp proteases can recognize target protein through broad range of target signals and with the aid of different adaptor proteins, which can explain the involvement of this ATP-dependent protease in responses to different environmental and metabolic changes (Sauer *et al.*, 2004). In *B. subtilis* the only identified molecular chaperone adaptor proteins for ATP-dependent proteases is MecA and YpbH. The *mecA* gene is located in the vicinity of *spx* in the *B subtilis* genome (Fig. 4.1A). MecA targets ComK, the competence regulator in exponential phase cells to facilitate its turnover by ClpCP, and in response to environmental changes such as high cell density. MecA binds to the small peptide ComS (encoded by the *srf* operon), the production of which is activated by the ComPA two-component signal transduction system upon receiving high cell density signals through the extracellular signaling peptides ComX and CSF (Lazazzera & Grossman, 1998). ComS peptide release ComK from the ClpCP/MecA proteolytic complex (Turgay *et al.*, 1998). ComK positively autoregulates the *comK* gene and activates the transcription of late competence genes (Dubnau, 1999).

Aside from SsrA-tagged proteins (Wiegert & Schumann, 2001), only a few protein targets of ClpXP have been identified in *B. subtilis*. Recently, the 52 residue long Sda peptide, which blocks sporulation in response to defects in replication initiation in *B. subtilis* (Burkholder *et al.*, 2001), was found to be a substrate for ClpXP (Ruvolo *et al.*, 2006). ClpXP is also required for activation of the SigW regulon in *B. subtilis* by catalyzing the complete cleavage of the anti-sigma protein RsiW (Zellmeier *et al.*, 2006), as part of the cell's envelope stress response. ClpX also inhibits the assembly of the tubulin-like cytoskeletal protein FtsZ which is required for cell division in a ClpP-independent manner in both *E. coli* and *B. subtilis* (Weart *et al.*, 2005). So far, there have been no adaptor proteins similar to SspB, reported that function in ClpXP substrate recognition in *B. subtilis*; however recent study suggested that YjbH is likely an adaptor protein for ClpXP-dependent Spx proteolysis (Larsson *et al.*, 2007).

In *B. subtilis*, the *yjbH* gene is co-transcribed with the upstream *yjbI* gene from the *yjbI* promoter (Rogstam *et al.*, 2007), and transcription is elevated in a strain

producing a protease-resistant form of Spx (Nakano *et al.*, 2003a). The *yjbHI* operon is located in the vicinity of *spx* in several Gram-positive genomes (Fig. 4.1). The *yjbI* gene encodes truncated hemoglobin (YjbI), and together with the flavohemoglobin Hmp to functions in the detoxification of reactive nitrogen species. (Choudhary *et al.*, 2005; Rogstam *et al.*, 2007). As part of *yjbIH* operon *yjbH* encodes a predicted 34 kDa cytosolic protein which might have an important role in the control of Spx in response to oxidative stress (Larsson *et al.*, 2007). YjbH contains seven cysteine residues including a highly conserved CXXC motif at the N terminal that might be involved in redox control.

In this chapter, we show that in *Bacillus subtilis* YjbH negatively controls Spx concentration and Spx-dependent transcriptional control. This negative effect could not be bypassed when Spx is under the control of an IPTG-inducible promoter. YjbH-dependent negative control is modulated in the presence of diamide. YjbH does not affect the concentration of another ClpXP substrate such as SsrA-tagged HrcA, suggesting that it might be specific for Spx. A mutation that changes the first cysteine residue of YjbH CXXC motif at the N terminus to alanine does not affect Spx-dependent transcriptional control and the control of Spx concentration in untreated and diamide-treated cells. Finally YjbH is proposed to post-transtranslationally modulate Spx level in *B. subtilis.*

4.2 RESULTS

4.2.1 Spx-dependent transcriptional control is enhanced in the absence of *yjbH*

In *B. subtilis*, Spx is a global transcriptional regulator that is active and abundant in cells suffering from disulfide stress. The *trxB* gene encoding thioredoxin reductase, which is activated by Spx (Nakano *et al.*, 2003a; Nakano *et al.*, 2005), was highly induced in both *yjbH*::*tet*^{F} and *yjbH*::*tet*^{R} insertion mutants (forward and reverse orientation of a Tetracycline-resistance gene cassette, respectively) (Fig. 4.2A and B). Spx negatively controls expression of the *srf* operon by interfering with ComA-dependent transcriptional activation of the *srf* promoter (Nakano *et al.*, 2003a; Nakano *et al.*, 2003b; Zhang *et al.*, 2006). The transcription of *srfA* was repressed in the *yjbH* insertion mutant strains (Fig. 4.2C and D). To determine if *yjbH* causes activation of *trxB* transcription and

repression of *srfA* through the regulation of Spx, the level of Spx protein in wild-type and *yjbH* mutant cells was examined.

4.2.2 Spx accumulated in the *yjbH* mutant strain.

In normal growing cells, Spx concentration is kept at a low level due to transcriptional repression exerted by PerR and YodB (Leelakriangsak *et al.*, 2007) and post-translational, proteolytic control by ClpXP. High levels of Spx accumulate in *clpX* and *clpP* mutant cells and the ATP-dependent protease ClpXP degrades Spx *in vivo* and *in vitro* (Nakano *et al.*, 2001; Nakano *et al.*, 2003a; Nakano *et al.*, 2003b). The *in vivo* effect of *yjbH* was examined to determine its contribution to the control of Spx concentration. *B. subtilis* cells of wild-type JH642 and the *yjbH* insertion mutants were grown in DSM medium until mid-log phase. One culture was treated with diamide and the other was left untreated for 30 min. Cell extracts were obtained by lysozyme treatment in protoplast buffer and were applied to an SDS-polyacrylamide gel for electrophoresis. Western blot analysis was performed using anti-Spx antiserum. Spx protein could be detected by western blot in cells of both *yjbH* strains but not wild-type cells (Fig. 4.2, lanes 1 and 3). This indicates that *in vivo*, *yjbH* mutation can cause up-regulation of Spx concentration.

4.2.3 YjbH controls Spx at the post-transcriptional level.

To determine which stage of *spx* expression was affected by YjbH, the *spx* gene promoter was replaced by an IPTG-inducible Phyperspank promoter to eliminate PerR/YodB negative transcriptional control of *spx*. The *trxB* promoter was fused with *lacZ* report gene, and the resulting construct was inserted at the *thrC* locus. The expression of *trxB*-*lacZ* was up-regulated when Spx was induced by IPTG (Fig. 4.4), but much higher activity was observed in *yjbH* mutation cells, indicating that YjbH exerts negative control on Spx at the post-transcriptional level.

4.2.4 An IPTG-inducible YjbH could complement loss of *yjbH*-dependent negative control of Spx.

The IPTG-inducible alleles encoding the wild YjbH proteins were introduced into the *amyE* locus of the *yjbH* insertion mutant, bearing either a *trxB-lacZ* fusion or a *srfA-lacZ* fusion, to determine if the alleles could complement *yjbH* with respect to Spx activity. The induction of an IPTG-controlled wild-type allele of *yjbH* resulted in reduced *trxB-lacZ* expression and increased *srfA-lacZ* relative to that of the *yjbH* mutant and non-induced control group and the promoter activities of *srfA* and *trxB* showed the same level of activity as observe in the wild-type background (Fig. 4.2 A, B, C, D). Furthermore, Spx protein concentration was reduced when the expression of the complementing allele of *yjbH* was induced (Fig. 4.3, lanes 1, 3, 5 and 7). The induction of the wild-type allele of *yjbH* from an ectopic position (the *amyE* locus) within the *yjbH* mutant genome can complement the loss of YjbH-dependent negative control of Spx.

4.2.5 Diamide abolishes negative control of YjbH on Spx.

The thiol-specific oxidant diamide causes high level Spx to accumulate to high levels [result in Fig. 4.3, lanes 1 and 2 (Nakano *et al.*, 2003a)] due to derepression of *spx* transcription from PerR and YodB (Leelakriangsak *et al.*, 2007) and decreased ClpXP proteolytic control. Microarray analysis showed that *yjbH* expression is induced 5-fold upon diamide treatment (Leichert *et al.*, 2003) and that induction of an *spx* allele encoding a protease-resistant form of Spx caused induction of *yjbIH* operon (Nakano *et al.*, 2003a). Upon diamide treatment though *yjbH* expression is increased, it failed to down-regulate Spx in wild-type cell (Fig. 4.3, lanes 1 and 2). When YjbH was overexpressed through an IPTG-controlled promoter, the negative effect on Spx was still abolished by diamide treatment (Fig. 4.3, lanes 7 and 8). This result indicated diamide-induced accumulation of Spx is not due to transcriptional regulation of *yjbH*.

4.2.6 Amino acid substitutions in the CXXC motif of YjbH do not significantly affect the negative control of Spx by YjbH.

The CXXC motif of YjbH is a likely target for oxidant-dependent inactivation of its negative control of Spx. To determine the role of the CXXC motif, an amino acid

substitution in the CXXC motif was generated by *in vitro* PCR mutagenesis and the product of the resulting allele was tested for activity *in vivo*. A cysteine to alanine substitution was created at position 31 of the first Cys of the YjbH CXXC motif. The IPTG-inducible alleles encoding the mutant YjbH proteins were introduced into the *amyE* locus of the *yjbH* insertion mutant, bearing either a *trxB-lacZ* fusion or a *srfA-lacZ* fusion, to determine if the alleles could complement *yjbH* with respect to Spx activity. The expression of the C31A allele of *yjbH* in the *yjbH*, *trxB-lacZ* strain resulted in reduced levels of expression, similar to that observed in the wild-type complemented strain (Fig. 4.2 A and B), indicating Cys31 of YjbH is not necessary for negative control of Spx. The *srf-lacZ* fusion expression which was repressed in both *yjbH* insertion mutants can be complemented by IPTG-induced wild-type or C31A mutant alleles of the *yjbH* gene, as shown by the increase in *srf-lacZ* expression (Fig. 4.2 C and D). The induction of C31A alleles of *yjbH* from the *amyE* locus within the *yjbH* mutant genome can increase *srf-lacZ* expression, due to the reduced Spx concentration. These results were confirmed by the western blot analysis of the *yjbH* insertion mutant strain bearing either C31A mutant inducible YjbH in Fig. 4.3, lanes 9 and 11.

The CXXC motif is sometimes involved in redox control. Western analysis also indicated accumulation of Spx upon diamide treatment is not affected by C31A mutant YjbH (Fig. 4.3, lane 12). So C31 of CXXC motif of YjbH might not be involved in the sensing of disulfide stress.

4.2.7 YjbH is not involved in negative control of other ClpXP substrate.

Besides Spx, the SsrA-tagged derivative of HrcA [HrcA-ssrA(AA)] is another substrate for ClpXP *in vivo* (Wiegert & Schumann, 2001). It is encoded by the *hrcA-ssrA* (AA) allele that is transcribed from a constitutively active promoter of the *dnaK* gene. Western-blot analysis showed that HrcA-ssrA(AA) (the high molecular weight band) is only detectable in *clpX* mutant cells [Fig. 4.5, lanes 3 and 7 and (Wiegert & Schumann, 2001)], but not in *yjbH*::*tet*^{F} and *yjbH*::*tet*^{R} strains (Fig. 4.5, lanes 9 and 11) before diamide treatment. HrcA-ssrA(DD) is resistant to proteolysis by ClpXP, and is present in high levels in untreated and diamide-treated cells (Fig. 4.5, lane 5). Diamide induces

endogenous *hrcA* expression (the low molecular weight band) from its own promoter and (Fig. 4.5, lanes 2, 4, 8 and 12).

4.3 DISCUSSION

In *Bacillus subtilis*, the global transcriptional regulator, Spx, is controlled at both transcriptional and post-translational levels. This control maintains the concentration of Spx protein at an undetectable level. At the transcriptional level, the *spx* gene expression is negatively controlled by YodB and PerR at the *spx* promoter (Lee & Helmann, 2006) which could be removed upon oxidative stress (Leelakriangsak *et al.*, 2007; Leelakriangsak & Zuber, 2007). The second level is achieved by the constitutive post-translational proteolytic control of Spx by ClpXP which is down-regulated in cells undergoing disulfide stress (Nakano *et al.*, 2003a) due to the aggregation of high-cysteine containing ATPase ClpX and releasing of Zn from its N-terminus Cys-4 Zinc-binding domain (ZBD) [(Zhang & Zuber, 7 September . 2007), Chapter 3]. Besides Spx, in *B. subtilis* the substrates of ClpXP also include SsrA-tagged proteins (Ruvolo *et al.*, 2006; Wiegert & Schumann, 2001) and RsiW (Zellmeier *et al.*, 2006). The recently discovered YjbH protein might play a role in ClpXP-dependent proteolytic control of Spx.

The 34 kDa protein YjbH encode by the *yjbIH* operon (Rogstam *et al.*, 2007) is suggested to play a role in the negative regulation of Spx in response to oxidative stress (Larsson *et al.*, 2007). Cells bearing the *yjbH* mutation display an apparent down-regulation of *srf* transcription and up-regulation of *trxB* transcription (Fig. 4.2 A-D), both of which belong to the Spx regulon. This is suggestive of a change in Spx concentration and/or activity when YjbH is absent. Spx-dependent transcriptional control is enhanced in the *yjbH* mutant strains, which can be explained by detection of Spx protein accumulation in the mutants (Fig. 4.3). Although we eliminated the transcriptional control region of Spx through its own promoter and place it under control by IPTG-inducible promoter, we still observed enhanced Spx-dependent activation of *trxB* transcription in the *yjbH* mutants. Thus, it is concluded that the YjbH protein functions to control Spx at the post-transcriptional level.

Residue 21 to 163 of YjbH is homologous to the DsbA_FrnE_like subfamily, with about 80% identity between them. DsbA functions to catalyze disulfide formation to accelerate folding of the periplasmic protein such as PhoA (Wunderlich *et al.*, 1995). Other members of DsbA_FrnE_like family include DsbC and DsbG that function as protein disulfide isomerases that serve to correct non-native disulfide bonds formed by DsbA and prevent aggregation of incorrectly folded proteins (Bessette *et al.*, 1999). Similarities to these proteins suggest that YjbH might function at the post-translational level to impart protein conformation changes that might destabilize Spx to provoke or facilitate the proteolytic elimination of Spx via ATP-dependent protease ClpXP.

Expression of *yjbH* is induced 5-folds upon diamide treatment in microarray analysis (Leichert *et al.*, 2003). Overexpression of YjbH from an ectopic *amyE* locus could not prevent Spx accumulation in the cell upon diamide treatment (Fig. 4.3), which might be partially due to loss of negative regulation by YjbH in response to diamide. These results also suggest that YjbH functions differently in normal reduced growth condition and oxidized stress response condition. Serveral hypothesis are raised here. YjbH might function as a disulfide isomerase to keep Spx in the stable active form during oxidative stress through its CXXC redox center, while in the reduced state it could function to destabilize Spx and tether it to ClpXP. Another possibility YjbH just loses the adaptor functions upon diamide treatment. In Chapter 3 we suggest that Zinc release and aggregation of ClpX is partly the reason of down-regulation of ClpXP protease activity upon diamide treatment. To further study that diamide either directly or indirectly affects YjbH function; we can exam the protein status of YjbH upon diamide treatment. We could not exclude the possibility YjbH is also a metal binding protein with its CXXC motif and histidine rich N-terminal domain. We have no evidence that there is direct contact between YjbH and Spx or YjbH and ClpX. *In vivo* yeast two hybrid experiment and *in vitro* protein pull down experiment need to be employed to test their interaction.

YjbH contains the highly conserved redox active CXXC sequence motif of the thioredoxin superfamily at the N-terminus. The CXXC motif is proposed to be the target site for redox control and active site of YjbH. The first Cys of the CXXC motif in YjbH, according to studies of DsbA, should be a redox active site (Wunderlich *et al.*, 1995). The enhanced repression of the *srf* promoter and activation of the *trxB* promoter due to up-

regulation of Spx caused by the *yjbH* insertion mutants could be complemented by expression of either wild-type or the C31A allele of *yjbH* (Fig. 4.2 and Fig. 4.3). The alanine substitution of the first cysteine residue, which might expose to the surface according to the crystal structure of *E. coli* DsbA (Martin *et al.*, 1993), did not abolish its negative control of Spx *in vivo*. Since YjbH contains seven cysteine residues (C13, C31XXC33, C89, C175, C236, C297), it is possible that other cysteines rather than the CXXC is required for its redox control. Amino sequence alignment shows C13 at the N-terminal and C236, C297 at the C-terminal are only found in *B. subtilis* YjbH. C89, C175 conserved only in *B. subtilis*, *Bacillus licheniformis*, *Geobacillus kaustophilus*, *Bacillus sp.* NRRL B-14911, *Oceanobacillus iheyensis*, *Bacillus clausii KSM-K16*, and *Bacillus halodurans.* Residues other than cysteine such as the Pro 32 residue in CXXC motif and Pro 188, are also highly conserved in all organisms and according to the crystal structure of *E. coli* DsbA (Martin *et al.*, 1993). The Pro 188 is near the CXXC motif and exposed at the surface of the protein. Alanine substitution of this cis-proline of DsbA destabilizes the protein and induces a significant conformational change at the active site, which is due to the loss of ver de Waals interaction between the proline containing loop and disulfide bond (Charbonnier *et al.*, 1999). The rearrangement of the active site might affect the redox control.

Interestingly, Fig. 4.2E showed that both *yjbH* insertion mutants display a lag phase during growth in DSM media, and the *yjbH*::*tet*^{R} showed a more severely defective growth phenotype compared with *yjbH*::*tet*^{F}. The ectopically expressed wild-type *yjbH* could partially alleviate the growth defect in *yjbH*::*tet*^{F} strain but not the *yjbH*::*tet*^{R} strain. The *yjbH*$^{\mathbf{C31A}}$ could not complement both insertion mutants with respect to the poor growth phenotype. Addition of IPTG did not show significant differences, which is likely explained by leaky expression of yjbH from the Phyperspank promoter. In the *E. coli* an unfoldable form of DsbA' fused with PhoA target to the periplasmic face of the inner membrane to block the export mechanism. This toxicity caused by the DsbA′-PhoA hybrid complex could be suppressed by efficient degradation by DegP protease (Guigueno *et al.*, 1997) or by a small UptR RNA which could release hybrid complex from the membrane (Guigueno *et al.*, 2001). The difference between forward and reverse mutation, might due to a putative small RNA sequence located at downstream of the

yjbIH operon. The reverse mutant might disrupt the synthesis of this small RNA, which could be involved in the post-transcriptional regulation of *yjbH* gene or regulation of YjbH-Spx interaction.

YjbH might exert negative control through direct interaction with Spx, and offering Spx to the ClpXP protease. Another possibility is that YjbH interacts directly with ClpXP and controlling which substrates are chosen for degradation. Fig. 4.5 showed degradation of SsrA-tagged HrcA by ClpXP is not affected by the absence of YjbH. So YjbH specifically target Spx for its negative control.

Structure alignment of YjbH (β1β2α1β3**α2α3α4α5α6α7**β4β5α8α9α10β6α11) to *E. coli* DsbA (β1β2α1α1′β3**α2α3α4α5α6**β4β5α7) reveals that YjbH contains the thioredoxin-like domain (the underline sequences) and more α-helical domains (the structures indicated by bold font) compared with DsbA, which is critical for stability of the protein (Hennecke *et al.*, 1999). Based on secondary structure predictions (Fig. 4.6), YjbH has an extended C-terminal with 2 long α helices α9 and α10 one β6 sheet followed a short α11 link, which is connected to the thioredoxin like domain by a proline-rich (P226, P228, P232, P233) loop conserved only in *Bacillus* species. Mutagenesis experiments have shown that DsbA is very robust towards amino acid substitutions and retains biological activity even if residues in or around the active-site disulfide bond are replaced (Wunderlich *et al.*, 1995). The N-terminus β1 sheet of YjbH is also largely different from DsbA, which contains a unique $H^{12}CHGHKKP^{20}$ sequence close to the C-terminal proline-rich loop, according to the crystal structure of *E. coli* DsbA (Guddat *et al.*, 1997; Martin *et al.*, 1993). The histidine residues in this unique sequence, which is also found in AraC transcriptional regulator in *Pseudomonas stutzeri*, and Threonyl-tRNA synthetase in *Hyperthermus butylicus* (Fig. 4.6), might involve in the metal binding. Dr. Saurabh Garg in our lab also observed that the translation of Spx is not affected by the *yjbH* insertion mutants.

So we proposed that YjbH specifically exerts negative control on Spx protein at the post-translational level and this negative regulation is abolished upon diamide treatment.

4.4 MATERIALS AND METHODS

4.4.1 Bacterial strains and growth conditions

Bacillus subtilis strains used in this study are derivatives of JH642 and are listed in Table 4.1. *B. subtilis* cells were cultured in a shaking water bath at 37°C in Difco Sporulation medium (DSM) (Schaeffer *et al.*, 1965) for β-galactosidase assays and diamide treatment experiments and genotype verification. Diamide was purchased from SIGMA.

4.4.2 Construction of insertion mutant of *yjbH*

According to (Rogstam *et al.*, 2007) an alternative start codon (TTG) proceeded by a putative ribosome-binding site (GGAGG) will encode additional 24 amino acid for YjbH protein compare with the prediction ((Kunst *et al.*, 1997). Primers oZY07-42 and oZY07-43 (Table 4.3) were used to amplify the *yjbH* gene from *B. subtilis* strain JH642 chromosomal DNA. The PCR fragment (from +62 to +936) was digested with *Sal*I, and then ligated with pUC19 that had been digested with the same enzyme, to generate pZY36. Plasmid pZY36 was cleaved with *Bgl*II before treatment with T4 DNA polymerase (New England BioLabs) to create blunt ends, and then further ligated with Tetracycline-resistance cassette from pDG1515 (Guerout-Fleury *et al.*, 1995) (cleaved with *Bam*HI, *Eco*RI fragment treated with T4 DNA polymerase to create blunt ends) to generate pZY38 (*yjbH*::*tet*^{F}) and pZY30 (*yjbH*::*tet*^{R}, F: forward, R: reverse). The plasmids were introduced by transformation into *B. subtilis* strain JH642 with selection for Tetracycline-resistance to obtain integrants bearing the Tetracycline-resistance cassette at position +486 of *yjbH* to generate ORB6952 and ORB.6953.

4.4.3 IPTG-induced expression of *yjbH*

Primers oZY07-51 and oZY07-43 (Table 4.3) were used to amplify the *yjbH* gene from *B. subtilis* strain JH642 chromosomal DNA. The PCR fragment (from -36 to +926, about 962 bp, including 897 bp of the coding region of *yjbH* as well as 36 bp of upstream sequence containing the ribosome binding site) was digested with *Sal*I and *Hin*dIII, then ligated with IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible pDR111

[Phyperspank (spank-hy) fusion vector (Guerout-Fleury *et al.*, 1996)], that had been digested with the same enzymes, to generate pZY41(pDR111 with *yjbH* allele). The *yjbH* sequence in plasmid pZY41 was verified by DNA sequencing.

The mutant *yjbH*^{C31A} allele was constructed by PCR-based site-directed mutagenesis. The first round of PCR was performed by using *B. subtilis* strain JH642 chromosome DNA as template with primers oZY07-51 and oZY07-50 for the upstream fragment of *yjbH*^{C31A}, and primer oZY07-44 and oZY07-43 for the downstream fragment of *yjbH*^{C31A}. The two resulting PCR fragments, purified on low-melting agarose gels, were mixed and used as templates for the second PCR with primers oZY07-51 and oZY07-43 to generate the full-length fragment (from -36 to +926, about 962 bp, including 897 bp of the coding region of *yjbH* as well as 36 bp of upstream sequence that includes the ribosomal binding site) bearing the desired mutant allele. The same procedure was used to insert the fragment into pDR111 to create pZY42 (pDR111with *yjbH*^{C31A} allele).

The plasmids pZY41 and pZY42 were introduced by transformation into JH642 to create the *amyE*::pZY41 (pDR111::*yjbH*) and *amyE*::pZY42 (pDR111::*yjbH*^{C31A}) - bearing strains ORB6991 and ORB6992. These strains were then transformed with DNA from the *yjbH*::*tet* null mutants, strain ORB6952 and ORB6953, yielding the mutant complementation strains ORB6997 (pDR111::*yjbH*, *yjbH*::*tet*^{F}), ORB6998 (pDR111::*yjbH*^{C31A}, *yjbH*::*tet*^{F}), ORB6999 (pDR111::*yjbH*, *yjbH*::*tet*^{R}), ORB7000 (pDR111::*yjbH*^{C31A}, *yjbH*::*tet*^{R}).

4.4.4 Transformation and transduction.

Isolation of *B. subtilis* chromosomal DNA, preparation of competent cells of *B. subtilis* and transformation of *B. subtilis* strains by chromosomal or plasmid DNA was performed as described previously (Dubnau & Davidoff-Abelson, 1971; Hoch *et al.*, 1967; Niaudet & Ehrlich, 1979). Specialized transduction using SPβ phage constructs was carried out as described previously (Zuber & Losick, 1987).

4.4.5 Assay of β-galactosidase activity

β-galactosidase activity was determined as previously described (Nakano *et al.*, 1988) and is presented as Miller units (Miller, 1972).

4.4.6 Western blot analysis

The total protein extracts were prepared from cells of *B. subtilis* cultures grown in DSM. Samples (1 ml) were taken at the indicated time points and centrifuged. Cells were then treated with 1 mg/ml lysozyme in protoplast buffer (20 mM K-phosphate pH 7.5; 15 mM $MgCl_2$; 20% sucrose) for 30 min and centrifuged. The protoplasts were then suspended in lysis buffer (30 mM Tris-HCl, 1 mM EDTA, pH 8.0). Total protein (30 µg) from each sample was applied to a 15% SDS-polyacrylamide gel and electrophoresis was performed. The protein level of Spx was examined by western blot analysis using anti-Spx (Nakano *et al.*, 2001) antiserum, followed by incubating with the secondary antibody conjugated to alkaline phosphatase.

Table 4.1 *Bacillus subtilis* strains

Strain	Relevant genotype or properties	Source and/or reference
ORB6952	*trpC2 pheA1 yjbH*::pZY38-5(*yjbH*::*tet*^{F})	This study
ORB6953	*trpC2 pheA1 yjbH*::pZY39-3(*yjbH*::*tet*^{R})	This study
ORB6991	*trpC2 pheA1 amyE*::pZY41-1 (pDR111::*yjbH*)	This study
ORB6992	*trpC2 pheA1 amyE*::pZY42-2 (pDR111::*yjbH*^{C31A})	This study
ORB6997	*trpC2 pheA1 amyE*::pZY41-1 (pDR111::*yjbH*) *yjbH*::*tet*^{F}	This study
ORB6998	*trpC2 pheA1 amyE*::pZY42-2 (pDR111::*yjbH*^{C31A}) *yjbH*::*tet*^{F}	This study
ORB6999	*trpC2 pheA1 amyE*::pZY41-1 (pDR111::*yjbH*) *yjbH*::*tet*^{R}	This study
ORB7000	*trpC2 pheA1 amyE*::pZY42-2 (pDR111::*yjbH*^{C31A}) *yjbH*::*tet*^{R}	This study
ORB3247	*trpC2 pheA srfA-lacZ* (pMMN84, Cm)	(Nakano *et al.*, 2000)
ORB6985	*trpC2 pheA1 yjbH*::pZY38-5(*yjbH*::*tet*^{F}) *srfA*::pMMN84(*srfA-lacZ*)	This study
ORB6986	*trpC2 pheA1 yjbH*::pZY39-3(*yjbH*::*tet*^{R}) *srfA*::pMMN84(*srfA-lacZ*)	This study
ORB7005	*trpC2 pheA1 amyE*::pZY41-1 (pDR111::*yjbH*) *yjbH*::*tet*^{F} *srfA*::pMMN84(*srfA-lacZ*)	This study
ORB7006	*trpC2 pheA1 amyE*::pZY42-2 (pDR111::*yjbH*^{C31A}) *yjbH*::*tet*^{F} *srfA*::pMMN84(*srfA-lacZ*)	This study
ORB7007	*trpC2 pheA1 amyE*::pZY41-1 (pDR111::*yjbH*) *yjbH*::*tet*^{R} *srfA*::pMMN84(*srfA-lacZ*)	This study
ORB7008	*trpC2 pheA1 amyE*::pZY42-2 (pDR111::*yjbH*^{C31A}) *yjbH*::*tet*^{R} *srfA*::pMMN84(*srfA-lacZ*)	This study
ORB4541	*trpC2 pheA1 trxB-lacZ* (pSN67, Cm)	(Nakano *et al.*,

		2003a)
ORB6975	*trpC2 pheA1 yjbH*::pZY38-5(*yjbH*::*tet*^{F}) *trxB-lacZ* (pSN67)	This study
ORB6976	*trpC2 pheA1 yjbH*::pZY39-3(*yjbH*::*tet*^{R}) *trxB-lacZ* (pSN67)	This study
ORB7001	*trpC2 pheA1 amyE*::pZY41-1 (pDR111::*yjbH*) *yjbH*::*tet*^{F} *trxB*::pSN67 (*trxB-lacZ*)	This study
ORB7002	*trpC2 pheA1 amyE*::pZY42-2 (pDR111::*yjbH*^{C31A}) *yjbH*::*tet*^{F} *trxB*::pSN67 (*trxB-lacZ*)	This study
ORB7003	*trpC2 pheA1 amyE*::pZY41-1 (pDR111::*yjbH*) *yjbH*::*tet*^{R} *trxB*::pSN67 (*trxB-lacZ*)	This study
ORB7004	*trpC2 pheA1 amyE*::pZY42-2 (pDR111::*yjbH*^{C31A}) *yjbH*::*tet*^{R} *trxB*::pSN67 (*trxB-lacZ*)	This study
ORB4574	*trpC2 pheA1 thrC*::pSN78(*trxB-lacZ*) *amyE*::pMMN521(Phyper-*spx*) *spx*::*neo*	(Nakano *et al.*, 2003a)
ORB6972	*trpC2 pheA1 thrC*::pSN78(*trxB-lacZ*) *amyE*::pMMN521(Phyper-*spx*) *spx*::*neo yjbH*::*tet*^{F}	This study
ORB6973	*trpC2 pheA1 thrC*::pSN78(*trxB-lacZ*) *amyE*::pMMN521(Phyper-*spx*) *spx*:: *neo yjbH*::*tet*^{R}	This study
ORB4381	*trpC2 pheA1 lacA*::*hrcA-ssrA*(*AA*)	This study
ORB4382	*trpC2 pheA1 lacA*::*hrcA-ssrA*(*DD*)	This study
ORB4384	*trpC2 pheA1 lacA*::*hrcA-ssrA*(*AA*) *clpX*::Spc	This study
ORB7055	*trpC2 pheA1 lacA*::*hrcA-ssrA*(*AA*) *yjbH*::pZY38-5(*yjbH*::*tet*^{F})	This study
ORB7056	*trpC2 pheA1 lacA*::*hrcA-ssrA*(*AA*) *yjbH*::pZY39-3(*yjbH*::*tet*^{R})	This study

Table 4.2 Plasmids

pTKlac	Plasmid for construction of *lacZ* transcriptional fusion	(Kenney & Moran Jr, 1991)
pDR111	IPTG-controlled Phyperspank(spank-hy) fusion vector for construction of transcriptional fusion into *amyE* locus	(Guerout-Fleury *et al.*, 1996)
pDG795	Integration plasmid at the *thrC* locus	(Guerout-Fleury *et al.*, 1996)
pDG1515	Plasmid bears Tetracycline-resistance cassette for Bacillus, flanked by multiple restriction sites	(Guerout-Fleury *et al.*, 1995)
pMMN521	pDR111 with *spx*	(Nakano *et al.*, 2003a)
pSN78	pDG795 with *trxB-lacZ*	(Nakano *et al.*, 2003a)
pMMN84	pTK*lacZ* with *srfA-lacZ*	(Nakano *et al.*, 2000)
pSN67	pTK*lacZ* with *trxB-lacZ*	(Nakano *et al.*, 2003a)
pZY41	pDR111 with *yjbH*	This study
pZY42	pDR111 with $yjbH^{C31A}$	This study

Table 4.3 Oligonucleotides

Oligo	Oligo Sequence	Position	Note[†]
oZY07-42	cag cgt cga cat gtt tgt aga c cc	*yjbH* (pUC19)	Fw +62 ~ +85
oZY07-51	cag caa gct tta tgg tga atc aaa cgg	*yjbH* (pDR111)	Fw –46 ~ –20
oZY07-43	cat aag tcg aca gcc tca agc ata tgc cc	*yjbH* (pUC19/pDR111)	Rv +908 ~ +936
oZY07-44	cag cgt cga cat gtt tgt aga ccc ttt ggc ccc tg	$yjbH^{C31A}$	Fw +58 ~ +96
oZY07-50	taa gga cca gca ttc agg ggc caa agg gtc	$yjbH^{C31A}$	Rv +81 ~ +110

[†] Fw = forward primer, Rv = reverse primer

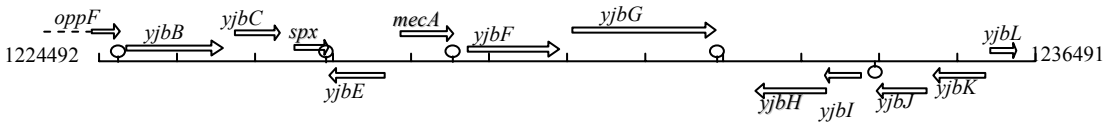


Figure 4.1 Drawing of the region of *yjbH* (from 1221.5 kb to 1241.5 kb) (20000 bp)

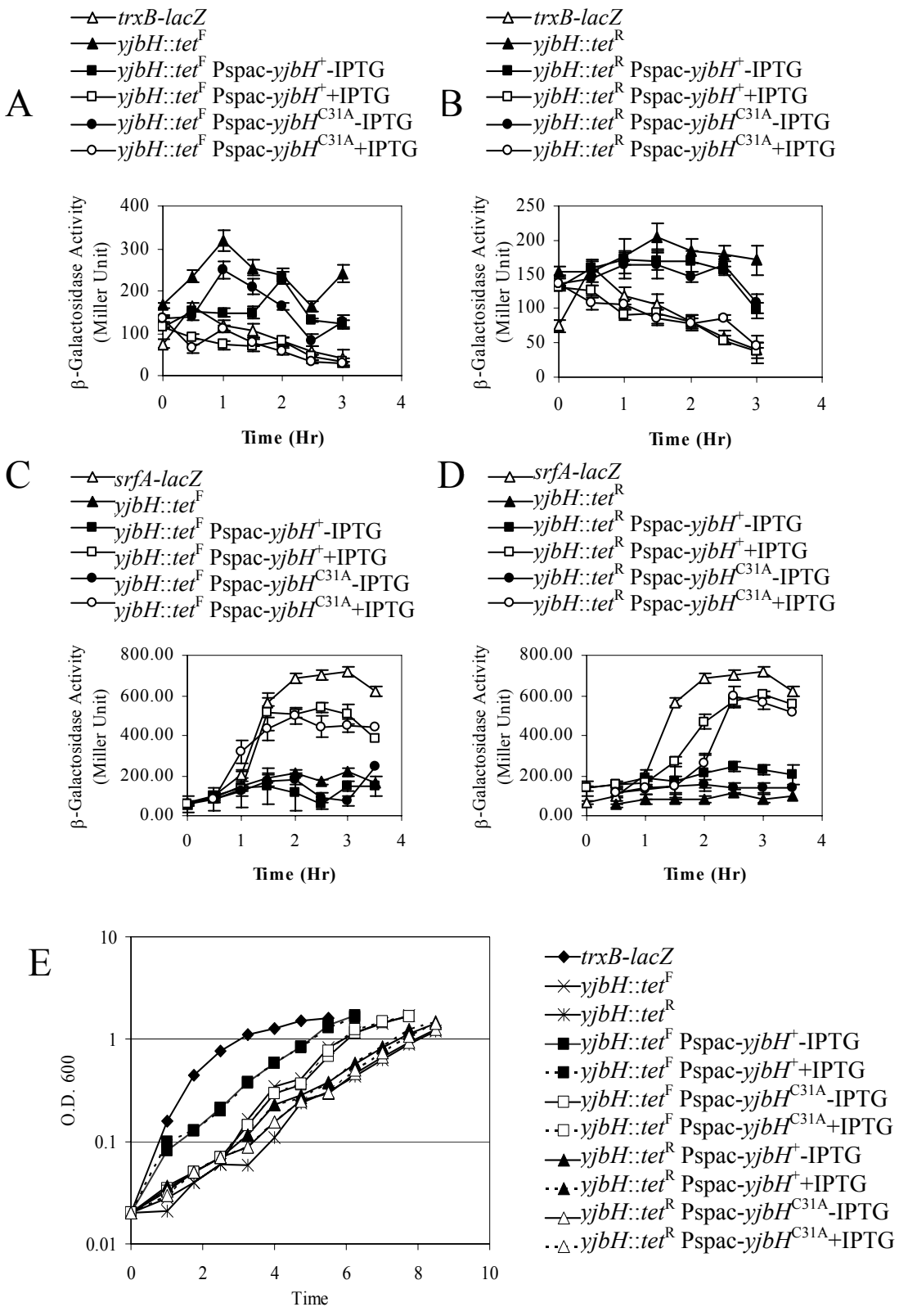

A
trxB-lacZ
yjbH::tet^F
yjbH::tet^F Pspac-yjbH^+-IPTG
yjbH::tet^F Pspac-yjbH^+IPTG
yjbH::tet^F Pspac-yjbH^C31A-IPTG
yjbH::tet^F Pspac-yjbH^C31A+IPTG
β-Galactosidase Activity (Miller Unit)
Time (Hr)
B
trxB-lacZ
yjbH::tet^R
yjbH::tet^R Pspac-yjbH^+-IPTG
yjbH::tet^R Pspac-yjbH^+IPTG
yjbH::tet^R Pspac-yjbH^C31A-IPTG
yjbH::tet^R Pspac-yjbH^C31A+IPTG
β-Galactosidase Activity (Miller Unit)
Time (Hr)
C
srfA-lacZ
yjbH::tet^F
yjbH::tet^F Pspac-yjbH^+-IPTG
yjbH::tet^F Pspac-yjbH^+IPTG
yjbH::tet^F Pspac-yjbH^C31A-IPTG
yjbH::tet^F Pspac-yjbH^C31A+IPTG
β-Galactosidase Activity (Miller Unit)
Time (Hr)
D
srfA-lacZ
yjbH::tet^R
yjbH::tet^R Pspac-yjbH^+-IPTG
yjbH::tet^R Pspac-yjbH^+IPTG
yjbH::tet^R Pspac-yjbH^C31A-IPTG
yjbH::tet^R Pspac-yjbH^C31A+IPTG
β-Galactosidase Activity (Miller Unit)
Time (Hr)
E
O.D. 600
Time
trxB-lacZ
yjbH::tet^F
yjbH::tet^R
yjbH::tet^F Pspac-yjbH^+-IPTG
yjbH::tet^F Pspac-yjbH^+IPTG
yjbH::tet^F Pspac-yjbH^C31A-IPTG
yjbH::tet^F Pspac-yjbH^C31A+IPTG
yjbH::tet^R Pspac-yjbH^+-IPTG
yjbH::tet^R Pspac-yjbH^+IPTG
yjbH::tet^R Pspac-yjbH^C31A-IPTG
yjbH::tet^R Pspac-yjbH^C31A+IPTG

Figure 4.2 Effect of insertion mutation and CXXC motif mutation of *yjbH* on Spx-dependent regulation of *srf* and *trxB* transcription.

(A) and (B): Measurement of β-galactosidase activity in a time course experiment of cultures of *trxB-lacZ* bearing cells in either wild-type (Δ), *yjbH* (▲), *yjbH*::*tet* pDR111::*yjbH*-IPTG(■), *yjbH*::*tet* pDR111::*yjbH*+IPTG(□), *yjbH*::*tet* pDR111::$yjbH^{C31A}$-IPTG(●), *yjbH*::*tet* pDR111::$yjbH^{C31A}$+IPTG(○) background. Except wild-type, all strains in (A) contain *yjbH*::tet^{F} mutation and strains in (B) contain *yjbH*::tet^{R} mutation. Data were from three independent experiments.

(C) and (D): Measurement of β-galactosidase activity in a time course experiment of cultures of *srf-lacZ* bearing cells in either wild-type (Δ), *yjbH* (▲), *yjbH*::*tet* pDR111::*yjbH*-IPTG(■), *yjbH*::*tet* pDR111::*yjbH*+IPTG(□), *yjbH*::*tet* pDR111::$yjbH^{C31A}$-IPTG(●), *yjbH*::*tet* pDR111::$yjbH^{C31A}$+IPTG(○) background. Except wild-type, all strains in (C) contain *yjbH*::tet^{F} mutation and strains in (D) contain *yjbH*::tet^{R} mutation. Data were from three individual experiments.

(E) Growth curve of wild-type and the *yjbH* insertion mutant strains in DSM media.

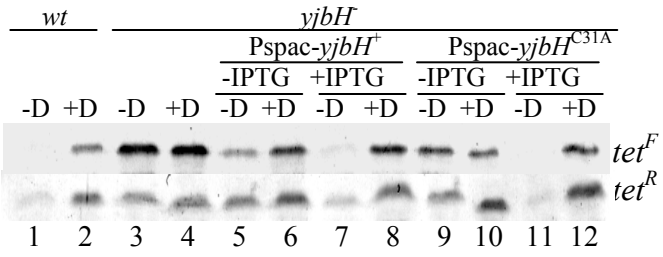


Figure 4.3 Western blot analyses of Spx levels in cells treated with diamide and IPTG.

Strains JH642(wild-type), ORB6952(*yjbH*::*tet*^{F}), ORB6953(*yjbH*::*tet*^{R}), ORB6997(*yjbH*::*tet*^{F}, pDR111::*yjbH*), ORB6998(*yjbH*::*tet*^{F}, pDR111::*yjbH*^{C31A}), ORB6999(*yjbH*::*tet*^{R}, pDR111::*yjbH*), ORB7000 (*yjbH*::*tet*^{R}, pDR111::*yjbH*^{C31A}) were grown in DSM media until OD600=0.5 in 37°C. Then each culture was treated with or without 1 mM diamide and 1 mM IPTG as indicated in the figure. Samples were taken 30 min after diamide treatment. Cells were lysed with 1 mg/ml lysozyme in protoplast buffer and were suspended in lysis buffer (See Experimental Procedures). Thirty μg protein from each sample was applied to an SDS polyacrylamide gel for electrophoresis. The protein levels of Spx were examined by western blot analysis using anti-Spx antibody. Lane 1 and 2 showed the wild-type strain. Lanes 3-12 of B up panel showed strains bearing *yjbH*::*tet*^{F} background and down panel for straining bearing *yjbH*::*tet*^{R} background.

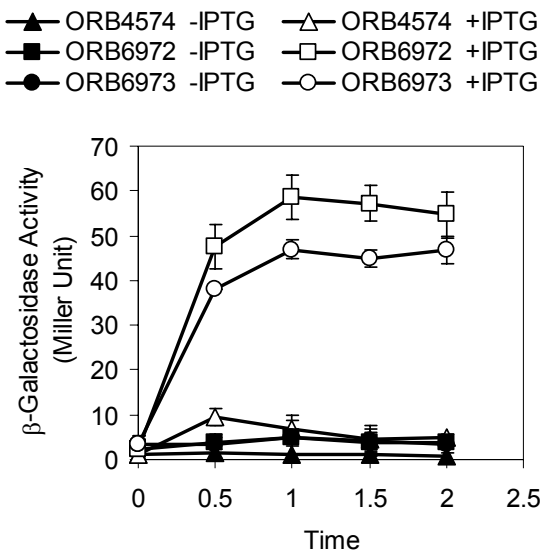


Figure 4.4 Post-transcriptional effect of YjbH on Spx-dependent regulation of *trxB* transcription

Measurement of β-galactosidase activity in a time course experiment of cultures of (*thrC*::*trxB-lacZ amyE*::Phyper-*spx spx*::*neo*) bearing cells in either ORB4574 (wild-type), -IPTG (▲) -IPTG (Δ); ORB6972 (*yjbH*::*tet*^{F}), -IPTG (■),+IPTG(□); ORB6973 (*yjbH*::*tet*^{R}), -IPTG (●), +IPTG(○) background. Data were from three independent experiments. 1 mM IPTG was added to the culture when O.D.600=0.5 at the 0 time point and start to take the sample for lac-assay.

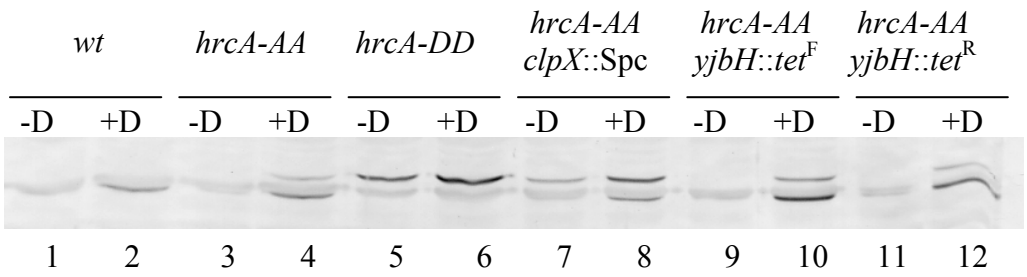
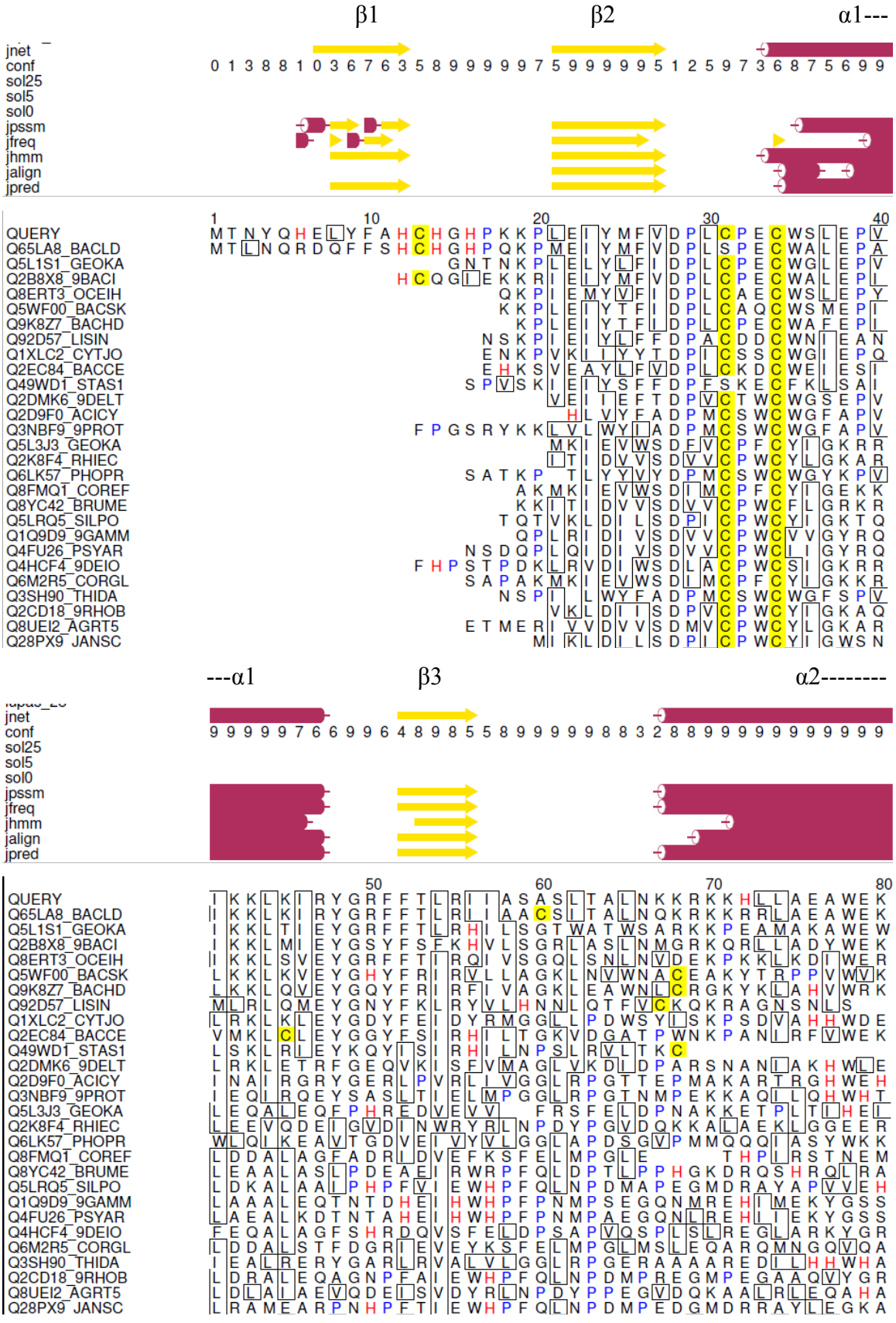
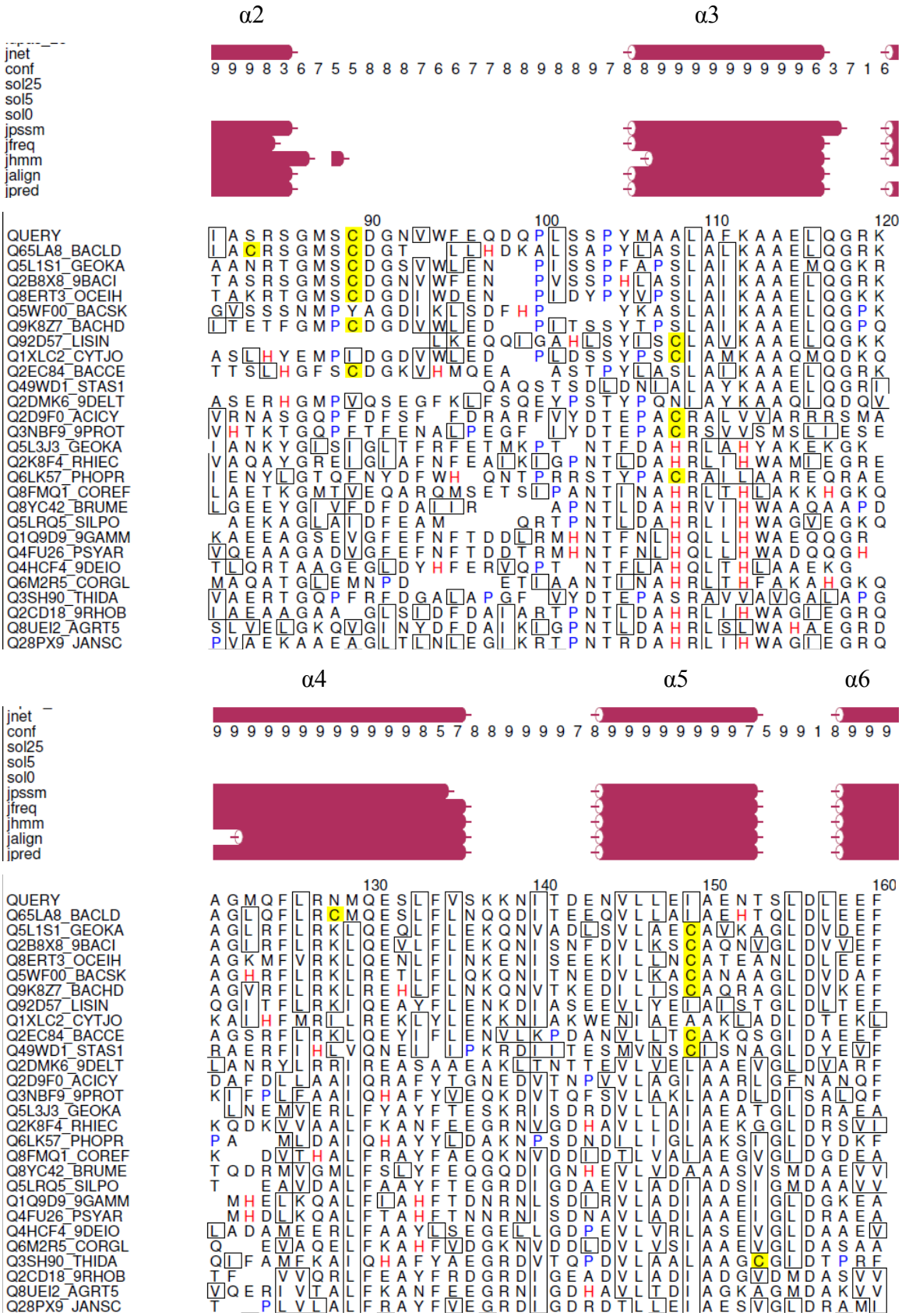
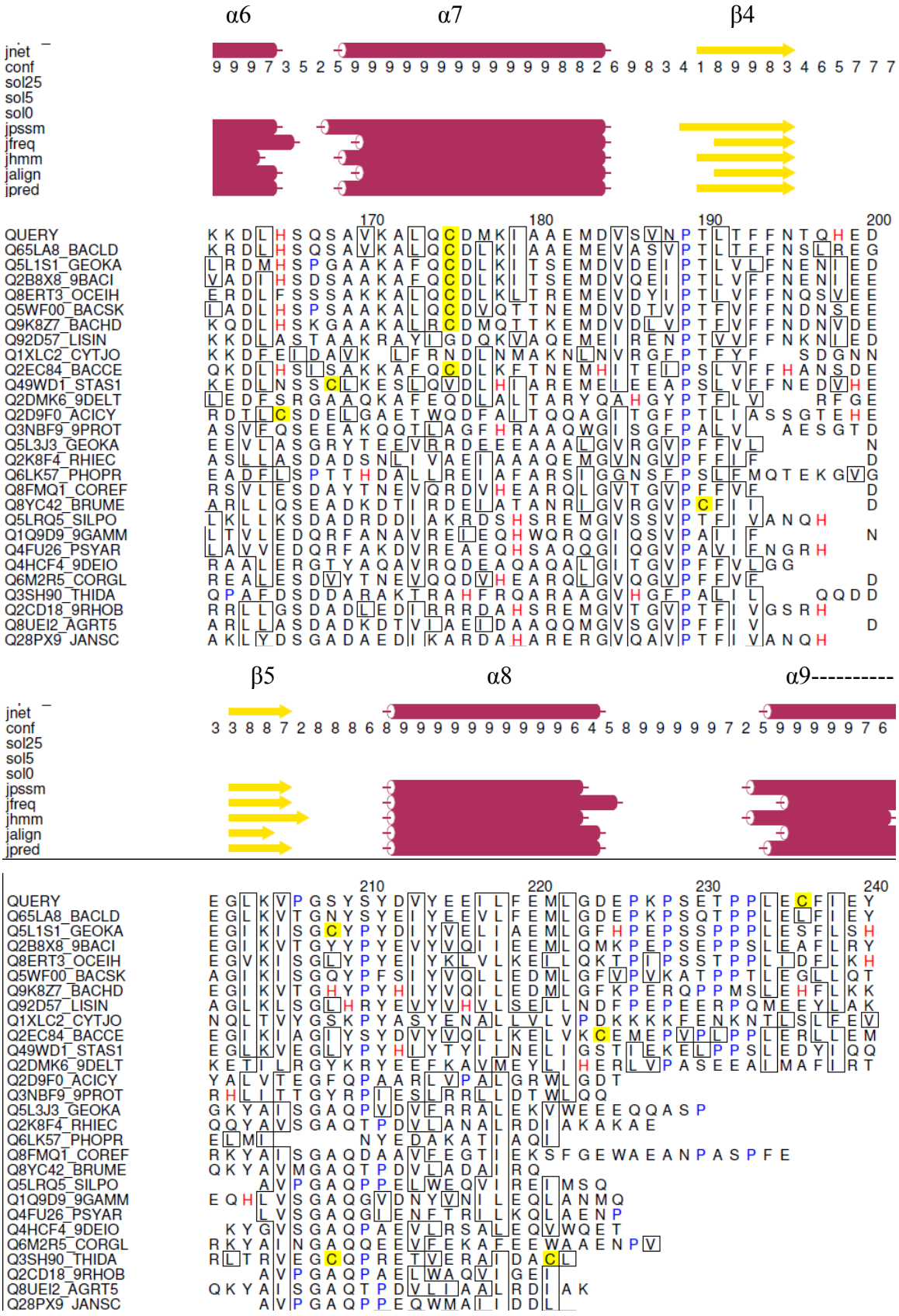


Figure 4.5 Western blot analysis of HrcA levels in cells treated with diamide.

Strains JH642 (*wild-type*), ORB4381 (*hrcA-ssrA [AA]*), ORB4382 (*hrcA-ssrA [DD]*), ORB4383 (*hrcA-ssrA* [AA], *clpX*::Spc), ORB7055 (*hrcA-ssrA [AA]*, *yjbH*::*tet*^{F}) and ORB7056 (*hrcA-ssrA* [AA], *yjbH*::*tet*^{R}) were grown in DSM media at 37°C with shaking. Samples were taken when OD600=0.5. And each culture was treated with 1 mM diamide. Samples were taken 30 min after diamide treatment. Cells were lysed with 1 mg/ml lysozyme in protoplast buffer and were suspended in lysis buffer (See Experimental Procedures). 30μg proteins from each sample were applied to an SDS polyacrylamide gel for electrophoresis. The protein levels of Spx were examined by western blot analysis using anti-HrcA antibody.


β1
β2
α1---
jnet
conf
sol25
sol5
sol0
jpssm
jfreq
jhmm
jalign
jpred
QUERY
Q65LA8_BACLD
Q5L1S1_GEOKA
Q2B8X8_9BACI
Q8ERT3_OCEIH
Q5WF00_BACSK
Q9K8Z7_BACHD
Q92D57_LISIN
Q1XLC2_CYTJO
Q2EC84_BACCE
Q49WD1_STAS1
Q2DMK6_9DELT
Q2D9F0_ACICY
Q3NBF9_9PROT
Q5L3J3_GEOKA
Q2K8F4_RHIEC
Q6LK57_PHOPR
Q8FMQ1_COREF
Q8YC42_BRUME
Q5LRQ5_SILPO
Q1Q9D9_9GAMM
Q4FU26_PSYAR
Q4HCF4_9DEIO
Q6M2R5_CORGL
Q3SH90_THIDA
Q2CD18_9RHOB
Q8UEI2_AGRT5
Q28PX9_JANSC
---α1
β3
α2--------


α2
α3
jnet
conf
sol25
sol5
sol0
jpssm
jfreq
jhmm
jalign
jpred
9 9 9 8 3 6 7 5 5 8 8 8 7 6 6 7 7 8 8 9 8 8 9 7 8 8 9 9 9 9 9 9 9 6 3 7 1 6
90
100
110
120
QUERY
Q65LA8_BACLD
Q5L1S1_GEOKA
Q2B8X8_9BACI
Q8ERT3_OCEIH
Q5WF00_BACSK
Q9K8Z7_BACHD
Q92D57_LISIN
Q1XLC2_CYTJO
Q2EC84_BACCE
Q49WD1_STAS1
Q2DMK6_9DELT
Q2D9F0_ACICY
Q3NBF9_9PROT
Q5L3J3_GEOKA
Q2K8F4_RHIEC
Q6LK57_PHOPR
Q8FMQ1_COREF
Q8YC42_BRUME
Q5LRQ5_SILPO
Q1Q9D9_9GAMM
Q4FU26_PSYAR
Q4HCF4_9DEIO
Q6M2R5_CORGL
Q3SH90_THIDA
Q2CD18_9RHOB
Q8UEI2_AGRT5
Q28PX9_JANSC
α4
α5
α6
jnet
conf
sol25
sol5
sol0
jpssm
jfreq
jhmm
jalign
jpred
9 9 9 9 9 9 9 9 9 9 9 8 5 7 8 8 9 9 9 9 7 8 9 9 9 9 9 9 9 9 7 5 9 9 1 8 9 9 9
130
140
150
160
QUERY
Q65LA8_BACLD
Q5L1S1_GEOKA
Q2B8X8_9BACI
Q8ERT3_OCEIH
Q5WF00_BACSK
Q9K8Z7_BACHD
Q92D57_LISIN
Q1XLC2_CYTJO
Q2EC84_BACCE
Q49WD1_STAS1
Q2DMK6_9DELT
Q2D9F0_ACICY
Q3NBF9_9PROT
Q5L3J3_GEOKA
Q2K8F4_RHIEC
Q6LK57_PHOPR
Q8FMQ1_COREF
Q8YC42_BRUME
Q5LRQ5_SILPO
Q1Q9D9_9GAMM
Q4FU26_PSYAR
Q4HCF4_9DEIO
Q6M2R5_CORGL
Q3SH90_THIDA
Q2CD18_9RHOB
Q8UEI2_AGRT5
Q28PX9_JANSC


α6
α7
β4
jnet
conf
sol25
sol5
sol0
jpssm
jfreq
jhmm
jalign
jpred
9 9 9 7 3 5 2 5 9 9 9 9 9 9 9 9 9 9 8 8 2 6 9 8 3 4 1 8 9 9 8 3 4 6 5 7 7 7
170
180
190
200
QUERY
Q65LA8_BACLD
Q5L1S1_GEOKA
Q2B8X8_9BACI
Q8ERT3_OCEIH
Q5WF00_BACSK
Q9K8Z7_BACHD
Q92D57_LISIN
Q1XLC2_CYTJO
Q2EC84_BACCE
Q49WD1_STAS1
Q2DMK6_9DELT
Q2D9F0_ACICY
Q3NBF9_9PROT
Q5L3J3_GEOKA
Q2K8F4_RHIEC
Q6LK57_PHOPR
Q8FMQ1_COREF
Q8YC42_BRUME
Q5LRQ5_SILPO
Q1Q9D9_9GAMM
Q4FU26_PSYAR
Q4HCF4_9DEIO
Q6M2R5_CORGL
Q3SH90_THIDA
Q2CD18_9RHOB
Q8UEI2_AGRT5
Q28PX9_JANSC
β5
α8
α9----------
jnet
conf
sol25
sol5
sol0
jpssm
jfreq
jhmm
jalign
jpred
3 3 8 8 7 2 8 8 8 6 8 9 9 9 9 9 9 9 9 9 6 4 5 8 9 9 9 9 9 7 2 5 9 9 9 9 9 7 6
210
220
230
240
QUERY
Q65LA8_BACLD
Q5L1S1_GEOKA
Q2B8X8_9BACI
Q8ERT3_OCEIH
Q5WF00_BACSK
Q9K8Z7_BACHD
Q92D57_LISIN
Q1XLC2_CYTJO
Q2EC84_BACCE
Q49WD1_STAS1
Q2DMK6_9DELT
Q2D9F0_ACICY
Q3NBF9_9PROT
Q5L3J3_GEOKA
Q2K8F4_RHIEC
Q6LK57_PHOPR
Q8FMQ1_COREF
Q8YC42_BRUME
Q5LRQ5_SILPO
Q1Q9D9_9GAMM
Q4FU26_PSYAR
Q4HCF4_9DEIO
Q6M2R5_CORGL
Q3SH90_THIDA
Q2CD18_9RHOB
Q8UEI2_AGRT5
Q28PX9_JANSC

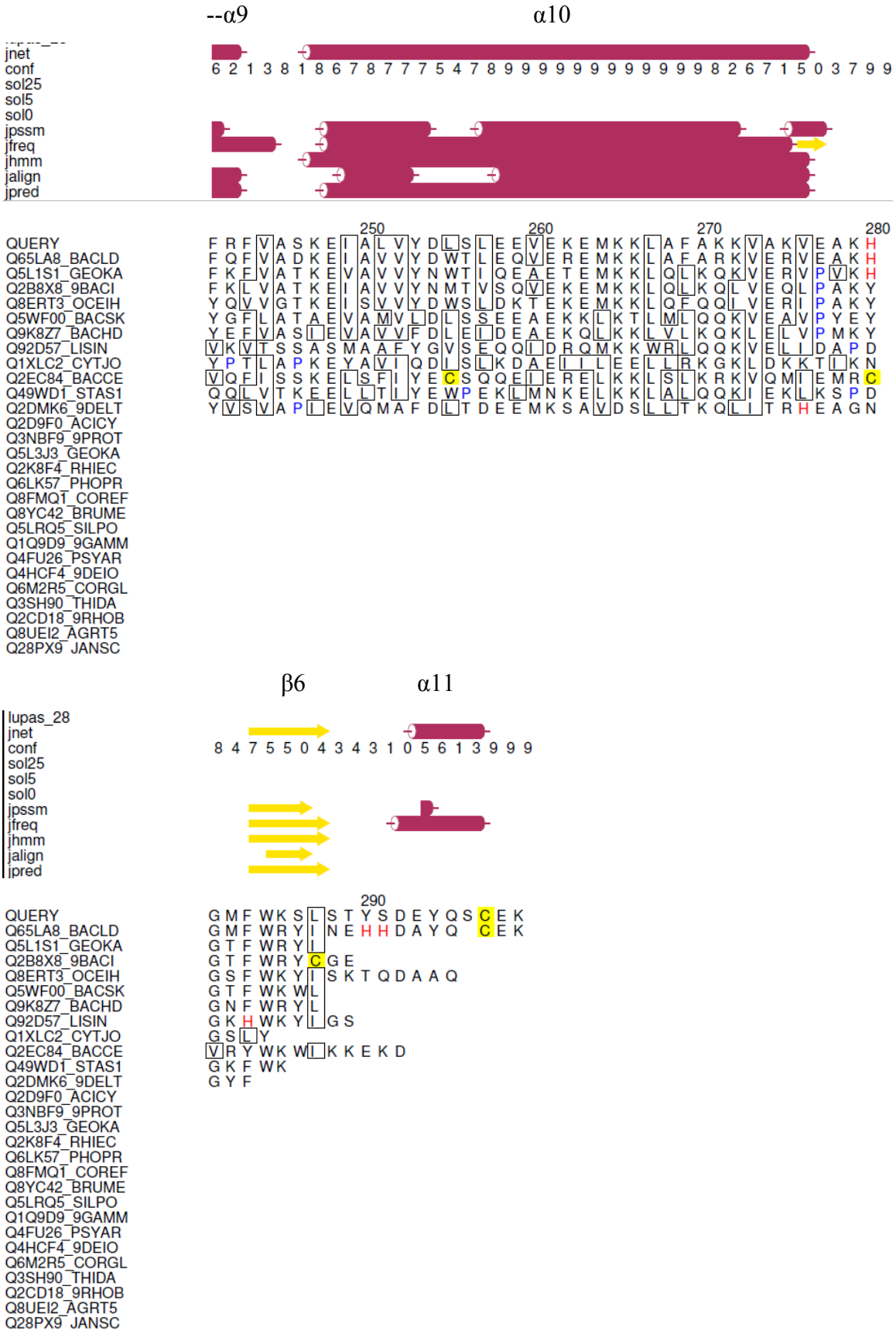

--α9
α10
jnet
conf
sol25
sol5
sol0
jpssm
jfreq
jhmm
jalign
jpred
6 2 1 3 8 1 8 6 7 8 7 7 7 5 4 7 8 9 9 9 9 9 9 9 9 9 9 8 2 6 7 1 5 0 3 7 9 9
250
260
270
280
QUERY
Q65LA8_BACLD
Q5L1S1_GEOKA
Q2B8X8_9BACI
Q8ERT3_OCEIH
Q5WF00_BACSK
Q9K8Z7_BACHD
Q92D57_LISIN
Q1XLC2_CYTJO
Q2EC84_BACCE
Q49WD1_STAS1
Q2DMK6_9DELT
Q2D9F0_ACICY
Q3NBF9_9PROT
Q5L3J3_GEOKA
Q2K8F4_RHIEC
Q6LK57_PHOPR
Q8FMQ1_COREF
Q8YC42_BRUME
Q5LRQ5_SILPO
Q1Q9D9_9GAMM
Q4FU26_PSYAR
Q4HCF4_9DEIO
Q6M2R5_CORGL
Q3SH90_THIDA
Q2CD18_9RHOB
Q8UEI2_AGRT5
Q28PX9_JANSC
β6
α11
lupas_28
jnet
conf
sol25
sol5
sol0
jpssm
jfreq
jhmm
jalign
jpred
8 4 7 5 5 0 4 3 4 3 1 0 5 6 1 3 9 9 9
290
QUERY
Q65LA8_BACLD
Q5L1S1_GEOKA
Q2B8X8_9BACI
Q8ERT3_OCEIH
Q5WF00_BACSK
Q9K8Z7_BACHD
Q92D57_LISIN
Q1XLC2_CYTJO
Q2EC84_BACCE
Q49WD1_STAS1
Q2DMK6_9DELT
Q2D9F0_ACICY
Q3NBF9_9PROT
Q5L3J3_GEOKA
Q2K8F4_RHIEC
Q6LK57_PHOPR
Q8FMQ1_COREF
Q8YC42_BRUME
Q5LRQ5_SILPO
Q1Q9D9_9GAMM
Q4FU26_PSYAR
Q4HCF4_9DEIO
Q6M2R5_CORGL
Q3SH90_THIDA
Q2CD18_9RHOB
Q8UEI2_AGRT5
Q28PX9_JANSC

Figure 4.6 Sequence alignment of YjbH and secondary structure prediction

The sequence alignment and prediction of secondary structure of YjbH is performed by the Barton Group Of University of Dundee, Nethergate, Dundee, DD1 4HN, Scotland, UK through the secondary structure prediction server http://www.compbio.dundee.ac.uk/~www-jpred/. The secondary structure of *yjbH* (β1β2α1β3**α2α3α4α5α6α7**β4β5α8α9α10β6α11) was displayed above the alignment. QUERY is the amino acid sequence of YjbH. The yellow arrows represent the β sheet structure and pink ribbons represent the α helix.

CHAPTER 5 CONCLUSIONS AND FUTURE DIRECTIONS

5.1 SUMMARY OF RESEARCH

5.1.1 Overlapping Spx-RNAP and ComA-RNAP interaction surface at the α1 helix of αCTD

The response regulator, ComA interacts with *srf* promoter DNA and this interaction is enhanced by the presence of RNA polymerase as shown by DNase I footprinting analysis. WT Spx, but not $Spx^{Cxs\text{-}16}$ can reduce both RNAP and ComA binding to the *srf* promoter. Spx-dependent release of *rpoA*(Y263C) RNAP from the *srf* promoter is reduced. Solid-phase promoter retention (SPPR) experiments indicated αCTD interaction with the *srf* promoter requires the presence of ComA and was inhibited by addition of Spx. Therefore, Spx-RNAP interaction reduces ComA-assisted binding of RNAP to the *srf* promoter.

Alanine-scanning mutagenesis of *B. subtilis* αCTD uncovered residues required for Spx function and ComA-dependent *srf* transcriptional activation. Analysis of *srf-lacZ* fusion expression, DNase I footprinting, and SPPR experiments indicated that *rpoA*(C265A) specifically reduces ComA-activated *srf* transcription and RNAP binding to the *srf* promoter *in vitro* and *rpoA*(K267A) mutation affects ComA- and Spx-activated transcription and Spx-dependent negative control *in vitro*. The *rpoA*(K267A) mutant is hypersensitive to diamide-induced thiol-specific oxidative stress *in vivo*. Spx blocks productive interaction between ComA and RNAP at the *srf* promoter by occupying an overlapping surface on αCTD involving residues Y263, C265, and K267 of the α1 region.

The requirement of the oxidized Spx for Spx-dependent activation of *trxA* and *trxB* expressions was demonstrated in previous work (Nakano *et al.*, 2005). Evidence is presented here that oxidized Spx, while enhancing interference of activator-RNAP interaction, is not essential for negative control.

SPPR experiments using biotinylated *trxA* and *trxB* promoter DNA indicated enhanced binding of RNAP ($\alpha\beta\beta'\sigma^A$) to the promoter in the presence of Spx. The binding of RNAP and Spx complex to the promoter *trxA* is reduced with increasing DTT. But 10 mM DTT could not completely abolish interaction. SPPR experiments showed slightly increasing Spx binding with *trxA* and *trxB* promoters in the absence of RNAP. This result conflicts to our knowledge, it might due to the Spx non-specific binding to the streptavidin bead.

5.1.2 ZBD domain of ClpX is required by for repression of ClpXP proteolytic control of Spx upon disulfide stress.

ClpXP-catalyzed degradation of either Spx or a green fluorescent protein (GFP) derivative bearing an SsrA tag recognized by ClpXP, was inhibited by diamide treatment *in vitro*. Spx is also a substrate for MecA/ClpCP-catalyzed proteolysis *in vitro*, but diamide used at the concentrations that inhibited ClpXP had little observable effect on MecA/ClpCP activity. ClpX bears a Cys4 Zinc-binding domain (ZBD), which in other Zinc-binding proteins is vulnerable to thiol-reactive electrophiles. Diamide treatment caused partial release of Zn from ClpX and the formation of high molecular weight species, as observed by electrophoresis through non-reducing gels. Reduced Spx proteolysis *in vitro* and elevated Spx concentration *in vivo* resulted when two of the Zn-coordinating Cys residues of the ClpX ZBD were changed to Ser. This was reflected in enhanced Spx activity, both in transcription activation and repression in cells expressing the Cys to Ser mutants. The results are consistent with the hypothesis that inhibition of ClpXP by disulfide stress is due to structural changes to the N-terminal ZBD of ClpX.

5.1.3 YjbH affects the concentration of the Spx protein

In *B. subtilis* YjbH negatively controls Spx concentration and Spx-dependent transcriptional control. This negative effect could not be bypassed when Spx is under the control of an IPTG-inducible promoter. YjbH-dependent negative control is modulated in the presence of diamide. YjbH does not affect the concentration of another ClpXP substrate, SsrA-tagged HrcA, suggesting that negative regulation exerted by YjbH might be specific for Spx. A mutation that changes the first cysteine residue of YjbH CXXC

motif at the N terminus to alanine did not affect Spx-dependent transcriptional control and the control of Spx concentration in untreated and diamide-treated cells. Dr. Saurabh Garg in our lab also observed that the translation of Spx is not affected by the *yjbH* insertion mutants. YjbH is proposed to post-translationally modulate Spx levels in *B. subtilis.*

5.2 FUTURE DIRECTIONS

Diamide sensitivity screening of all *B. subtilis* strains bearing alanine codon substitutions in the αCTD-coding region of the *rpoA* gene indicated that *rpoA*(Y263A) exhibited 1000-fold, *rpoA*(E254A) and *rpoA*(K267A) showed 100-fold and *rpoA*(*D257A*) and *rpoA*(K294A) showed 10-fold sensitivity to 0.1 mM diamide compared with wild-type. According to the *B. subtilis* crystal structure (Newberry *et al.*, 2005) Y263, K267 and E254 are located close to each other so they might form a Spx-contacting surface on αCTD (Fig. 5.1). Together with C265, the alanine substitution of these four residues showed reduced ComA-dependent *srfA-lacZ* activity, indicating that they constitute the binding surface for ComA. The corresponding residue of D257 and E255 in the *E. coli* is the contact position for σ^{70} R603 of region 4, which is highly conserved in bacteria and corresponds to R362 of *B. subtilis* σ^{A}. *sigA*(K356A), which confers defects in both Spx-dependent repression and activation, is proposed here to be in contact with Spx. Thus, Spx might interact with both σ^{A} and αCTD, which together have been shown to serve as a scaffold to stable the σ^{A}-αCTD interaction in certain transcription initiation complexes.

Michiko Nakano identified the interaction between Spx and β subunit via yeast two-hybrid experiment, thus identifying a third potential contact point on RNAP holoenzyme for Spx interaction. Spx might function as a subunit for RNAP holoenzyme to promote formation of a transcriptional initiation complex at the Spx-controlled genes. The repression of ComA-dependent transcription could be explained since *srfA* promoter is not a strong σ^{A}-dependent promoter as *trxA* and *trxB*. The recruitment of RNA polymerase to the *srf* promoter is performed through the *srf* promoter-binding activator ComA. Spx does not have specific DNA-binding activity, but by stabilizing the holoenzyme, the promoter recognition function could be performed by σ^{A}. The recent

chemical crosslink analysis conducted by Dindo Reyes in our lab indicated that the crosslink of σ^A of RNAP to *trxA* or *trxB* promoter DNA only occurs in the presence of Spx. To test the hypothesis Spx promotes the αCTD and σ^A interaction, the chemical crosslink experiment could be performed with only αCTD and σ^A rather than the holoenzyme in the presence or absence of Spx with *trxA* promoter. SPPR and EMSA could also be used to test this hypothesis.

A study could be conducted that focuses on the residues of RNA polymerase β subunit involved in the interaction between the β subunit and Spx. This could be achieved by UV or chemical mutagenesis of strain MH5636 which carry His10-*rpoC*::cat. The chromosome DNA from the resulting mutant library could be used to transform the [*srfA-lacZ*, *amyE*::pDR111(*spxDD*) *spx*::*neo*] strain or [*trxB-lacZ*, *amyE*::pDR111(*spxDD*), *spx*::*neo*] strain and selecting for colony bearing chloramphenicol-resistant to make sure the mutation site is linked to the *rpoC* locus and showing same Lac phenotype on agar containing X-gal with and without IPTG (which is used to induce ectopic expression of SpxDD from the *amyE* locus). With and without IPTG the mutant strains exhibit both blue for carrying *srfA-lacZ* or both white for carrying *trxB-lacZ* might interrupt the interaction between β subunit and Spx.

With similar strategy, we could also use strain ORB5925 bearing $sigA^{WT}$(pJB2), in which the *sigA* locus is linked with Neo-resistant cassette. The chromosome DNA from UV or chemical random mutagenesis treated strain ORB5925 can serve as a *sigA* random mutation library to transform [*amyE*::pSN56(pDR111-*spxLDD*), *thrC*::pSN78 (*trxB-lacZ*)]. The *sigA* mutants that disrupt Spx-σ^A interaction might exhibit white colony on x-gal containing plate with and without IPTG.

Other regulatory factors that interact with Spx during transcriptional control could be searched by mini-Tn10 transposon mutagenesis to select suppressor mutations which could carry out Spx-dependent transcriptional activation of *trxB* with mutant Spx(C10A). Suppressor mutations for spx^{C10A} that restore Spx-dependent activation of *trxB* transcription could be identified by the screening for both blue colony phenotype in the presence or absence of IPTG-inducible Spx(C10A)LDD and *trxB-lacZ*. Since the major target of Spx during transcriptional control is the holoenzyme, the suppresser mutation might reside in the gene encoding the β or σ^A subunit of RNAP. UV or chemical

mutagenesis created the His10-*rpoC*::cat or *sigA*(neo) mutation libraries which directly target *rpoC* or *sigA* locus could also be used to screen the spx^{C10A} suppressor mutation with similar strategy.

Transposon mutagenesis could also be used to identify other members of the Spx regulon by selecting for variants bearing mutations that confer survival in the presence of a high concentration of diamide when Spx is absent or in cells expressing only the C10A allele of *spx*, since the Spx protein is indispensable for survival of *B. subtilis* under disulfide stress. These mutants might constitutively express Spx protein which might eliminate the transcriptional repressions from PerR and YodB or block other post-transcriptional or post-translational regulators such as YjbH or ClpX or the Spx regulon such as *trxA* or *trxB* with a mutated promoter which could bypass the Spx-dependent activation.

LDD form of Spx(C13A) is not stable in *B. subtilis*, but upon diamide treatment accumulation of Spx, Spx(C10A) and Spx(C13A), all in the SpxLAN form, was observed (Nakano *et al.*, 2005). Our evidence also indicated that *in vitro* degradation of wild-type and Spx(C10A) by ClpXP and their resistance to proteolytic control upon diamide treatment were similar (Fig. 3.7). All these results suggest that the Spx(C13A) is stabilized by diamide treatment but is more sensitive to ClpXP degradation than wild-type and C10A Spx in diamide-untreated cell. Therefore, we might expect to observe similar levels of Spx, Spx(C10A) and Spx(C13A) in a *clpX* or in *yjbH* mutant cells.

Our experiment showed protein YjbH negatively controls Spx protein concentration at the post-transcriptional level *in vivo*. In order to elucidate at which step YjbH functions in Spx control, translational fusion of Spx will be used to check whether the *yjbH* mutation affects Spx expression at the translational level. YjbH is a cysteine-rich protein with a highly conserved CXXC motif at the N-terminus. According to the secondary structure prediction it is composed of a thioredoxin-like domain and an α helical domain similar to *E. coli* DsbA, which functions as a disulfide isomerase that targets periplasmic and secreted proteins. Thus, *yjbH* might function as a chaperone to destabilize the Spx protein or tether it to the degradation apparatus. *In vitro* proteolysis experiment could be performed to determine whether YjbH elevates ClpXP-dependent proteolysis of Spx. In order to identify the direct target of the YjbH protein, a pull-down

experiment using His-tagged YjbH with either ClpX or Spx could be employed to answer this question. We could revisit the role of the CXXC motif of YjbH by mutationally changing both cysteines and observing the effect of the double mutant on Spx concentration control in the presence of diamide. Other residues such as Pro32 in the CXXC motif and Pro188 at the vicinity of CXXC motif could also be the target for study the role of YjbH in redox control since the corresponding Pro 151 in *E. coli* DsbA functions to stabilize the active site of the protein through van der Waals interaction with the disulfide bond (Charbonnier *et al.*, 1999; Kadokura *et al.*, 2005; Ondo-Mbele *et al.*, 2005). Deletion analysis could be performed on the specific high-His sequence at N-terminus or proline rich linker at the C-terminus of YjbH since the N-terminal and C-terminal regions are not shared among the thioredoxin-like domain family. They might not be essential or could be required for other specific function such as stabilizing the thioredoxin-like domain, or facilitating Spx and/or ClpX contact. Mutant proteins could be tested with yeast two-hybrid or pull-down experiments to test the latter functions. A series thioredoxin domain containing proteins, such as DsbA with its thiol-regenerating, membrane binding protein DsbB, TrxA (in cytoplasm) and DsbC and DsbD (in periplasm), assist necessary disulfide bond formation for secreted and membrane protein in the *E. coli*. CXXC motif containing proteins ClpX, TrxA, TrxB, Spx and YjbH might serve as an alternative pathway for disulfide bond reduction in *B. subtilis* cytoplasm.

yjbG is located downstream *yjbH* gene and transcribe from the opposite orientation. There is a 524 bp non-coding sequence between the two genes and the growth deficiency, which only is observed in the reverse-oriented *yjbH*::*tet* insertion mutant, could not be complemented by full length wild-type *yjbH*. This observation suggests that there is some downstream element that might function in YjbH activity related to the growth and Spx activity/concentration. The attachment of an unfoldable DsbA/PhoA complex to membrane could be released by a small RNA (Guigueno *et al.*, 2001). If the complementary construct including the downstream non-coding sequence can recover the growth deficiency, we could further search for regulator of *yjbH* activity in this downstream non-coding region.

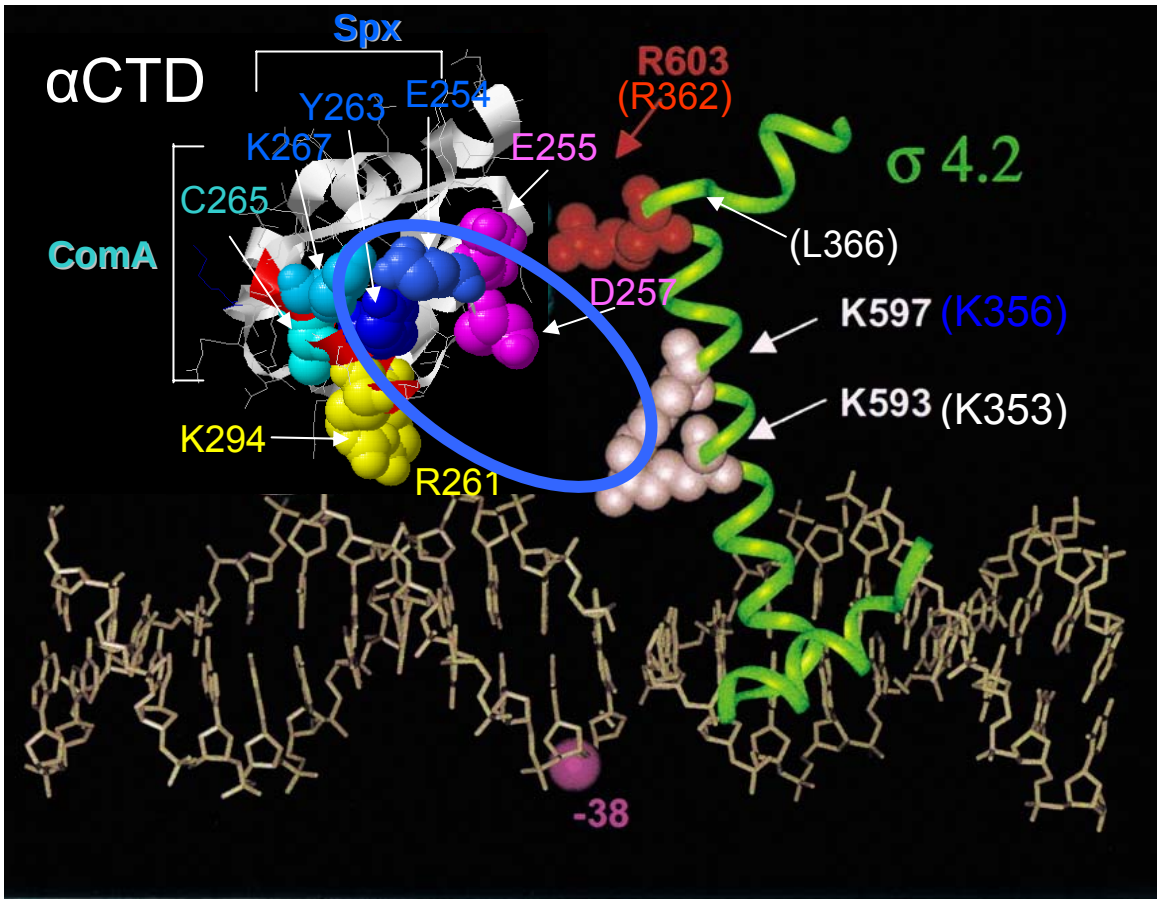


Figure 5.1 Structure-based model of *B. subtilis* RNA polymerase αCTD and region 4.2. of σ^A.

αCTD crystal structure is based on (Newberry *et al.*, 2005). *E. coli* "265" determinant is corresponded in *B subtilis* to R261 involved binding of DNA. *E. coli* "261" determinant is corresponded in *B subtilis* to D257 and E255 involved in binding region 4.2. of σ^A. *T. aquaticus* R603, K597 and K593 is corresponded in *B subtilis* to R362, K356 and K352 (Ross *et al.*, 2003). White ribbon structures denote peptide backbone and amino acid side chains of αCTD. The red ribbon indicates the α1 helix. The side chains of αCTD residues R261 and K294 involved in DNA contact are presented as yellow balls, C265, K267, Y263 and E254 involved in ComA interaction are presented as blue balls, E255 and D257 involved in σ^A interaction are present as pink balls. R362 of σ^A likely involved in interaction with αCTD are presented in red and K356 of σ^A and K267, Y263, E254, of αCTD which likely involved in interaction with Spx are

indicated with dark blue characters and the possible position of Spx is indicated by the blue ellipse.

LITERATURE CITED

Adam, Z., Adamska, I., Nakabayashi, K. & other authors (2001). Chloroplast and Mitochondrial Proteases in Arabidopsis. A Proposed Nomenclature. *Plant Physiol 125*, 1912-1918.

Akanuma, G., Nanamiya, H., Natori, Y., Nomura, N. & Kawamura, F. (2006). Liberation of zinc-containing L31 (RpmE) from ribosomes by its paralogous gene product, YtiA, in *Bacillus subtilis*. *J Bacteriol 188*, 2715-2720.

Antelmann, H., Engelmann, S., Schmid, R. & Hecker, M. (1996). General and oxidative stress responses in *Bacillus subtilis*: cloning, expression, and mutation of the alkyl hydroperoxide reductase operon. *J Bacteriol 178*, 6571-6578.

Antelmann, H., Scharf, C. & Hecker, M. (2000). Phosphate starvation-inducible proteins of *Bacillus subtilis*: proteomics and transcriptional analysis. *J Bacteriol 182*, 4478-4490.

Banner, C. D., Moran, C. P., Jr. & Losick, R. (1983). Deletion analysis of a complex promoter for a developmentally regulated gene from *Bacillus subtilis*. *J Mol Biol 168*, 351-365.

Bardwell, J. C. (1994). Building bridges: disulphide bond formation in the cell. . *Mol Microbiol 14*, 199-205.

Barnett, M. E., Nagy, M., Kedzierska, S. & Zolkiewski, M. (2005). The amino-terminal domain of ClpB supports binding to strongly aggregated proteins. *J Biol Chem 280*, 34940-34945.

Baud, L., Hagege, J., Sraer, J., Rondeau, E., Perez, J. & Ardaillou, R. (1983). Reactive oxygen production by cultured rat glomerular mesangial cells during phagocytosis is associated with stimulation of lipoxygenase activity. *J Exp Med 158* 1836-1852.

Becker, G., Klauck, E. & Hengge-Aronis, R. (1999). Regulation of RpoS proteolysis in *Escherichia coli*: the response regulator RssB is a recognition factor that interacts with the turnover element in RpoS. *Proc Natl Acad Sci U S A 96*, 6439-6444.

Becker, G., Klauck, E. & Hengge-Aronis, R. (2000). The response regulator RssB, a recognition factor for sigmaS proteolysis in *Escherichia coli*, can act like an anti-sigmaS. *Mol Microbiol 35*, 657-666.

Berka, R. M., Hahn, J., Albano, M., Draskovic, I., Persuh, M., Cui, X., Sloma, A., Widner, W. & Dubnau, D. (2002). Microarray analysis of the *Bacillus subtilis* K-state: genome-wide expression changes dependent on ComK. *Mol Microbiol 43*, 1331-1345.

Bessette, P. H., Cotto, J. J., Gilbert, H. F. & Georgiou, G. (1999). *In Vivo* and *in Vitro* Function of the *Escherichia coli* Periplasmic Cysteine Oxidoreductase DsbG. *J Biol Chem 274*, 7784 - 7792.

Bougdour, A. & Gottesman, S. (2007). ppGpp regulation of RpoS degradation via anti-adaptor protein IraP. *Proc Natl Acad Sci U S A 104*, 12896-12901.

Boylan, S. A., Redfield, A. R. & Price, C. W. (1993). Transcription factor sigma B of *Bacillus subtilis* controls a large stationary-phase regulon. *J Bacteriol 175*, 3957-3963.

Britton, R. A., Eichenberger, P., Gonzalez-Pastor, J. E., Fawcett, P., Monson, R., Losick, R. & Grossman, A. D. (2002). Genome-wide analysis of the stationary-phase sigma factor (sigma-H) regulon of *Bacillus subtilis*. *J Bacteriol 184*, 4881-4890.

Brune, B. & Mohr, S. (2001). Protein Thiol Modification of Glyceraldehyde-3-phosphate Dehydrogenase and Caspase-3 by Nitric Oxide. *Curr Protein Pept Sci 2*, 61-72.

Buck, M., Gallegos, M.-T., Studholme, D. J., Guo, Y. & Gralla, J. D. (2000). The Bacterial Enhancer-Dependent sigma 54 (sigma N) Transcription Factor. *J Bacteriol 182*, 4129-4136.

Burbulys, D., Trach, K. A. & Hoch, J. A. (1991). Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. *Cell 64*, 545-552.

Burkholder, W. F., Kurtser, I. & Grossman, A. D. (2001). Replication initiation proteins regulate a developmental checkpoint in *Bacillus subtilis*. *Cell 104*, 269-279.

Burton, B. M. & Baker, T. A. (2003). Mu transpososome architecture ensures that unfolding by ClpX or proteolysis by ClpXP remodels but does not destroy the complex. *Chem Biol 10*, 463-472.

Busby, S. & Ebright, R. H. (1999). Transcription activation by catabolite activator protein (CAP). *J Mol Biol 293*, 199-213.

Campbell, E. A., Muzzin, O., Chlenov, M., Sun, J. L., Olson, C. A., Weinman, O., Trester-Zedlitz, M. L. & Darst, S. A. (2002). Structure of the bacterial RNA polymerase promoter specificity [sigma] subunit. *Mol Cell 9*, 527-539.

Candiano, G., Bruschi, M., Musante, L., Santucci, L., Ghiggeri, G. M., Carnemolla, B., Orecchia, P., Zardi, L. & Righetti, P. G. (2004). Blue silver: a very sensitive colloidal coomassie G-250 staining for proteome analysis. *Electrophoresis 25*, 1327-1333.

Cashel, M., Gentry, D. R., Hernandez, V. H. & Vinella, D. (1996). The stringent response. In *Escherichia Coli* and *Salmonella Typhimurium*, Neidhardt FC (ed). *Washington, DC: ASM Press*, 1458-1496.

Charbonnier, J. B., Belin, P., Moutiez, M., Stura, E. A. & Quemeneur, E. (1999). On the role of the cis-proline residue in the active site of DsbA. *Protein Sci 8*, 96-105.

Chatterjee, S. S., Hossain, H., Otten, S., Kuenne, C., Kuchmina, K., Machata, S., Domann, E., Chakraborty, T. & Hain, T. (2006). Intracellular gene expression profile of *Listeria monocytogenes*. *Infect Immun 74*, 1323-1338.

Choudhary, M. L., Jawaid, S., Ahuja, M. K., Shiva, N. K., Gupta, P., Bhuyan, A. K. & Khatri, G. S. (2005). Open reading frame yjbI of *Bacillus subtilis* codes for truncated hemoglobin. *Protein expression and purification 41*, 363-372.

Comella, N. & Grossman, A. D. (2005). Conservation of genes and processes controlled by the quorum response in bacteria: characterization of genes controlled by the quorum-sensing transcription factor ComA in *Bacillus subtilis*. *Mol Microbiol 57*, 1159-1174.

Costanzo, A. & Ades, S. E. (2006). Growth phase-dependent regulation of the extracytoplasmic stress factor, σ^E, by guanosine 3′,5′-bispyrophosphate (ppGpp). *J Bacteriol 188*, 4627-4634.

D'Souza, C., Nakano, M. M. & Zuber, P. (1994). Identification of *comS*, a gene of the *srfA* operon that regulates the establishment of genetic competence in *Bacillus subtilis*. *Proc Natl Acad Sci U S A 91*, 9397-9401.

Deneke, S. M. (2000). Thiol-based antioxidants. *Curr Top Cell Regul 36*, 151-180.

Derre, I., Rapoport, G., Devine, K., Rose, M. & Msadek, T. (1999a). ClpE, a novel type of HSP100 ATPase, is part of the CtsR heat shock regulon of *Bacillus subtilis*. *Mol Microbiol 32*, 581-593.

Derre, I., Rapoport, G. & Msadek, T. (1999b). CtsR, a novel regulator of stress and heat shock response, controls clp and molecular chaperone gene expression in gram-positive bacteria. *Mol Microbiol 31*, 117-131.

Dreyfus, M. & Regnier, P. (2002). The poly(A) tail of mRNAs: bodyguard in eukaryotes, scavenger in bacteria. *Cell 111*, 611-613.

Dubnau, D. & Davidoff-Abelson, R. (1971). Fate of transforming DNA following uptake by competent *Bacillus subtilis*. I. formation and properties of the donor-recipient complex. *J Mol Biol 56*, 209-221.

Dubnau, D., Hahn, J., Roggiani, M., Piazza, R. & Weinrauch, Y. (1994). Two-component regulators and genetic competence in *Bacillus subtilis*. *Res Microbiol 145*, 403-411.

Dubnau, D. (1999). DNA uptake in bacteria. *Annu Rev Microbiol 53*, 217-244.

Dubnau, D. & Lovett Jr., C. M. (2002). Transformation and recombination. In *Bacillus subtilis and its closest relatives: from Genes to Cells*, pp. 453-471. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, D. C.: American Society for Microbiology.

Duwat, P., Ehrlich, S. D. & Gruss, A. (1999). Effects of metabolic flux on stress response pathways in *Lactococcus lactis*. *Mol Microbiol 31*, 845-858.

Errington, J. (1991). Possible intermediate steps in the evolution of a prokaryotic developmental system. *Proceedings: Biological Sciences 244*, 117-121.

Frees, D., Varmanen, P. & Ingmer, H. (2001). Inactivation of a gene that is highly conserved in Gram-positive bacteria stimulates degradation of non-native proteins and concomitantly increases stress tolerance in *Lactococcus lactis*. *Mol Microbiol 41*, 93-103.

Fuchs, R. T., Grundy, F. J. & Henkin, T. M. (2007). S-adenosylmethionine directly inhibits binding of 30S ribosomal subunits to the SMK box translational riboswitch RNA. *Proc Natl Acad Sci U S A 104*, 4876-4880.

Gaballa, A. & Helmann, J. D. (1998). Identification of a Zinc-Specific Metalloregulatory Protein, Zur, Controlling Zinc Transport Operons in *Bacillus subtilis*. *J Bacteriol 180*, 5815-5821.

Gaballa, A. & Helmann, J. D. (2002). A peroxide-induced zinc uptake system plays an important role in protection against oxidative stress in *Bacillus subtilis*. *Mol Microbiol 45*, 997-1005.

Gaballa, A., Wang, T., Ye, R. W. & Helmann, J. D. (2002). Functional analysis of the *Bacillus subtilis* Zur regulon. *J Bacteriol 184*, 6508-6514.

Geng, H., Zhu, Y., Mullen, K., Zuber, C. S. & Nakano, M. M. (2006). Characterization of ResDE-dependent *fnr* transcription in *Bacillus subtilis*. *J Bacteriol*.

Gerth, U., Wipat, A., Harwood, C. R., Carter, N., Emmerson, P. T. & Hecker, M. (1996). Sequence and transcriptional analysis of *clpX*, a class-III heat-shock gene of *Bacillus subtilis*. *Gene 181*, 77-83.

Gerth, U., Kruger, E., Derre, I., Msadek, T. & Hecker, M. (1998). Stress induction of the *Bacillus subtilis clpP* gene encoding a homologue of the proteolytic component of the Clp protease and the involvement of ClpP and ClpX in stress tolerance. *Mol Microbiol 28*, 787-802.

Ghezzi, P. (2005). Oxidoreduction of protein thiols in redox regulation. *Biochem Soc Trans 33*, 1378-1381.

Gonciarz-Swiatek, M., Wawrzynow, A., Um, S. J., Learn, B. A., McMacken, R., Kelley, W. L., Georgopoulos, C., Sliekers, O. & Zylicz, M. (1999). Recognition, targeting, and hydrolysis of the lambda O replication protein by the ClpP/ClpX protease. *J Biol Chem 274*, 13999-14005.

Gottesman, S. (1996). Proteases and their targets in *Escherichia coli*. *Annu Rev Genet 30*, 465-506.

Gottesman, S., Roche, E., Zhou, Y. & Sauer, R. T. (1998). The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes Dev 12*, 1338-1347.

Gregory, B. D., Nickels, B. E., Garrity, S. J. & other authors (2004). A regulator that inhibits transcription by targeting an intersubunit interaction of the RNA polymerase holoenzyme. *Proc Natl Acad Sci U S A 101*, 4554-4559.

Gross, C. A. (1996). Function and regulation of the heat shock proteins. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, pp. 1382-1399. Edited by F. C. Neidhardt. Washington D. C.: American Society for Microbiology.

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

Gruber, T. M. & Gross, C. A. (2003). Multiple sigma subunts and the partitioning of bacterial transcription space. *Annu Rev Microbiol 57*, 441-466.

Grundy, F. J. & Henkin, T. M. (1992). Characterization of the *Bacillus subtilis rpsD* regulatory target site. *J Bacteriol 174*, 6763-6770.

Grundy, F. J., Rollins, S. M. & Henkin, T. M. (1994). Interaction between the acceptor end of tRNA and the T box stimulates antitermination in the *Bacillus subtilis tyrS* gene: a new role for the discriminator base. *J Bacteriol 176*, 4518-4526.

Guerout-Fleury, A. M., Shazand, K., Frandsen, N. & Stragier, P. (1995). Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene 167*, 335-336.

Guerout-Fleury, A. M., Frandsen, N. & Stragier, P. (1996). Plasmids for ectopic integration in *Bacillus subtilis*. *Gene 180*, 57-61.

Guigueno, A., Belin, P. & Boquet, P. L. (1997). Defective export in *Escherichia coli* caused by DsbA'-PhoA hybrid proteins whose DsbA' domain cannot fold into a conformation resistant to periplasmic proteases. *J Bacteriol 179*, 3260-3269.

Guigueno, A., Dassa, J., Belin, P. & Boquet, P. L. (2001). Oversynthesis of a new *Escherichia coli* small RNA suppresses export toxicity of DsbA'-PhoA unfoldable periplasmic proteins. *J Bacteriol 183*, 1147-1158.

Guo, F., Esser, L., Singh, S. K., Maurizi, M. R. & Xia, D. (2002). Crystal structure of the heterodimeric complex of the adaptor, ClpS, with the N-domain of the AAA+ chaperone, ClpA. *J Biol Chem 277*, 46753-46762.

Hahn, J., Roggiani, M. & Dubnau, D. (1995). The major role of Spo0A in genetic competence is to downregulate *abrB*, an essential competence gene. *J Bacteriol 177*, 3601-3605.

Hahn, J., Luttinger, A. & Dubnau, D. (1996). Regulatory inputs for the synthesis of ComK, the competence
transcription factor of *Bacillus subtili*s. *Mol Microbiol 21*, 763-775.

Haldenwang, W. G. & Losick, R. (1980). A novel RNA polymerase sigma factor from *Bacillus subtilis*. *Proc Natl Acad Sci 77*, 7000-7005.

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

Hamoen, L. W., Eshuis, H., Jongbloed, J., Venema, G. & van Sinderen, D. (1995). A small gene, designated *comS*, located within the coding region of the fourth amino acid-activation domain of *srfA*, is required for competence development in *Bacillus subtilis*. *Mol Microbiol 15*, 55-63.

Hamoen, L. W., Venema, G. & Kuipers, O. P. (2003). Controlling competence in *Bacillus subtilis*: shared use of regulators. *Microbiology 149*, 9-17.

Hanukoglu, I. (2006). Antioxidant protective mechanisms against reactive oxygen species (ROS) generated by mitochondrial P450 dystems in steroidogenic cells. *Drug Metabolism Reviews 38*, 171 - 196.

Harwood, C. R. (1992). *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. *Trends Biotechnol 10*, 247-256.

Hecker, M. & Volker, U. (1998). Non-specific, general and multiple stress resistance of growth-restricted *Bacillus subtilis* cells by the expression of the sigmaB regulon. *Mol Microbiol 29*, 1129-1136.

Henkin, T. M. & Yanofsky, C. (2002). Regulation by transcription attenuation in bacteria: how RNA provides instructions for transcription termination/antitermination decisions. *BioEssays 24*, 700-707.

Hennecke, J., Sebbel, P. & Glockshuber, R. (1999). Random circular permutation of DsbA reveals segments that are essential for protein folding and stability. *J Mol Biol 286*, 1197-1215.

Hille, R. & Massey, V. (1981). Studies on the oxidative half-reaction of xanthine oxidase. *J Biol Chem 256*, 9090-9095.

Hinnerwisch, J., Reid, B. G., Fenton, W. A. & Horwich, A. L. (2005). Roles of the N-domains of the ClpA unfoldase in binding substrate proteins and in stable complex formation with the ClpP protease. *J Biol Chem 280*, 40838-40844.

Hoch, J. A., Barat, M. & Anagnostopoulos, C. (1967). Transformation and transduction in recombination-defective mutants of *Bacillus subtilis*. *J Bacteriol 93*, 1925-1937.

Hochgrafe, F., Mostertz, J., Pother, D.-C., Becher, D., Helmann, J. D. & Hecker, M. (2007). S-cysteinylation is a general mechanism for thiol protection of *Bacillus subtilis* proteins after oxidative stress. *J Biol Chem*, C700105200.

Hondorp, E. R. & Matthews, R. G. (2004). Oxidative stress inactivates cobalamin-independent methionine synthase (MetE) in *Escherichia coli*. *PLoS Biol 2*, e336.

Igarashi, K., Hanamura, A., Makino, K., Aiba, H., Aiba, H., Mizuno, T., Nakata, A. & Ishihama, A. (1991). Functional map of the α subunit of *Escherichia coli* RNA polymerase: Two modes of transcription activation by positive factors. *Proc Natl Acad Sci 88*, 8958-8962.

Igarashi, K. & Ishihama, A. (1991). Bipartite functional map of the *E. coli* RNA polymerase alpha subunit: involvement of the C-terminal region in transcription activation by cAMP-CRP. *Cell 65*, 1015-1022.

Ilbert, M., Graf, P. C. & Jakob, U. (2006). Zinc center as redox switch--new function for an old motif. *Antioxid Redox Signal 8*, 835-846.

Ilbert, M., Horst, J., Ahrens, S., Winter, J., Graf, P. C. F., Lilie, H. & Jakob, U. (2007). The redox-switch domain of Hsp33 functions as dual stress sensor. *Nat Struct Mol Biol 14*, 556-563.

Imlay, J. A. (2003). Pathways of oxidative damage. *Annu Rev Microbiol 57*, 395-418.

Ingmer, H., Vogensen, F. K., Hammer, K. & Kilstrup, M. (1999). Disruption and analysis of the *clpB*, *clpC*, and *clpE* genes in *Lactococcus lactis*: ClpE, a new Clp family in Gram-positive bacteria. *J Bacteriol 181*, 2075-2083.

Ishikawa, S., Core, L. & Perego, M. (2002). Biochemical Characterization of Aspartyl Phosphate Phosphatase Interaction with a Phosphorylated Response Regulator and Its Inhibition by a Pentapeptide. *J Biol Chem 277*, 20483-20489.

Jakob, U., Eser, M. & Bardwell, J. C. (2000). Redox switch of Hsp33 has a novel zinc-binding motif. *J Biol Chem 275*, 38302-38310.

Jenkins, L. M., Durell, S. R., Maynard, A. T., Stahl, S. J., Inman, J. K., Appella, E., Legault, P. & Omichinski, J. G. (2006). Comparison of the specificity of interaction of cellular and viral zinc-binding domains with 2-mercaptobenzamide thioesters. *J Am Chem Soc 128*, 11964-11976.

Jeon, Y. H., Negishi, T., Shirakawa, M., Yamazaki, T., Fujita, N., Ishihama, A. & Kyogoku, Y. (1995). Solution structure of the activator contact domain of the RNA polymerase alpha subunit. *Science 270*, 1495-1497.

Jiang, M., Shao, W., Perego, M. & Hoch, J. A. (2000). Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. *Mol Microbiol 38*, 535-542.

Jones, J. M., Welty, D. J. & Nakai, H. (1998). Versatile action of *Escherichia coli* ClpXP as protease or molecular chaperone for bacteriophage Mu transposition. *J Biol Chem 273*, 459-465.

Juang, Y.-L. & Helmann, J. D. (1994). The [delta] subunit of *Bacillus subtilis* RNA polymerase : an allosteric effector of the initiation and core-recycling phases of transcription. *J Mol Biol 239*, 1-14.

Kadokura, H., Nichols, L., II & Beckwith, J. (2005). Mutational alterations of the key cis proline residue that cause accumulation of enzymatic reaction intermediates of DsbA, a member of the thioredoxin superfamily. *J Bacteriol 187*, 1519-1522.

Kang, M. S., Lim, B. K., Seong, I. S., Seol, J. H., Tanahashi, N., Tanaka, K. & Chung, C. H. (2001). The ATP-dependent CodWX (HslVU) protease in *Bacillus subtilis* is an N-terminal serine protease. *EMBO J 20*, 734-742.

Katayama-Fujimura, Y., Gottesman, S. & Maurizi, M. R. (1987). A multiple-component, ATP-dependent protease from *Escherichia coli*. *J Biol Chem 262*, 4477-4485.

Keiler, K. C., Waller, P. R. & Sauer, R. T. (1996). Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science 271*, 990-993.

Kenney, L. J., Bauer, M. D. & Silhavy, T. J. (1995). Phosphorylation-dependent conformational changes in OmpR, an osmoregulatory DNA-binding protein of *Escherichia coli*. *Proc Natl Acad Sci U S A 92*, 8866-8870.

Kenney, T. J. & Moran Jr, C. P. (1991). Genetic evidence for interaction of sigma A with two promoters in *Bacillus subtilis*. *J Bacteriol 173*, 3282-3290.

Kenniston, J. A., Baker, T. A., Fernandez, J. M. & Sauer, R. T. (2003). Linkage between ATP consumption and mechanical unfolding during the protein processing reactions of an AAA+ degradation machine *Cell 114*, 511-520.

Kessel, M., Maurizi, M. R., Kim, B., Kocsis, E., Trus, B. L., Singh, S. K. & Steven, A. C. (1995). Homology in structural organization between *E. coli* ClpAP protease and the eukaryotic 26 S proteasome. *J Mol Biol 250*, 587-594.

Kim, Y.-I., Levchenko, I., Fraczkowska, K., Woodruff, R. V., Sauer, R. T. & Baker, T. A. (2001). Molecular determinants of complex formation between Clp/Hsp100 ATPases and the ClpP peptidase. *Nat Struct Mol Biol 8*, 230-233.

Kirstein, J., Schlothauer, T., Dougan, D. A., Lilie, H., Tischendorf, G., Mogk, A., Bukau, B. & Turgay, K. (2006). Adaptor protein controlled oligomerization activates the AAA+ protein ClpC. *EMBO J 25*, 1481-1491.

Kock, H., Gerth, U. & Hecker, M. (2004). The ClpP peptidase is the major determinant of bulk protein turnover in *Bacillus subtilis*. *J Bacteriol 186*, 5856-5864.

Krásný, L. & Gourse, R. L. (2004). An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation. *EMBO J 23*, 4473-4483.

Kruger, E., Volker, U. & Hecker, M. (1994). Stress induction of *clpC* in *Bacillus subtilis* and its involvement in stress tolerance. *J Bacteriol 176*.

Kruger, E. & Hecker, M. (1998). The first gene of the *Bacillus subtilis clpC* operon, *ctsR*, encodes a negative regulator of its own operon and other class III heat shock genes. *J Bacteriol 180*, 6681-6688.

Kruger, E., Witt, E., Ohlmeier, S., Hanschke, R. & Hecker, M. (2000). The Clp proteases of *Bacillus subtilis* are directly involved in degradation of misfolded proteins. *J Bacteriol 182*, 3259-3265.

Kunst, F., Ogasawara, N., Moszer, I. & other authors (1997). The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature 390*, 249-256.

Kurnasov, O., Jablonski, L., Polanuyer, B., Dorrestein, P., Begley, T. & Osterman, A. (2003). Aerobic tryptophan degradation pathway in bacteria: novel kynurenine formamidase. *FEMS Microbiol Lett 227*, 219-227.

Kuznedelov, K., Minakhin, L., Niedziela-Majka, A., Dove, S. L., Rogulja, D., Nickels, B. E., Hochschild, A., Heyduk, T. & Severinov, K. (2002). A role for interaction of the RNA polymerase flap domain with the sigma subunit in promoter recognition. *Science 295*, 855-857.

Lacal, J., Busch, A., Guazzaroni, M.-E., Krell, T. & Ramos, J. L. (2006). The TodS-TodT two-component regulatory system recognizes a wide range of effectors and works with DNA-bending proteins. *Proc Natl Acad Sci U S A 103*, 8191-8196.

Landick, R., Turnbough, C. L. J. & Yanofsky., C. (1996). Transcription attenuation. In *Escherichia coli and Salmonella:cellular and molecular biology*, pp. 1263-1286. Edited by F. C. Neidhardt, R. Curtiss III, J. L. Ingraham & other authors: ASM Press, Washington, D.C.

Larsson, J. T., Rogstam, A. & Wachenfeldt, C. v. (2007). YjbH is a novel negative effector of the disulphide stress regulator, Spx, in *Bacillus subtilis*. *Mol Microbiol*.

Lazazzera, B. A., Solomon, J. M. & Grossman, A. D. (1997). An exported peptide functions intracellularly to contribute to cell density signaling in *B. subtilis*. *Cell 89*, 917-925.

Lazazzera, B. A. & Grossman, A. D. (1998). The ins and outs of peptide signaling. *Trends Microbiol 6*, 288-294.

Lease, R. A. & Belfort, M. (2000). From the cover: a trans-acting RNA as a control switch in *Escherichia coli*: DsrA modulates function by forming alternative structures. *Proc Natl Acad Sci U S A 97*, 9919-9924.

Lee, J. W. & Helmann, J. D. (2006). The PerR transcription factor senses H_2O_2 by metal-catalysed histidine oxidation. *Nature 440*, 363-367.

Leelakriangsak, M., Kobayashi, K. & Zuber, P. (2007). Dual negative control of *spx* transcription initiation from the P3 promoter by repressors PerR and YodB in *Bacillus subtilis*. *J Bacteriol 189*, 1736-1744.

Leelakriangsak, M. & Zuber, P. (2007). Transcription from the P3 promoter of the *Bacillus subtilis spx* gene is induced in response to disulfide stress. *J Bacteriol 189*, 1727-1735.

Leichert, L. I., Scharf, C. & Hecker, M. (2003). Global characterization of disulfide stress in *Bacillus subtilis*. *J Bacteriol 185*, 1967-1975.

Leroyer, V., Werner, L., Shaughnessy, S., Goddard, G. J. & Orr, F. W. (1987). Chemiluminescence and oxygen radical generation by walker carcinosarcoma cells following chemotactic stimulation. *Cancer Res 47*, 4771-4775.

Levchenko, I., Yamauchi, M. & Baker, T. A. (1997). ClpX and MuB interact with overlapping regions of Mu transposase: implications for control
of the transposition pathway. *Genes Dev 11*, 1561-1572.

Levchenko, I., Seidel, M., Sauer, R. T. & Baker, T. A. (2000). A specificity-enhancing factor for the ClpXP degradation machine. *Science 289*, 2354-2356.

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

Liu, J. & Zuber, P. (2000). The ClpX protein of *Bacillus subtilis* indirectly influences RNA polymerase holoenzyme composition and directly stimulates sigmaH-dependent transcription. *Mol Microbiol 37*, 885-897.

Lloyd, G. S., Niu, W., Tebbutt, J., Ebright, R. H. & Busby, S. J. W. (2002). Requirement for two copies of RNA polymerase alpha subunit C-terminal domain for synergistic transcription activation at complex bacterial promoters. *Genes Dev 16*, 2557-2565.

Lonetto, M., Gribskov, M. & Gross, C. A. (1992). The sigma 70 family: sequence conservation and evolutionary relationships. *J Bacteriol 174*, 3843-3849.

Macek, B., Mijakovic, I., Olsen, J. V., Gnad, F., Kumar, C., Jensen, P. R. & Mann, M. (2007). The serine/threonine/tyrosine phosphoproteome of the model bacterium *Bacillus subtilis*. *Mol Cell Proteomics 6*, 697-707.

Madigan, M. & Martinko, J. (2005). Brock Biology of Microorganisms, 11th ed.

Martin, J. L., Bardwell, J. C. A. & Kuriyan, J. (1993). Crystal structure of the DsbA protein required for disulphide bond formation *in vivo*. *Nature 365*, 464-468.

Martin, P., DeMel, S., Shi, J., Gladysheva, T., Gatti, D. L., Rosen, B. P. & Edwards, B. F. (2001). Insights into the structure, solvation, and mechanism of ArsC arsenate reductase, a novel arsenic detoxification enzyme. *Structure (Camb) 9*, 1071-1081.

Maurizi, M. R., Clark, W. P., Kim, S. H. & Gottesman, S. (1990). ClpP represents a unique family of serine proteases. *J Biol Chem 265*, 12546-12552.

McDaniel, B. A. M., Grundy, F. J., Artsimovitch, I. & Henkin, T. M. (2003). Transcription termination control of the S box system: Direct measurement of S-adenosylmethionine by the leader RNA. *Proc Natl Acad Sci U S A 100*, 3083-3088.

Meijer, W. J. J. & Salas, M. (2004). Relevance of UP elements for three strong *Bacillus subtilis* phage {phi}29 promoters. *Nucl Acids Res 32*, 1166-1176.

Mejlhede, N. & Neuhard, J. (2000). The role of zinc in *Bacillus subtilis* cytidine deaminase. *Biochemistry 39*, 7984-7989.

Mencia, M., Monsalve, M., Rojo, F. & Salas, M. (1998). Substitution of the C-terminal domain of the *Escherichia coli* RNA polymerase alpha subunit by that from *Bacillus*

subtilis makes the enzyme responsive to a *Bacillus subtilis* transcriptional activator. *J Mol Biol 275*, 177-185.

Miethke, M., Hecker, M. & Gerth, U. (2006). Involvement of *Bacillus subtilis* ClpE in CtsR degradation and protein quality control. *J Bacteriol 188*, 4610-4619.

Miller, J. H. (1972). *Experiments in molecular genetics*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

Missiakas, D. & Raina, S. (1998). The extracytoplasmic function sigma factors: role and regulation. *Mol Microbiol 28*, 1059-1066.

Mizuno, T., Chou, M. Y. & Inouye, M. (1984). A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). *Proc Natl Acad Sci U S A 81*
1966-1970.

Mogk, A., Homuth, G., Scholz, C., Kim, L., Schmid, F. X. & Schumann, W. (1997). The GroE chaperonin machine is a major modulator of the CIRCE heat shock regulon of *Bacillus subtilis*. *EMBO J 16*, 4579-4590.

Moll, I., Afonyushkin, T., Vytvytska, O., Kaberdin, V. R. & Bläsi, U. (2003). Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs. *RNA 9*, 1308-1314.

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

Mukherji, M. (2005). Phosphoproteomics in analyzing signaling pathways. *Expert Review of Proteomics 2*, 117-128.

Nagai, H. & Shimamoto, N. (1997). Regions of the *Escherichia coli* primary sigma factor sigma70 that are involved in interaction with RNA polymerase core enzyme. *Genes Cells 2*, 725-734.

Nakano, M. M., Marahiel, M. A. & Zuber, P. (1988). Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. *J Bacteriol 170*, 5662-5668.

Nakano, M. M. & Zuber, P. (1989). Cloning and characterization of *srfB*: A regulatory gene involved in surfactin production and competence in *Bacillus subtilis*. *J Bacteriol 171*, 5347-5353.

Nakano, M. M., Magnuson, R., Myers, A., Curry, J., Grossman, A. D. & Zuber, P. (1991). *srfA* is an operon required for surfactin production, competence development, and efficient sporulation in *Bacillus subtilis*. *J Bacteriol 173*, 1770-1778.

Nakano, M. M. & Zuber, P. (1993). Mutational analysis of the regulatory region of the *srfA* operon in *Bacillus subtilis*. *J Bacteriol 175*, 3188-3191.

Nakano, M. M., Zuber, P., Glaser, P., Danchin, A. & Hulett, F. M. (1996). Two-component regulatory proteins ResD-ResE are required for transcriptional activation of *fnr* upon oxygen limitation in *Bacillus subtilis*. *J Bacteriol 178*, 3796-3802.

Nakano, M. M., Zhu, Y., Liu, J., Reyes, D. Y., Yoshikawa, H. & Zuber, P. (2000). Mutations conferring amino acid residue substitutions in the carboxy-terminal domain of RNA polymerase α can suppress *clpX* and *clpP* with respect to developmentally regulated transcription in *Bacillus subtilis*. *Mol Microbiol 37*, 869-884.

Nakano, M. M., Hajarizadeh, F., Zhu, Y. & Zuber, P. (2001). Loss-of-function mutations in *yjbD* result in ClpX- and ClpP-independent competence development of *Bacillus subtilis*. *Mol Microbiol 42*, 383-394.

Nakano, M. M., Nakano, S. & Zuber, P. (2002a). Spx (YjbD), a negative effector of competence in *Bacillus subtilis*, enhances ClpC-MecA-ComK interaction. *Mol Microbiol 44*, 1341-1349.

Nakano, S., Zheng, G., Nakano, M. M. & Zuber, P. (2002b). Multiple pathways of Spx (YjbD) proteolysis in *Bacillus subtilis*. *J Bacteriol 184*, 3664-3670.

Nakano, S., Küster-Schöck, E., Grossman, A. D. & Zuber, P. (2003a). Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*. *Proc Natl Acad Sci U S A 100*, 13603-13608.

Nakano, S., Nakano, M. M., Zhang, Y., Leelakriangsak, M. & Zuber, P. (2003b). A regulatory protein that interferes with activator-stimulated transcription in bacteria. *Proc Natl Acad Sci U S A 100*, 4233-4238.

Nakano, S., Erwin, K. N., Ralle, M. & Zuber, P. (2005). Redox-sensitive transcriptional control by a thiol/disulphide switch in the global regulator, Spx. *Mol Microbiol 55*, 498-510.

Natori, Y., Nanamiya, H., Akanuma, G., Kosono, S., Kudo, T., Ochi, K. & Kawamura, F. (2007). A fail-safe system for the ribosome under zinc-limiting conditions in *Bacillus subtilis*. *Mol Microbiol 63*, 294-307.

Neher, S. B., Villen, J., Oakes, E. C., Bakalarski, C. E., Sauer, R. T., Gygi, S. P. & Baker, T. A. (2006). Proteomic profiling of ClpXP substrates after DNA damage reveals extensive instability within SOS regulon. *Mol Cell 22*, 193-204.

Neuwald, A. F., Aravind, L., Spouge, J. L. & Koonin, E. V. (1999). AAA^{+}: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res 9*, 27-41.

Newberry, K. J., Nakano, S., Zuber, P. & Brennan, R. G. (2005). Crystal structure of the *Bacillus subtilis* anti-alpha, global transcriptional regulator, Spx, in complex with the alpha C-terminal domain of RNA polymerase. *Proc Natl Acad Sci U S A 102*, 15839-15844.

Newton, G. L., Arnold, K., Price, M. S. & other authors (1996). Distribution of thiols in microorganisms: mycothiol is a major thiol in most actinomycetes. *J Bacteriol 178*, 1990-1995.

Niaudet, B. & Ehrlich, S. D. (1979). *In vitro* genetic labeling of *Bacillus subtilis* cryptic plasmid pHV400. *Plasmid 2*, 48-58.

Nickels, B. E., Garrity, S. J., Mekler, V., Minakhin, L., Severinov, K., Ebright, R. H. & Hochschild, A. (2005). The interaction between {sigma}70 and the {beta}-flap of *Escherichia coli* RNA polymerase inhibits extension of nascent RNA during early elongation. *Proc Natl Acad Sci U S A 102*, 4488-4493.

Ondo-Mbele, E., Vivès, C., Koné, A. & Serre, L. (2005). Intriguing conformation changes associated with the trans/cis isomerization of a prolyl residue in the active site of the DsbA C33A mutant. *J Mol Biol 347*, 555-563.

Paget, M. & Helmann, J. (2003). The sigma70 family of sigma factors. *Genome Biology 4*, 203.

Pamp, S. J., Frees, D., Engelmann, S., Hecker, M. & Ingmer, H. (2006). Spx is a global effector impacting stress tolerance and biofilm formation in *Staphylococcus aureus*. *J Bacteriol 188*, 4861-4870.

Pan, Q., Garsin, D. A. & Losick, R. (2001). Self-reinforcing activation of a cell-specific transcription factor by proteolysis of an anti-σ factor in *B. subtilis*. *Mol Cell 8*, 873-883.

Pande, S., Makela, A., Dove, S. L., Nickels, B. E., Hochschild, A. & Hinton, D. M. (2002). The bacteriophage T4 transcription activator MotA interacts with the far-C-terminal region of the sigma70 subunit of *Escherichia coli* RNA polymerase. *J Bacteriol 184*, 3957-3964.

Park, E. Y., Lee, B. G., Hong, S. B., Kim, H. W., Jeon, H. & Song, H. K. (2007). Structural basis of SspB-tail recognition by the zinc binding domain of ClpX. *J Mol Biol DOI*.

Parkinson, J. S. (1993). Signal transduction schemes of bacteria. *Cell 73*, 857-871.

Pawson, T. & Scott, J. D. (2005). Protein phosphorylation in signaling - 50 years and counting. *Trends in Biochemical Sciences 30*, 286-290.

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

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

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

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

Persuh, M., Mandic-Mulec, I. & Dubnau, D. (2002). A MecA paralog, YpbH, binds ClpC, affecting both competence and sporulation. *J Bacteriol 184*, 2310-2313.

Piggot, P. J. & Coote, J. G. (1976). Genetic aspects of bacterial endospore formation. *Bacteriol Rev 40*, 908-962.

Punchard, N. A. & Kelly, F. J. e. (1996). Free radicals: a practical approach *Oxford:IRL Press*.

Qi, Y. & Hulett, F. M. (1998). PhoP-P and RNA polymerase sigmaA holoenzyme are sufficient for transcription of Pho regulon promoters in *Bacillus subtilis*: PhoP-P activator sites within the coding region stimulate transcription *in vitro. Mol Microbiol 28*, 1187-1197.

Reischl, S., Wiegert, T. & Schumann, W. (2002). Isolation and analysis of mutant alleles of the *Bacillus subtilis* HrcA repressor with reduced dependency on GroE function. *J Biol Chem 277*, 32659-32667.

Riethdorf, S., Volker, U., Gerth, U., Winkler, A., Engelmann, S. & Hecker, M. (1994). Cloning, nucleotide sequence, and expression of the *Bacillus subtilis lon* gene. *J Bacteriol 176*, 6518-6527.

Ritz, D. & Beckwith, J. (2001). Roles of thiol-redox pathways in bacteria. *Annu Rev Microbiol 55*, 21-48.

Roggiani, M. & Dubnau, D. (1993). ComA, a phosphorylated response regulator protein of *Bacillus subtilis*, binds to the promoter region of *srfA*. *J Bacteriol 175*, 3182-3187.

Rogstam, A., Larsson, J. T., Kjelgaard, P. & von Wachenfeldt, C. (2007). Mechanisms of Adaptation to Nitrosative Stress in *Bacillus subtilis*. *J Bacteriol 189*, 3063-3071.

Rosenkrantz, M. S., Dingman, D. W. & Sonenshein, A. L. (1985). *Bacillus subtilis citB* gene is regulated synergistically by glucose and glutamine. *J Bacteriol 164*, 155-164.

Ross, W., Gosink, K. K., Salomon, J., Igarashi, K., Zou, C., Ishihama, A., Severinov, K. & Gourse, R. L. (1993). A third recognition element in bacterial promoters: DNA binding by the alpha subunit of RNA polymerase. *Science 262*, 1407-1413.

Ross, W., Schneider, D. A., Paul, B. J., Mertens, A. & Gourse, R. L. (2003). An intersubunit contact stimulating transcription initiation by *E coli* RNA polymerase: interaction of the alpha C-terminal domain and sigma region 4. *Genes Dev 17*, 1293-1307.

Ross, W. & Gourse, R. L. (2005). Sequence-independent upstream DNA-{alpha}CTD interactions strongly stimulate *Escherichia coli* RNA polymerase-lacUV5 promoter association. *Proc Natl Acad Sci U S A 102*, 291-296.

Rowland, S. L., Burkholder, W. F., Cunningham, K. A., Maciejewski, M. W., Grossman, A. D. & King, G. F. (2004). Structure and mechanism of action of Sda, an inhibitor of the histidine kinases that regulate initiation of sporulation in *Bacillus subtilis*. *Mol Cell 13*, 689-701.

Russel, M., Model, P. & Holmgren, A. (1990). Thioredoxin or glutaredoxin in *Escherichia coli* is essential for sulfate reduction but not for deoxyribonucleotide synthesis. *J Bacteriol 172*, 1923-1929.

Ruvolo, M. V., Mach, K. E. & Burkholder, W. F. (2006). Proteolysis of the replication checkpoint protein Sda is necessary for the efficient initiation of sporulation after transient replication stress in *Bacillus subtilis*. *Mol Microbiol 60*, 1490-1508.

Ryan, K. J. & Ray, C. G. (2004). Sherris Medical Microbiology, 4th ed.

Sagara, Y., Dargusch, R., Chambers, D., Davis, J., Schubert, D. & Maher, P. (1998). Cellular mechanisms of resistance to chronic oxidative stress. *Free Radic Biol Med 24*, 1375-1389.

Sauer, R. T., Bolon, D. N., Burton, B. M. & other authors (2004). Sculpting the proteome with AAA(+) proteases and disassembly machines. *Cell 119*, 9-18.

Savijoki, K., Ingmer, H., Frees, D., Vogensen, F. K., Palva, A. & Varmanen, P. (2003). Heat and DNA damage induction of the LexA-like regulator HdiR from *Lactococcus lactis* is mediated by RecA and ClpP. *Mol Microbiol 50*, 609-621.

Schaeffer, P., Millet, J. & J.-P., A. (1965). Catabolic repression of bacterial sporulation. *Proc Natl Acad Sci 54*, 704-711.

Scharf, C., Riethdorf, S., Ernst, H., Engelmann, S., Volker, U. & Hecker, M. (1998). Thioredoxin is an essential protein induced by multiple stresses in *Bacillus subtilis*. *J Bacteriol 180*, 1869-1877.

Sies, H. (1985). Oxidative stress. *Academic, London*.

Simeonov, M. F., Bieber Urbauer, R. J., Gilmore, J. M., Adelman, K., Brody, E. N., Niedziela-Majka, A., Minakhin, L., Heyduk, T. & Urbauer, J. L. (2003). Characterization of the interactions between the bacteriophage T4 AsiA protein and RNA polymerase. *Biochemistry 42*, 7717-7726.

Solomon, J. M., Magnuson, R., Srivastava, A. & Grossman, A. D. (1995). Convergent sensing pathways mediate response to two extracellular competence factors in *Bacillus subtilis*. *Genes Dev 9*, 547-558.

Stadtman, E. R. (1993). Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annual Review of Biochemistry 62*, 797-821.

Stragier, P. & Losick, R. (1996). Molecular genetics of sporulation in *Bacillus subtilis*. *Annu Rev Genet 30*, 297-341.

Szalewska-Palasz, A., Johansson, L. U. M., Bernardo, L. M. D., Skarfstad, E., Stec, E., Brannstrom, K. & Shingler, V. (2007). Properties of RNA polymerase bypass mutants:implications for the role of ppGpp and its co-factor DksA in controlling transcription dependent on sigma-54. *J Biol Chem 282*, 18046-18056.

Thackray, P. D. & Moir, A. (2003). SigM, an extracytoplasmic function sigma factor of *Bacillus subtilis*, is activated in response to cell wall antibiotics, ethanol, heat, acid, and superoxide stress. *J Bacteriol 185*, 3491-3498.

Thibault, G., Tsitrin, Y., Davidson, T., Gribun, A. & Houry, W. A. (2006a). Large nucleotide-dependent movement of the N-terminal domain of the ClpX chaperone. *EMBO J 25*, 3367-3376.

Thibault, G., Yudin, J., Wong, P., Tsitrin, V., Sprangers, R., Zhao, R. & Houry, W. A. (2006b). Specificity in substrate and cofactor recognition by the N-terminal domain of the chaperone ClpX. *Proc Natl Acad Sci U S A 103*, 17724-17729.

Turgay, K., Hamoen, L. W., Venema, G. & Dubnau, D. (1997). Biochemical characterization of a molecular switch involving the heat shock protein ClpC, which controls the activity of ComK, the competence transcription factor of *Bacillus subtilis*. *Genes Dev 11*, 119-128.

Turgay, K., Hahn, J., Burghoorn, J. & Dubnau, D. (1998). Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. *EMBO J 17*, 6730-6738.

Tzeng, Y. L., Feher, V. A., Cavanagh, J., Perego, M. & Hoch, J. A. (1998). Characterization of interactions between a two-component response regulator, Spo0F, and its phosphatase, RapB. *Biochemistry 37*, 16538-16545.

Valentin-Hansen, P., Eriksen, M. & Udesen, C. (2004). The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol Microbiol 51*, 1525-1533.

Vallee, B. L. & Falchuk, K. H. (1993). The biochemical basis of zinc physiology. *Physiol Rev 73*, 79-118.

van Sinderen, D., Galli, G., Cosmina, P., de Ferra, F., Withoff, S., Venema, G. & Grandi, G. (1993). Characterization of the *srfA* locus of *Bacillus subtilis*: only the valine-activating domain of *srfA* is involved in the establishment of genetic competence. *Mol Microbiol 8*, 833-841.

van Sinderen, D. & Venema, G. (1994). *comK* acts as an autoregulatroy control switch in the signal transduction route to competence in *Bacillus subtilis*. *J Bacteriol 176*, 5762-5770.

Varmanen, P., Vogensen, F. K., Hammer, K., Palva, A. & Ingmer, H. (2003). ClpE from *Lactococcus lactis* promotes repression of CtsR-dependent gene expression. *J Bacteriol 185*, 5117-5124.

Veiga, P., Bulbarela-Sampieri, C., Furlan, S. & other authors (2007). SpxB regulates O-acetylation-dependent resistance of *Lactococcus lactis* peptidoglycan to hydrolysis. *J Biol Chem 282*, 19342-19354.

Wagner, E. G. H., Altuvia, S. & Romby, P. (2002). Antisense RNAs in bacteria and their genetic elements. *Adv Genet 46*, 361-398.

Wang, J. & Fung, D. Y. C. (1996). Alkaline-fermented foods: A review with emphasis on pidan fermentation. *Crit Rev Microbiol 22*, 101-138.

Wang, J., Hartling, J. A. & Flanagan, J. M. (1997). The structure of ClpP at 2.3 A resolution suggests a model for ATP-dependent proteolysis. *Cell 91*, 447-456.

Wang, J. D., Sanders, G. M. & Grossman, A. D. (2007). Nutritional control of elongation of DNA replication by (p)ppGpp. *Cell 128*, 865-875.

Wassarman, K. M. & Saecker, R. M. (2006). Synthesis-mediated release of a small RNA inhibitor of RNA polymerase. *Science 314*, 1601-1603.

Watson, W. H., Yang, X., Choi, Y. E., Jones, D. P. & Kehrer, J. P. (2004). Thioredoxin and its role in toxicology. *Toxicol Sci 78*, 3-14.

Weart, R. B., Nakano, S., Lane, B. E., Zuber, P. & Levin, P. A. (2005). The ClpX chaperone modulates assembly of the tubulin-like protein FtsZ *Mol Microbiol 57*, 238–249.

Weinrauch, Y., Penchev, R., Dubnau, E., Smith, I. & Dubnau, D. (1990). A *Bacillus subtilis* regulatory gene product for genetic competence and sporulation resembles sensor protein members of the bacterial two-component signal-transduction systems. *Genes Dev 4*, 860-872.

Wickner, S., Maurizi, M. R. & Gottesman, S. (1999). Posttranslational quality control: folding, refolding, and degrading proteins. *Science 286*, 1888-1893.

Wiegert, T. & Schumann, W. (2001). SsrA-mediated tagging in *Bacillus subtilis*. *J Bacteriol 183*, 3885-3889.

Wojtyra, U. A., Thibault, G., Tuite, A. & Houry, W. A. (2003). The N-terminal zinc binding domain of ClpX is a dimerization domain that modulates the chaperone function. *J Biol Chem in press*.

Wu, C. C., Kuo, S. C., Lee, F. Y. & Teng, C. M. (1999). YC-1 potentiates the antiplatelet effect of hydrogen peroxide via sensitization of soluble guanylate cyclase. *Eur J Pharmacol 381*, 185-191.

Wunderlich, M., Otto, A., Maskos, K., Mu, M., Seckler, R. & Glockshuber, R. (1995). Efficient catalysis of disulfide formation during protein folding with a single active-site cysteine. *J Mol Biol 247*, 28-33.

Xia, Y., Dawson, V. L., Dawson, T. M., Snyder, S. H. & Zweier, J. L. (1996). Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc Natl Acad Sci U S A 93*, 6770-6774.

Yamato, M., Shiba, T., Yoshida, M., Ide, T., Seri, N., Kudou, W., Kinugawa, S. & Tsutsui, H. (2007). Fatty acids increase the circulating levels of oxidative stress factors in mice with diet-induced obesity via redox changes of albumin. *FEBS Journal 274*, 3855-3863.

Yang, D.-H., von Kalckreuth, J. & Allmansberger, R. (1999). Synthesis of the sigma D protein is not sufficient to trigger expression of motility functions in *Bacillus subtilis*. *J Bacteriol 181*, 2942-2946.

Youngman, P., Poth, H., Green, B., York, K., Olmedo, G. & Smith, K. (1989). Methods for genetic manipulation, cloning, and functional analysis of sporulation genes in *Bacillus*

subtilis. In *Regulaiton of Procaryotic Development*, pp. 65-87. Edited by I. Smith, R. A. Slepecky & P. Setlow. Washington, D. C.: American Society for Microbiology.

Zellmeier, S., Schumann, W. & Wiegert, T. (2006). Involvement of Clp protease activity in modulating the *Bacillus subtilis* sigmaB stress response. *Mol Microbiol 61*, 1569-1582.

Zhang, A., Altuvia, S., Tiwari, A., Argaman, L., Hengge-Aronis, R. & Storz, G. (1998). The OxyS regulatory RNA represses *rpoS* translation and binds the Hfq(HF-1) protein. *EMBO J 17*, 6061-6068.

Zhang, Y. & Zuber, P. (7 September . 2007). Requirement of the Zinc-binding domain of ClpX for Spx proteolysis in *Bacillus subtilis* and Effects of disulfide stress on ClpXP Activity. *J Bacteriol*, 10.1128/JB.00745-00707.

Zhang, Y., Zhang, Z., Ling, L., Shi, B. & Chen, R. (2004). Conservation analysis of small RNA genes in *Escherichia coli*. *Bioinformatics 20*, 599-603.

Zhang, Y., Nakano, S., Choi, S. Y. & Zuber, P. (2006). Mutational analysis of the *Bacillus subtilis* RNA polymerase alpha C-terminal domain supports the interference model of Spx-dependent repression. *J Bacteriol 188*, 4300-4311.

Zhou, Y. & Gottesman, S. (1998). Regulation of proteolysis of the stationary-phase sigma factor RpoS. *J Bacteriol 180*, 1154-1158.

Zuber, P. & Losick, R. (1987). Role of AbrB in the Spo0A- and Spo0B-dependent utilization of a sporulation promoter in *Bacillus subtilis*. *J Bacteriol 169*, 2223-2230.

Zuber, P. (2004). Spx-RNA polymerase interaction and global transcriptional control during oxidative stress. *J Bacteriol 186*, 1911-1918.

Zuber, U., Drzewiecki, K. & Hecker, M. (2001). Putative sigma factor SigI (YkoZ) of *Bacillus subtilis* is induced by heat shock. *J Bacteriol 183*, 1472-1475.

BIOGRAPHICAL SKETCH

Ying Zhang was born in Shanghai, P.R.China on October 10, 1978. In 2001, she received her B.S. in Genetics from the Fudan University in Shanghai, P.R.China. She decided to continue with her Ph.D degree in Biochemistry and Molecular Biology at the Oregon Health and Science University under the supervision and research of Dr. Peter A. Zuber.

Publications

Nakano, S., Nakano, M. M., Zhang, Y., Leelakriangsak, M. & Zuber, P. (2003). A regulatory protein that interferes with activator-stimulated transcription in bacteria. *Proc Natl Acad Sci U S A* **100**, 4233-4238.

Zhang, Y., Nakano, S., Choi, S. Y. & Zuber, P. (2006). Mutational analysis of the *Bacillus subtilis* RNA polymerase alpha C-terminal domain supports the interference model of Spx-dependent repression. *J Bacteriol* **188**, 4300-4311

Zhang, Y., and P. Zuber 7 September 2007. Requirement of the Zinc-binding domain of ClpX for Spx proteolysis in *Bacillus subtilis* and Effects of disulfide stress on ClpXP Activity. *J. Bacteriol.* doi:10.1128/JB.00745-07

Conference presentation

Zhang, Y., and P. Zuber. A transcriptional regulatory protein that interferes with activator-stimulated transcription in *Bacillus subtilis*. Wind River Conference on Prokaryotic Biology 48th Annual Meeting, June, 2004

Zhang, Y., Nakano, S. and P.A. Zuber. RNA polymerase interaction with Spx and ComA~P in the control of ComA-dependent transcription in *Bacillus subtilis*. 3rd Conference on Functional Genomics of Gram-Positive Microorganisms 13th International Conference on *Bacilli*, June 12-16, 2005, San Diego, California