

Redox Control and Mechanism of Spx-activated Transcription
in *Bacillus subtilis*

By

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Abstract

Redox Control and Mechanism of Spx-activated Transcription in Bacillus subtilis

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Bacillus subtilis Spx is a global transcriptional regulator that is conserved among gram-positive bacteria, in which Spx is required for preventing oxidatively induced proteotoxicity. Upon stress induction, Spx engages RNA polymerase (RNAP) at least partially through interaction with the C-terminal domain of the *rpoA*-encoded RNAP α subunit (α CTD).

Results from previous studies indicate that Spx contact with α CTD is essential during Spx-activated transcription. Previous mutational analysis of *rpoA* revealed that substitutions of Y263 in α CTD severely impair Spx-activated transcription. Attempts to generate several other alanine codon substitution mutations in the part of *rpoA* encoding α CTD were unsuccessful, suggesting that the corresponding residues are essential. To determine whether these RpoA residues are required for

productive Spx-RNAP interaction, we ectopically expressed the putatively lethal *rpoA* mutant alleles in the *rpoAY263C* mutant. By complementation analysis, we determined that several Spx-bound α CTD amino acid residues are not essential for Spx-activated transcription *in vivo*, but some of the *rpoA* mutants confer a partial defect in NaCl-stress induction of Spx-controlled genes. Our findings suggest that, while α CTD is essential for Spx-activated transcription, Spx is the primary DNA-binding determinant of the Spx- α CTD complex. Also, a *sigAR362A* mutation negatively affects expression of Spx-activated genes, which suggests that region 4.2 of the σ^A subunit is required during Spx-activated transcription.

Intrinsic structural requirements for Spx-RNAP interaction have not been fully elucidated. α CTD may serve as one of multiple contact sites for Spx within RNAP and some Spx amino acid residues may facilitate Spx-RNAP interaction. Evidence suggests that some amino acid residues may facilitate Spx- α CTD interaction and possibly Spx- σ^A interaction. Structural modeling of the Spx- α CTD complex show that SpxK43, D40, and Q77 residues cluster at the Spx- α CTD interface and may be in close proximity to region 4.2 of σ^A . Spx-Ala codon substitution mutants were constructed to examine their effects on Spx-activated transcription. The results suggest that in addition to differential redox requirements of Spx, the functions of some Spx amino acid residues differ among Spx-regulated genes.

To alleviate oxidative stress, Spx activates thiol homeostasis genes including the thioredoxin (*trxA*), thioredoxin reductase (*trxB*), and bacillithiol (BSH) biosynthesis genes (*bshABC*). Findings from previous work support a model wherein Spx activity as a transcriptional activator is dependent on oxidation of its

C₁₀XXC₁₃ redox disulfide center. However, recent evidence suggests that expression of some members of the Spx regulon (e.g. *bsh* biosynthesis genes), do not required Spx in its oxidized disulfide form. Mutational analysis of promoters of *bsh* biosynthetic gene containing operons (*ypjD-bshBA*) were conducted to unveil the cause of this novel mechanism of Spx activation. Results revealed that *ypjD*, which exhibits a unique promoter architecture, harbors an extensive putative cis-regulatory region upstream of the core -35 hexamer, and that a co-regulator may contact the promoter. Finally, a double codon substitution of the CxxC redox-sensing Spx C10 and C13 residues rescues the expression level of genes that are less responsive to Spx mutants bearing a single C10 (or C13) codon substitution. The findings suggest that for some Spx-activated genes, a free Cys thiol inhibits Spx activity, and thus disulfide formation of the Spx redox center is required to relieve this inhibition.

CHAPTER I: INTRODUCTION

Bacillus subtilis

Bacillus subtilis is a Gram-positive, spore-forming, rod-shaped bacterium commonly found in the soil and upper gastrointestinal tract of humans and ruminants. As a member of the *Bacillus* genus within the phylum, *Firmicutes*, this model bacterium has provided a vast amount of information regarding the fundamental process governing physiology, development, and differentiation of Gram-positive species. Historically, *B. subtilis* has been used in fermented food production as an immunostimulatory aid in gastrointestinal and urinary diseases and also as a test species for spaceflight experimentation (Dose, Bieger-Dose et al. 1995, Wassmann, Moeller et al. 2012). Additionally, *B. subtilis* can secrete up to Gram per liter amounts of industrial enzymes and produce dozens of antibiotics (Stein 2005, van Dijl and Hecker 2013). Since *B. subtilis* can degrade biopolymers and outcompete plant pathogens through biofilm formation, the organism is often regarded as an integral component of the carbon- and nitrogen cycle and the plant rhizosome system (Allard-Massicotte, Tessier et al. 2016).

The 4.2 Mbp genome of *B. subtilis* comprises 4,100 coding genes, of which about 6% are considered indispensable or essential. A large proportion of important genes are involved in chemotaxis, cell morphology, and metabolism (Kunst, Ogasawara et al. 1997). While *B. subtilis* prefers aerobic growth, the facultative anaerobe can generate ATP by fermentation or by using nitrate or nitrite as a terminal electron acceptor (Nakano and Zuber 1998). *B. subtilis* can metabolize pyruvate using

pyruvate dehydrogenase to carry out fermentation in the absence of external electron acceptors, distinguishing the organism from other anaerobes. Glucose and malate are the preferred carbon sources of *B. subtilis* and genes encoding proteins that function in the transport and utilization of secondary carbon sources are under tight catabolite control (Meyer and Stulke 2013).

Nutritional and environmental stress are common in the environment and thus *B. subtilis* has evolved to employ strategies to adapt and survive under harsh and dynamic environmental conditions. Upon nutrient limitation, vegetative cells undergo sporulation, a widely studied developmental program involving a genome-wide re-organization of gene expression to transform into stress-resistant and metabolically inert endospores (Errington 1993). In response to other stress conditions like encounters with toxic agents, antibiotics and reactive oxygen species, *B. subtilis* employs additional complex signal transduction pathways.

ROS production and oxidative stress response

Many bacterial species, including *B. subtilis* and other members of the Firmicutes prefer to use molecular oxygen (O_2) for respiration, and oxidation of nutrients for energy generation. Paradoxically, aerobic organisms experience major cellular dysfunction upon exposure to elevated oxygen levels. The auto-oxidation of components of the electron transport chain leads to production of reactive oxygen species (ROS), byproducts of metabolism, which can damage nucleic acids, proteins, and cell membranes (Fig. 1.1). During the stepwise four-electron reduction of O_2 , superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are generated, largely due to

collisions between O_2 and dihydroflavin cofactors (Messner and Imlay 1999). Superoxide can bind and oxidize solvent-exposed Fe-S clusters of Fe-bearing enzymes, such as dehydratases, resulting in enzyme inactivation and release of reduced iron (Flint, Smyk-Randall et al. 1993). The unincorporated Fe^{2+} can react with H_2O_2 to form highly reactive hydroxyl radicals ($OH\cdot$) through Fenton chemistry.

Hydroxyl radicals cause cytotoxic effects mainly through oxidative DNA damage (Hutchinson 1985, Dizdaroglu, Rao et al. 1991). Mononuclear iron proteins are subject to H_2O_2 -induced inactivation either by Fenton reaction between H_2O_2 and the Fe-atom or by oxidation of the thiols in Fe-coordinating cysteine residues (Anjem and Imlay 2012).

ROS disrupt thiol homeostasis by forming adducts with low molecular weight (LMW) thiols and modifying the cysteine thiols of proteins (Fig. 1.2). Specifically, ROS reversibly oxidize cysteine thiols to a highly unstable Cys sulfenic acid intermediate (Cys-SOH), which is prone to react with proximal thiols to form intra- or inter- molecular disulfides (Loi, Rossius et al. 2015). The sulfenic acid moiety can be further oxidized to irreversible modifications sulfinic - (Cys-SO₂H) and sulfonic acid (Cys-SO₃H), leading to inactivation of enzymes and protein aggregation.

To adapt or survive ROS-induced oxidative stress, *B. subtilis* employs multiple strategies aimed at ROS detoxification and repair of damaged biomolecules. ROS scavenging enzymes play a crucial role in antioxidant defense. Superoxide dismutases catalyze the dismutation of $O_2\cdot^-$ to O_2 or H_2O_2 (Benov and Fridovich 1995). H_2O_2 degradation is catalyzed by various ROS scavenging enzymes including

catalases, and several classes of peroxidases (Mishra and Imlay 2013). In *E. coli*, alkyl hydroperoxide reductase (AhpCP) is the primary scavenger of endogenous hydrogen peroxide (Seaver and Imlay 2001). Organic hydroperoxide is detoxified by alkyl hydroperoxide reductases including and the OhrA peroxiredoxin (Fuangthong, Atichartpongkul et al. 2001, Mishra and Imlay 2013).

Oxidative stress response is primarily orchestrated by regulation of gene expression at the level of transcription initiation. DNA-dependent multisubunit RNA polymerase (RNAP) is the essential enzyme of gene expression and a target of transcriptional regulation in all kingdoms of life. This form of transcriptional control may require alternative forms of RNA polymerase (RNAP) and/or transcription factors that target RNA polymerase. Such transcription factors, including Spx, may gain or lose activity upon exposure to toxic oxidants.

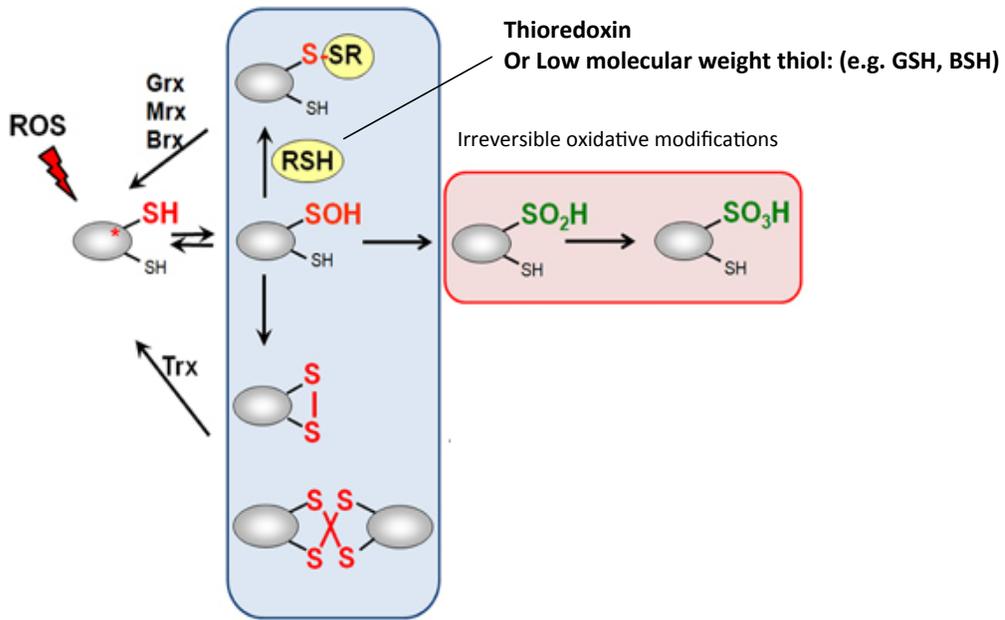


Figure 1.2. Reactive oxygen species and cysteine thiol oxidation.

Loi, V. V., et al. (2015). "Redox regulation by reversible protein S-thiolation in bacteria." *Front Microbiol* **6**: 187.

RNAP: structure and assembly

The conserved catalytic core bacterial DNA-dependent RNAP consists of two copies of α (encoded by *rpoA*), β (*rpoB*) β' (*rpoC*) and, ω (*yloH*). Additional subunits δ (*rpoE*) and ϵ (*rpoY*) function as small accessory subunits in *Bacillus* species and other Gram-positive bacteria. RNAP assembly initiates upon alpha dimerization, which provides an essential docking site for the β subunit, followed by the binding of the $\beta\omega$ inter-subunit complex (Murakami 2015).

The β and β' catalytic subunits form the characteristic crab-claw like structure in which the catalytic Mg^{2+} is located deep in the catalytic cleft, insulating the addition of nucleoside monophosphates to the nascent RNA transcript. Located at the back of the claw, the alpha subunits are each composed of an N-terminal domain (α NTD), a flexible linker, and a C-terminal domain (α CTD). Each α NTD is essential for RNAP assembly and in rare instances can serve as a target for some transcription factors (Niu, Kim et al. 1996). The α CTD can function in upstream promoter elements interactions, interactions with transcription factors, and RNAP inter-subunit contact during activator-stimulated transcription (Ross, Gosink et al. 1993, Ebright and Busby 1995, Gourse, Ross et al. 2000, Ross, Schneider et al. 2003, Lin and Zuber 2012). The omega subunit is highly conserved from bacteria to humans. In *E. coli*, omega has been suggested to function in sigma factor recruitment and together with β' forms a regulatory binding site for (p)ppGpp alarmone during amino acid starvation (Ross, Vrentas et al. 2013, Bhowmik, Bhardwaj et al. 2017). The epsilon subunit was proposed to promote protection from phage infection, as it bears homology to the Gp2 family of phage proteins involved in transcription inhibition

following infection (Keller, Yang et al. 2014). A consensus on the physiological role of the δ subunit has yet to be established in the literature. Several reports have implicated the Gram-positive conserved delta subunit in maintaining cell fitness, transcriptional repression, aiding in RNAP recycling, and sigma factor switching (Hyde, Hilton et al. 1986, Juang and Helmann 1994, Rabatinova, Sanderova et al. 2013, Prajapati, Sur et al. 2016).

The ~ 400 kDa catalytic core enzyme ($\alpha_2\beta\beta'\omega$) functions in non-specific binding DNA and subsequent RNA synthesis, and acquires promoter-specificity as a holoenzyme upon association with the sigma subunit, ($\alpha_2\beta\beta'\omega\sigma$). The major *B. subtilis* “housekeeping” RNAP (E) form bears the SigA subunit (σ^A), a member of the *E. coli* σ^{70} family of proteins, and maintains the constitutive expression of general “house keeping” genes (Fig. 1.3). SigA, composed of 4 functional regions, recognizes conserved -35 core promoter sequences (TTGACA) through region 4 and -10 (TATAAT) through region 2. *B. subtilis* bears 18 alternative sigma factors, which recognize distinct core promoter elements sequences (Haldenwang 1995). The sigma subunit is a target for Class II activators and interacts with α CTD in some cases of activator-stimulated transcription (Browning and Busby 2016).

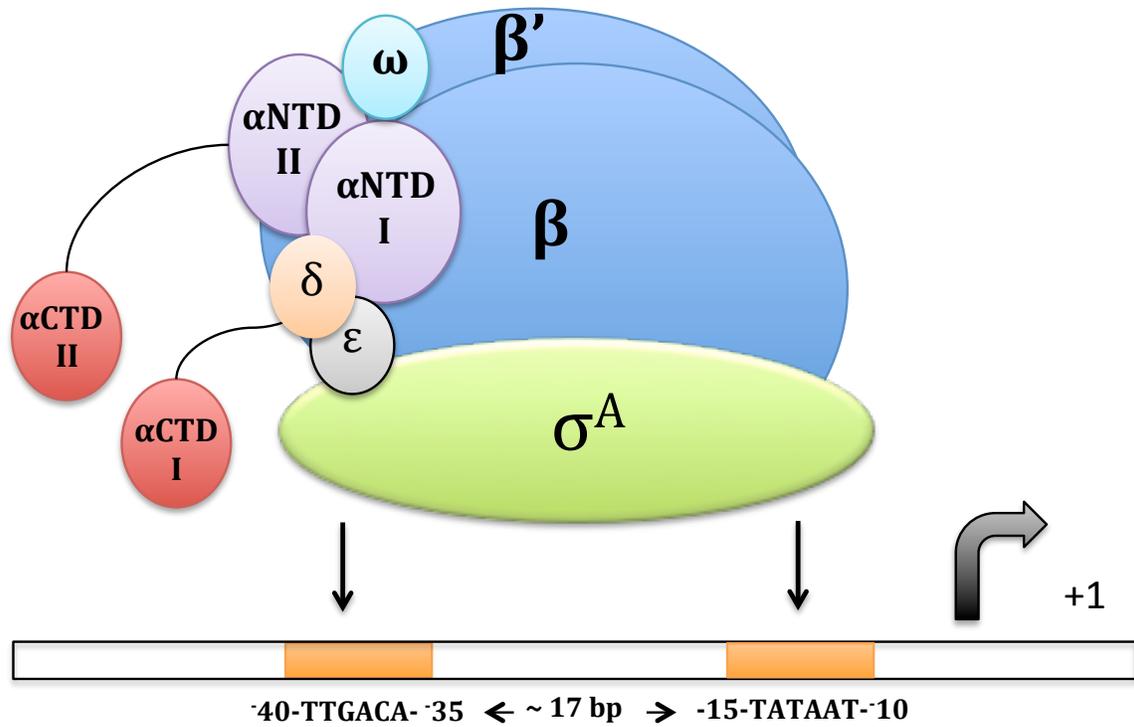


Figure 1.3. Depiction of *B. subtilis* "housekeeping" RNAP holoenzyme.

Note. The structural orientation of the δ and ϵ accessory subunits have not been experimentally determined.

Overview of Bacterial Transcription:

The cycle of transcription consists of three main stages: initiation, processive elongation and termination. The RNAP holoenzyme ($E\sigma$) binds to the conserved -35 and -10 hexamers forming the initial closed complex (RPc). The transcription complex undergoes significant conformational changes to bend and destabilize the helical environment of upstream promoter DNA, which is required for orienting the downstream duplex DNA into the active site cleft (Ruff, Record et al. 2015). Subsequent open complex formation is achieved by DNA melting, or unwinding of the duplex promoter DNA spanning approximately 13 bp from -11 to +3. RNAP then engages in de novo transcript synthesis by loading initiating nucleotide triphosphates (iNTPs) opposite the first and second template DNA bases to generate the first phosphodiester bond of RNA (Basu, Warner et al. 2014). Prior to promoter clearance, RNAP frequently produces short RNA transcripts, two to nine nucleotides in length, (abortive initiation). Once the RNA chain reaches ~12 nucleotides, the transcription complex stabilizes and the subsequent release of sigma facilitates the initiation-to-elongation transition (Johnston, Lewis et al. 2009). The elongation complex proceeds to extend the nascent RNA chain while translocating along the gene's coding region, typically with the aid of elongation factors such as NusA, GreA, and Mfd (Borukhov, Lee et al. 2005, Washburn and Gottesman 2015). Termination is initiated by halting and dissociation of the elongation complex from the template and is mainly initiated by either factor-mediated (Rho-dependent) or intrinsic (Rho-independent) mechanisms (Boudvillain, Figueroa-Bossi et al. 2013).

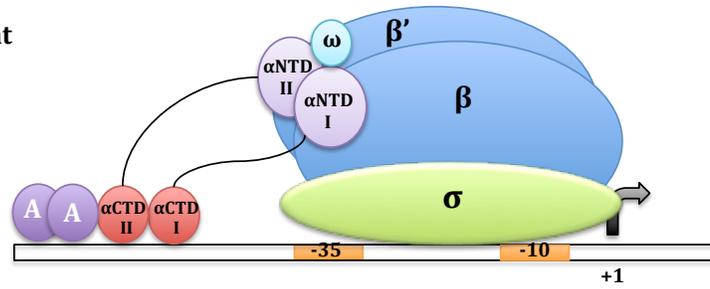
Recruitment-based transcriptional activation:

In response to dynamic environmental cues, regulation of gene expression is essential to the adaptation, differentiation and development of bacteria.

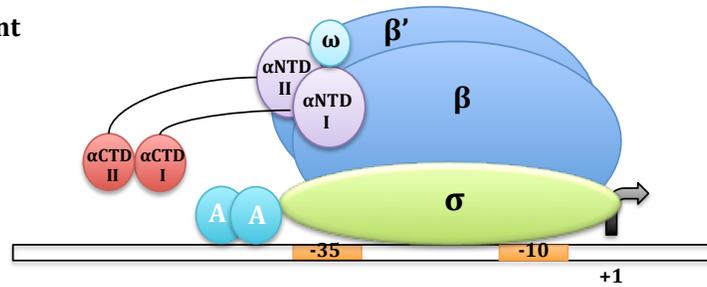
Transcriptional regulation can occur at all stages of RNA synthesis, however, most transcription factors regulate gene expression at the transcription initiation phase. The predominant mechanism of activator-stimulated transcription involves DNA-binding factors that function in promoter-centered regulation (recruitment) (Fig. 1.4). Common strategies to increase promoter activity include Class I activation, Class II activation, or activation by conformational change (Browning and Busby 2016). Some promoters harbor at least one region of sub-optimal RNAP affinity and thus bear compensatory *cis*-regulatory “operators” located distal, adjacent to, or in between the -35 and -10 core promoter elements that serve as sites for transcription factors.

The *E. coli* catabolite activator protein (CAP) is the prototypical Class I activator, which binds to operators significantly upstream of the -35 of Class I CAP-dependent promoters to recruit RNAP by direct interaction with α CTD (Benoff, Yang et al. 2002). Depending on the promoter type, CAP and other activators also employ class II activation by binding operators overlapping the -35 to recruit RNAP by targeting region 4 of sigma and/or the α NTD (Browning and Busby 2004). The distance between the -35 and -10 hexamers of some promoters is suboptimal and requires a distinct class of activators to modulate the promoter conformation to a more suited docking site for RNAP (Philips, Canalizo-Hernandez et al. 2015).

a. Class I recruitment



b. Class II recruitment



c. Recruitment by conformational change

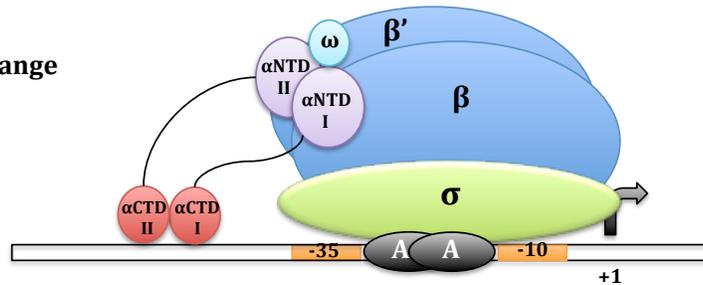


Figure 1.4. Common mechanism of recruitment-based activation. “A” denotes a transcriptional activator. a. Class I activators recruit RNAP by binding significantly and interact with alphaCTD. b. Class II activators recruit RNAP by binding adjacent to the -35 and interact the sigma subunit. c. Other activators modulate the spacing between the -35 and -10 to improve the docking site for sigma. Diagram is adapted from (Browning and Busby 2016)

Appropriation of RNAP

RNAP recruitment prevails as the dominant mechanism employed by transcription factors that activate transcription at the initiation phase. Conversely, RNAP “appropriators” can modulate gene expression by associating with holoenzyme and altering its promoter preference. During *E. coli* infection, the T4 phage protein AsiA remodels the conformation of the region 4 of σ^{70} to prevent its binding to the -35 elements of σ^{70} -dependent promoters and instead provides a contact site for the MotA transcription factor. The MotA-RNAP complex will then exclusively initiate transcription of phage genes expressed during the middle stage of the T4 life cycle (Hinton, March-Amegadzie et al. 1996, Lambert, Wei et al. 2004, Gregory, Deighan et al. 2005).

Spx and the *E. coli* SoxS, MarA, and Rob regulators are small monomeric bacterial encoded appropriators that have been proposed to function in RNAP “pre-recruitment” (Fig. 1.5). In *E. coli*, the SoxRS two-component system, MarA, and Rob activate overlapping regulons comprised of genes required for virulence, multi-drug resistance, superoxide stress resistance, and general stress response (Martin, Gillette et al. 1999, Duval and Lister 2013). Cis-acting elements within or proximal to the promoters of SoxS, MarA, and Rob activated genes are highly degenerate and denoted as sox-, mar-, and rob- boxes, respectively. Compounding evidence supports the notion that diverting RNAP from strong UP-element containing promoters to SoxS- (and likely MarA- and Rob-) – dependent promoters prioritizes the mobilization of stress resistance genes (Griffith, Shah et al. 2002, Martin, Gillette et al. 2002, Griffith and Wolf 2004, Shah and Wolf 2004). SoxS binds to sox boxes

located upstream of the -35 promoter hexamer of class I promoters, and to *sox* boxes overlapping the -35 promoter hexamer of class II promoters. It was proposed that *in vivo*, SoxS utilizes a single class I/II interfacing surface to form mutually exclusive SoxS-RNAP binary complexes by interacting with either α CTD or region 4 of σ^{70} (Shah 2004, Zafar 2010). In *E. coli*, the α CTD functions in binding to upstream promoter elements (UP) to drive transcription of ribosomal RNA promoters and protein-DNA binding is facilitated by R265 and other amino acid residues residing within the 265 DNA binding determinant region of α CTD (Ross 1993). SoxS binds to this region of α CTD and masks the ability of α CTD to contact UP-element DNA. Region 4 of σ^{70} binds to -35 promoter hexamers and interacts with class II activators that bind to cis-acting elements that overlap with the -35 hexamer (Rhodius 1998).

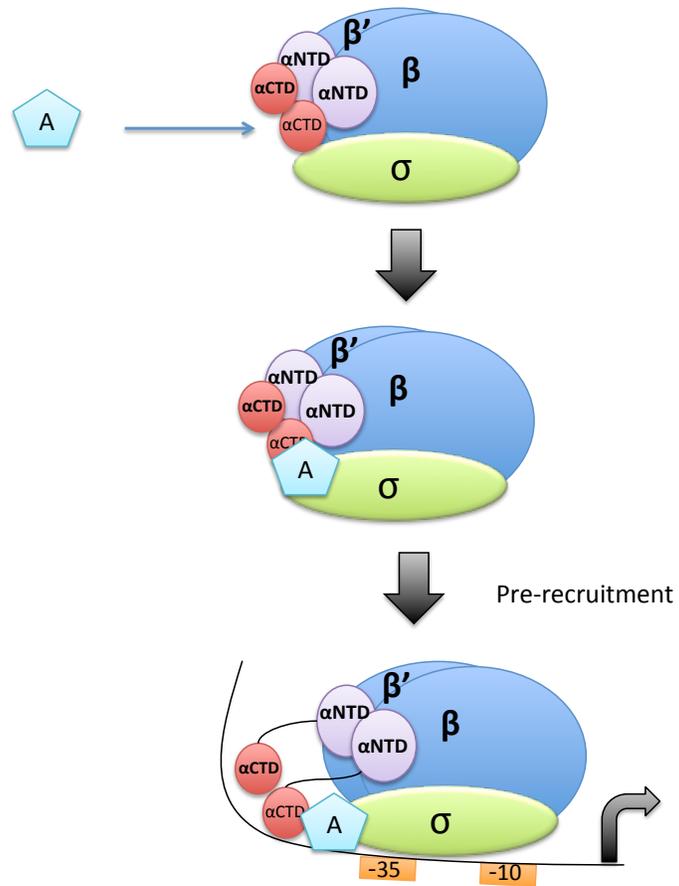


Figure 1.5. Activation by pre-recruitment. “A” denotes a transcriptional activator. Transcription factors that employ this mechanism form binary complexes with RNAP prior to contacting promoter DNA.

SigB, the global regulator of Bacillus general stress response

During vegetative growth, the housekeeping RNAP holoenzyme bears the σ^A promoter specificity factor, a member of the *E. coli* σ^{70} family of proteins, to maintain the constitutive expression of general “house keeping” genes. In addition to transcription factors, alternative sigma factors appropriate core RNAP to the promoters of their cognate regulons to govern stress resistance, physiological processes including chemotaxis, and progression and initiation of sporulation (Haldenwang 1995). The SigB-containing RNAP holoenzyme functions as a global regulator of non-specific, multiple, and general stress response in *B. subtilis* (Hecker, Pane-Farre et al. 2007). The σ^A -RNAP maintains basal transcription of many SigB-controlled genes, and upon oxidative stress, and perturbations in pH, temperature, osmolarity, and nutrient availability, the genes are transcriptionally activated by SigB (Petersohn 2001, Hecker 1998). SigB mutants confer heightened sensitivity to peroxide and paraquat, a superoxide-generating compound, suggesting that induction of the SigB regulon contributes to oxidative stress resistance (Reder, Hoper et al. 2012). The SigB regulon includes *sodA* (superoxide dismutase) and *dps* encoding a DNA binding protein functioning in the starvation-mediated oxidative stress response (Engelmann and Hecker 1996). SigB also activates the vegetative catalase *katE*, a paralog of PerR-regulated *kata*. However, *katE* null mutants retain peroxide-stress resistance (Engelmann and Hecker 1996). Stress-responsive genes of the Spx regulon may also be induced indirectly, as SigB activates expression of *spx*. SigB also activates MgsR (Modulator of general stress response), an Spx homolog required for regulating a subset of general stress response genes (Reder,

Hoper et al. 2008, Reder, Pother et al. 2012). Two genes, *trxA* (thioredoxin) and *yraA* (general stress protein), are under dual control by Spx and SigB and contribute to oxidative stress resistance (Reder, Hoper et al. 2012). Thiol disulfide exchange mediated by the Thioredoxin/Thioredoxin reductase/NADPH system alleviates disulfide stress and donates electrons to antioxidant enzymes. The Trx system donates electrons to the DNA-repair associated ribonucleotide reductases (*nrdEF*), some thiol dependent peroxidases, and the methionine disulfide reductase (*msrA*), that functions in protein repair (Lu and Holmgren 2014).

Redox control of oxidative stress response

Redox-sensing transcription factors generally control the production of ROS scavengers and expression of members of the oxidative stress response stimulon. In *B. subtilis*, two peroxide-sensing factors PerR and OhrR govern peroxide-induced stress response.

OhrR (Organic hydroperoxide regulator) is a MarR-type (Multiple antibiotic resistance regulator) transcriptional repressor of the peroxiredoxin-encoding *ohrA* gene. OhrR dimerizes and binds to an inverted repeat sequence overlapping the *ohrA* promoter region (Fuangthong, Atichartpongkul et al. 2001). A two-step oxidation pathway is required to disrupt DNA binding by OhrR. Cumene hydroperoxide and other organic hydroperoxides oxidize Cys15 of OhrR to form a Cys-sulfenic acid intermediate, which is further oxidized by mixed disulfide formation with cysteine or low molecular weight thiols Coenzyme A (CoASH) or bacillithiol (BSH). S-thiolation of the sulfenate intermediate is essential for full

dissociation of OhrR from operator DNA, prevention of irreversible oxidative damage to the Cys15-SOH, and regenerating active OhrR by thiol-disulfide exchange (Lee, Soonsanga et al. 2007, Soonsanga, Lee et al. 2008).

In contrast with most redox-sensing regulators that employ thiol-based redox switches, PerR (peroxide resistance regulator) senses peroxides by metal-catalyzed histidine oxidation. PerR is a member of the Fur family of metalloproteins and is subject to negative auto-regulation, while additional members of the PerR regulon include *fur* (ferric uptake regulator), *ahpCF* (alkyl hydroperoxide reductase), *katA* (vegetative catalase), and *hemAXCDBL* (heme biosynthesis). PerR bears a DNA binding domain at the N-terminus and a C-terminal dimerization domain which is structurally stabilized by the Zn(Cys₄) site. PerR also contains a C-terminal metal-binding regulatory site at which a cluster of histidine and aspartate residues preferentially coordinate Fe²⁺ and Mn²⁺ ions, which is essential for the PerR dimer to gain DNA binding activity (Lee and Helmann 2006). Full metallation establishes interaction between the DNA-binding and dimerization domains. PerR:Zn:Fe²⁺ then binds with high affinity to conserved *per* boxes, which are cis-acting elements irregularly distributed along regulatory regions within or proximal to PerR-repressed promoters (Herbig and Helmann 2001). Hydrogen peroxide reacts with regulatory Fe²⁺ ion site, leading to the oxidation of Fe-coordinating His37 and/or His91 to 8-oxo-His forms. Peroxide-induced formation of either 8-oxo-His destabilizes the PerR repressor complex, resulting in depression of PerR regulated genes PerR along with the reactive electrophilic species-sensing YodB repressor, negatively regulates *spx* gene expression (see section below).

Spx is a global transcriptional regulator

Spx, a member of the ArsC (Arsenate reductase) family of proteins functions as a global transcriptional regulator and is highly conserved among low GC Gram-positive bacteria. In *B. subtilis*, Spx regulates the expression of over 250 genes, or 144 transcription units in response to a wide assortment of stress conditions that potentiate proteotoxicity (Nakano, Kuster-Schock et al. 2003, Zuber 2004, Rochat, Nicolas et al. 2012).

Spx has very little intrinsic DNA-binding activity (Nakano, Erwin et al. 2005), which further distinguishes it from other transcription factors. Spx activates genes by the “pre-recruitment” mechanism. Spx preferentially forms a binary complex with the σ^A holoenzyme, at least partially through Spx- α CTD interaction, prior to contacting target promoter DNA (Lin and Zuber 2012) (Fig 1.6). To repress genes, Spx outcompetes other activators for contact with α CTD-binding surfaces and thus interferes with activator-stimulated transcription (Fig. 1.6).

The *spx* gene, formally denoted as *yjbD*, was identified as a suppressor of mutations in *clpP* and *clpX* encoding protease subunits. ClpXP dysfunction renders cells deficient in sporulation, competence development, and motility and

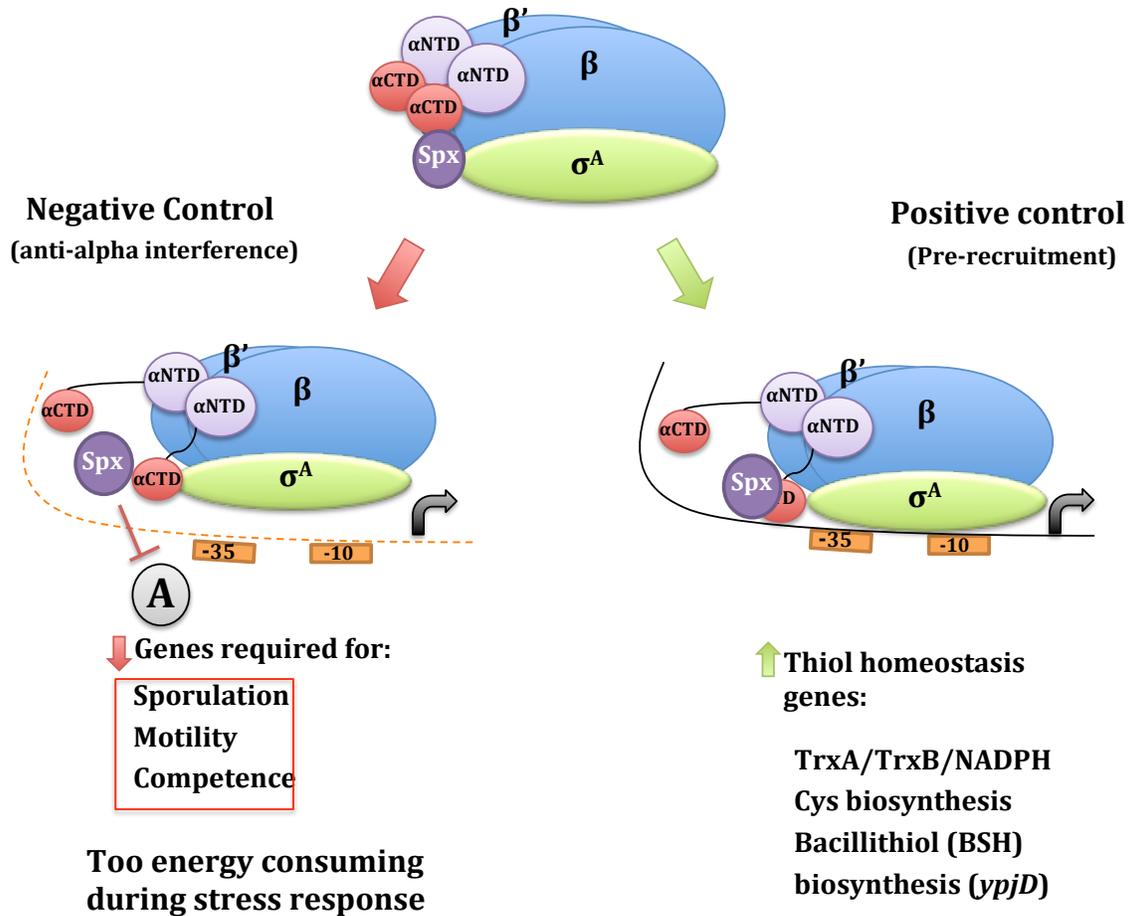


Figure 1.6. Spx functions as a transcriptional activator and repressor.

“A” denotes a transcriptional activator. Spx binds to housekeeping RNAP holoenzyme, at least partially by interacting with α CTD. To repress genes, Spx exhibits “anti-alpha” activity to interfere with activator-stimulated transcription. To activate genes, Spx pre-recruits RNAP to target promoters. The depicted classes of Spx-controlled genes comprise only an exemplary fraction of the Spx regulon.

compromises growth under some conditions, and the complex was later found to be required to prevent Spx-mediated inhibition of these processes (Nakano, Hajarizadeh et al. 2001, Nakano, Nakano et al. 2002). Upon accumulation and oxidation of its N-terminal CXXC redox center, Spx endows *B. subtilis* cells with disulfide and oxidative stress resistance, most notably through direct interaction with RNAP and transcriptional activation of thiol homeostasis genes thioredoxin (*trxA*) and thioredoxin reductase (*trxB*). Using NADPH as a cofactor, *TrxA* and *TrxB* function in thiol disulfide exchange to prevent stress-induced disulfide accumulation and modulate the activity of some proteins (You, Sekowska et al. 2008, Lu and Holmgren 2014, Castro-Cerritos, Yasbin et al. 2017). Other Spx-activated genes that function in maintenance of thiol homeostasis include methionine sulfoxide reductases (You, Sekowska et al. 2008), cysteine biosynthesis genes (Nakano, Kuster-Schock et al. 2003, Choi, Reyes et al. 2006), and genes that function in biosynthesis of bacillithiol (BSH), a redox buffering low molecular weight (LMW) thiol (Gaballa, Newton et al. 2010). *B. subtilis* Spx also regulates gene expression to prevent heat-shock induced protein aggregation and thus it is essential for conferring adaptive thermotolerance (Runde, Moliere et al. 2014). Additionally, Spx has been linked to iron homeostasis and implicated in the sulfate-dependent regulation of organosulfur metabolism, though the physiological significance of these functions require further clarification (Erwin, Nakano et al. 2005, Choi, Reyes et al. 2006, Zuber, Chauhan et al. 2011).

Spx in other bacteria

Spx often exists as multiple paralogs and have been described in *Lactococcus lactis* and pathogenic members of the Firmicutes phylum. *Bacillus anthracis*, the causative agent of Anthrax disease, bears *spxA1* and *spxA2* paralogs, of which only *spxA1*-linked genes show synteny with those in *B. subtilis*. (Barendt, Lee et al. 2013). SpxA1 and SpxA2 control overlapping regulons including *trxA* and, *trxB*, genes that function in either the synthesis or recycling of redox buffering low molecular weight thiols bacillithiol (BSH) or coenzyme A (CoASH). *B. anthracis* *spxA2* is highly induced upon spore germination in the host macrophage, suggesting a role for SpxA2 in pathogenesis (Bergman, Anderson et al. 2007). Phenotypic analysis of *spxA1* and *spxA2* null mutants suggests that both paralogs contribute to disulfide stress resistance, while SpxA1 is essential for peroxide stress resistance (Barendt, Lee et al. 2013).

Spx paralogs and Spx-liked proteins of *L. lactis* have been implicated in oxidative stress and cell envelope stress resistance (Turner, Tan et al. 2007, Veiga, Bulbarela-Sampieri et al. 2007). In *Staphylococcus aureus*, Spx functions as a global transcriptional regulator and is required for growth, cell wall antibiotic resistance and survival during various stress conditions (Pamp, Frees et al. 2006, Jousselin, Kelley et al. 2013). Spx proteins modulate stress response and virulence in several Streptococcal pathogens with an additional role in hydrogen peroxide production in *Streptococcus sanguinis* (Chen, Ge et al. 2012, Zheng, Xu et al. 2014, Galvao, Rosalen et al. 2017). Opportunistic pathogens *Listeria monocytogenes* and *Enterococcus faecalis* also require Spx paralogs for stress tolerance, aerobic growth and virulence

(Kajfasz, Mendoza et al. 2012, Whiteley, Ruhland et al. 2017). Spx proteins negatively regulate X-state competence development and biofilm formation in some *Staphylococci*, presumably by interfering with activator-stimulated transcription (Pamp, Frees et al. 2006, Turlan, Prudhomme et al. 2009, Wang, Fan et al. 2010).

Control of Spx

To prevent the pleiotropic effects of aberrant Spx induction, the regulator is subject to multilayered transcriptional, proteolytic, and thiol-based redox control. The gathering of insights into the function and control of the *spx* product began during phenotypic analysis of *B. subtilis* strains bearing null mutations in the *clpP* and *clpX* genes (Nakano, Hajarizadeh et al. 2001) encoding the ClpXP ATP-dependent protease complex. ClpC or ClpX ATPases form a multi-subunit complex with a ClpP protease, an assembly on which cells depend for stress response, sporulation, and the development of genetic competence (Nakano, Nakano et al. 2002).

The absence of ClpXP compromises ComPA-controlled expression of early competence genes. In high cell-density cultures, the cell density regulator ComX triggers the autophosphorylation of the ComP kinase of the two-component ComPA signal transduction system (Magnuson, Solomon et al. 1994). The phosphate is then acquired by the ComA response regulator, which then binds to RNAP and activates expression of the *srf* operon at the onset of competence development. The *srf* operon encodes surfactin synthase and the competence development regulator ComS (Nakano, Zhu et al. 2000). The key competence development transcription

factor ComK is subject to transcriptional auto-activation and regulated proteolysis by which the MecA adaptor protein complexes with ComK-ClpC to enhance ClpCP-catalyzed proteolysis (Turgay, Hahn et al. 1998). ComS accumulates in a cell-density dependent manner and disrupts the MecA-ComK-ClpC complex, activating ComK and stimulating ClpCP catalyzed proteolysis of MecA. In a *clpX* mutant, the *in vivo* expression of *comK-lacZ* and *srf-lacZ* fusions markedly diminished, suggesting that ClpXP somehow promotes ComA~P activated transcription of the *srf* operon and also indirectly stimulated ComK activity (Nakano, Hajarizadeh et al. 2001). A Y263C codon substitution in α CTD suppressed the low *srf* expression phenotype in the *clpP* mutant (Nakano, Zhu et al. 2000). Yeast two-hybrid analysis (Nakano, Nakano et al. 2003), and electrophoretic mobility shift assays (EMSA) indicated that Spx directly binds to α CTD and disrupts the interaction between RNAP and ComA as well as ResD, a major response regulator of aerobic and anaerobic respiration (Nakano, Nakano et al. 2003, Hartig and Jahn 2012). Loss of the Y263 residue of α CTD disrupts Spx- α CTD interaction, explaining the *clpX* suppressor phenotype of *rpoAY263C*. Spx was hence proposed to exert transcriptional repression as an “anti-alpha” factor that interfered with activator-stimulated transcription.

Inactivation of *spx* in a *clpP* mutant causes ClpP-independent competence development, restoration of ComK activity, and a partial restoration of sporulation. It was later shown that Spx antagonizes ComS dependent stimulation of ComK activity by enhancing the formation of the MecA-ComK-ClpC complex *in vitro* (Nakano, Nakano et al. 2002). The phenotypes of the *spx/clpP* double mutant and

the accumulation of Spx in the *clpP* null mutants indicated that ClpP-dependent proteolysis of Spx was required for competence development.

In summary, to prevent the pleiotropic effects of Spx accumulation during non-stress conditions, ClpXP is required for the post-translational repression of Spx (Nakano 2001, Nakano 2002). The YjbH adaptor protein enhances ClpXP-dependent proteolysis of Spx (Larsson 2007, Garg 2009) (Fig. 1.6). During proteotoxic stress, the proteolytic down-regulation of Spx is severely compromised, resulting in a drastic accumulation of Spx protein. Specifically, the YjbH protein concentration will plummet due to its aggregation under conditions that impair protein folding/stability, including heat-, ethanol- and diamide-induced disulfide stress (Engman and von Wachenfeldt 2015). Diamide also inactivates ClpXP by oxidizing the thiols of the Cys4 Zinc-binding domain of ClpX, resulting in the aggregation of the unfoldase (Zhang and Zuber 2007).

Redox-dependent activation provides an additional mechanism for post-transcriptional control of Spx. Primer extension analysis conducted using RNA from diamide-treated cells showed significant induction of thiol homeostasis genes *trxA* and *trxB*, and repression of *srfA*, which was dependent on Spx- α CTD interaction (Nakano, Kuster-Schock et al. 2003). Run-off *in vitro* transcription assays with purified RNAP and linear promoter DNA templates showed that WT Spx, but not SpxC10A or C13A directly activates transcription of *trxA* and *trxB* (Nakano, Erwin et al. 2005). Spx-dependent transcription was abolished upon addition of DTT, a thiol specific reductant. Furthermore, DNase I footprinting analysis showed that purified Spx altered the RNAP-dependent protection pattern of *P_{trxA}* and *P_{trxB}* in a DTT-

sensitive manner (Nakano, Erwin et al. 2005). The *in vitro* activity of Spx is essentially abolished upon incubation with DTT. SpxC10A and SpxC13A codon

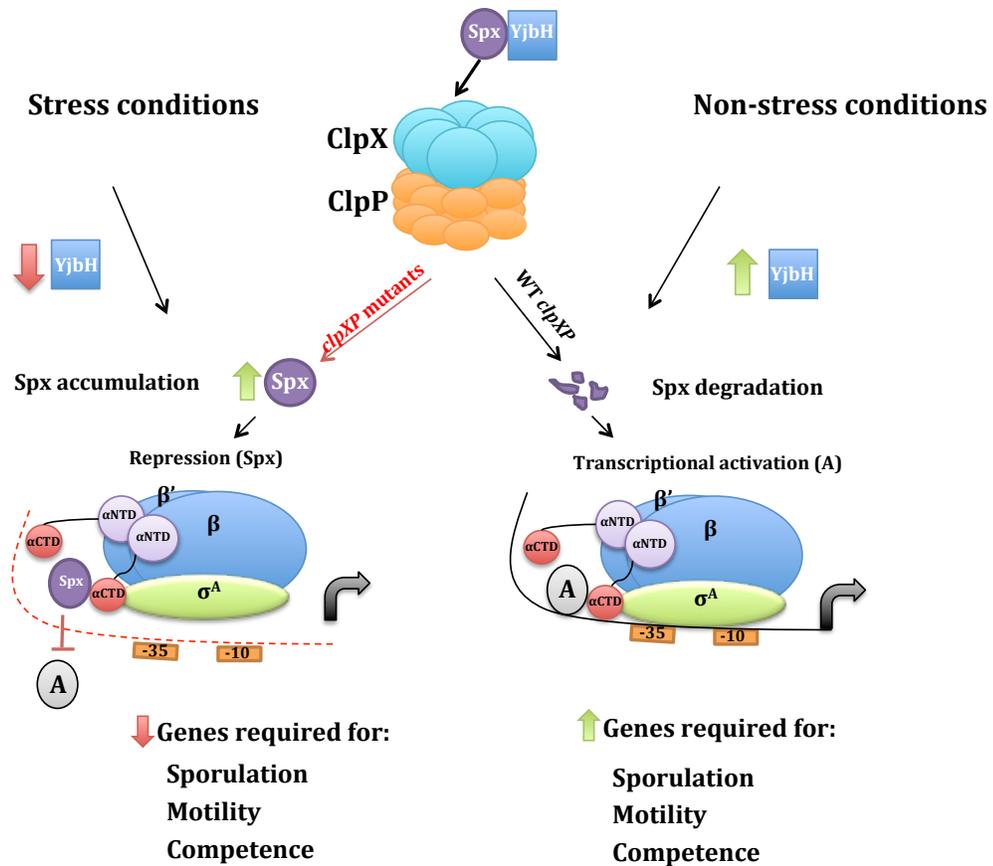


Figure 1.6. ClpXP-dependent proteolytic control of Spx. ClpXP-catalyzed degradation of Spx is enhanced by the yjbH adaptor protein. Spx can accumulate during proteotoxic stress -induced reduction in YjbH levels and also in clpX and clpP mutants. Upon accumulation, Spx can interfere with activator-stimulated transcription. ClpXP is required to prevent the pleiotropic effects of aberrant Spx accumulation by proteolysis.

substitution mutants, which are pseudo-mimetic of reduced Spx, abolish *in vitro* and *in vivo* Spx-activated transcription of *trxA* and *trxB*. Conversely, Spx activity as a negative effector of activated transcription is largely unaffected by its redox state as *srf-lacZ* expression was only moderately de-repressed upon induction of Spx CXXC mutants (Zhang, Nakano et al. 2006). The redox-sensitive nature of Spx-activated transcription and the defect in *trxA* and *trxB* activation conferred by the CXXC mutants provided evidence that Spx gains activity as a transcriptional regulator upon oxidation of its redox center. However, Gaballa et al. showed that an SpxC10A mutant can activate transcription at the *ypjD* promoter, from which transcription reads through a 7-gene operon containing genes that function in synthesis of bacillithiol (BSH), a redox buffering LMW thiol (Gaballa, Newton et al. 2010). A more detailed summary of intrinsic structural requirements for Spx activity and also efforts to reconcile differential redox requirements of Spx activity among the Spx regulon will be reported in upcoming chapters of this dissertation.

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purified Spx altered the RNAP-dependent protection pattern of *PtrxA* and *PtrxB* in a DTT-sensitive manner (Nakano, Erwin et al. 2005). The *in vitro* activity of Spx is essentially abolished upon incubation with DTT. SpxC10A and SpxC13A codon substitution mutants, which are pseudo-mimetic of reduced Spx, abolish *in vitro* and *in vivo* Spx-activated transcription of *trxA* and *trxB*. Conversely, Spx activity as a negative effector of activated transcription is largely unaffected by its redox state, as *srf-lacZ* expression was only moderately de-repressed upon induction of Spx CXXC mutants (Zhang, Nakano et al. 2006). The redox-sensitive nature of Spx-activated transcription and the defect in *trxA* and *trxB* activation conferred by the CXXC mutants provided evidence that Spx gains activity as a transcriptional regulator upon oxidation of its redox center. However, Gaballa et al. showed that an SpxC10A mutant can activate transcription at the *ypjD* promoter, from which transcription reads through a 7-gene operon containing genes that function in synthesis of bacillithiol (BSH), a redox buffering low molecular weight thiol (Gaballa, Newton et al. 2010). A more detailed summary of intrinsic structural requirements for Spx activity and also efforts to reconcile differential redox requirements of Spx activity among the Spx regulon will be reported in upcoming chapters of this dissertation.

The *spx* gene resides in a dicistronic operon downstream of *yjbC*, which encodes a putative acetyltransferase of unknown physiological function. Multiple stress conditions can trigger transcriptional activation of *spx*, as suggested by the presence of several promoters upstream of *yjbC-spx* and one in the *ypjC-spx* intergenic region. The housekeeping σ^A - form of RNAP utilizes a promoter farthest upstream of *yjbC*, followed by a promoter utilized by σ^B , the master regulator of general stress

response. The *yjbC-spx* operon and other σ^B -regulated genes are under dual control by σ^A form of RNAP, and are regulated in response to oxidative stress, and other environmental perturbations (Petersohn, Brigulla et al. 2001), (Hecker and Volker 1998). Downstream of the σ^B -dependent promoter, an additional promoter is subject to tripartite utilization by RNAP holoenzymes bearing extracytoplasmic σ factors (ECF) σ^W , σ^X and σ^M . σ^W , σ^X and σ^M exert transcriptional control over overlapping regulons to maintain cell envelope homeostasis under harsh environmental conditions (Jervis, Thackray et al. 2007). Indeed, SigW and SigM induction activates expression of *spx* and additional constituents of the σ^W / σ^M regulons during cell envelope stress induced by alkaline shock, heat, low pH, ethanol and several antibiotics that inhibit cell wall biosynthesis. Additionally, exposure to paraquat induces *sigM* expression and *sigM* null mutants conferred heightened sensitivity to the superoxide-generating compound (Cao, Moore et al. 2005). Heat stress and some antimicrobial peptides such as nisin, produced by *L. lactis* activate the *sigX* operon and σ^X -regulated genes (Huang, Decatur et al. 1997, Cao and Helmann 2004), and may trigger SigX utilization of the σ^W -, σ^X - and σ^M -recognized promoter of *yjbC-spx*.

Finally the reactive electrophilic species (RES)-sensing YodB repressor and the peroxide-sensing PerR repressor can both negatively regulate *spx* expression by interaction with the *spx* P3 promoter. DNase I footprinting assays show that peroxide and diamide, a thiol specific oxidant, disrupt YodB and PerR binding at the *spx* P3 promoter, reflecting the physiological requirement for depression of *spx*

during oxidative stress (Leelakriangsak, Kobayashi et al. 2007, Leelakriangsak and Zuber 2007).

Major aims

The broad aim of the Zuber lab is to elucidate the mechanism of Spx-activated transcription of thiol-specific oxidative stress response genes. The structure and redox state of Spx, interactions between Spx and RNAP, and the promoter architecture of Spx-controlled genes can influence Spx activity. Spx must productively interact with α CTD to engage promoter DNA and activate transcription. The contribution of the RNAP α subunit during nucleotide specific recognition of Spx-utilized promoters will be examined. It is possible that Spx can interact with other RNAP subunits and some Spx amino acid residues function at the putative contact sites. Mutational analysis of Spx amino acid residues located near the Spx- α CTD interface will be conducted to determine their role in Spx-activation transcription. Finally, the functional significance of each Spx amino acid residue has not been fully established. Furthermore, the longstanding paradigm regarding redox control of Spx asserts a stringent requirement of the oxidized disulfide form of Spx to contact promoter DNA for transcriptional activation. However, recent reports have suggested that not all members of the Spx regulon require Spx in its oxidized disulfide form for activation, but the underlying cause is not yet known (Gaballa, Antelmann et al. 2013). The following dissertation highlights efforts to further explore these determinants of Spx-dependent control.

CHAPTER 2, PART I:
EXPLORING THE ROLE OF THE RNAP α SUBUNIT
IN SPX-ACTIVATED TRANSCRIPTION

PART I: INTRODUCTION

Regulation of gene expression at the level of transcription initiation is required for bacteria to maintain fitness and survival in a dynamic environment (e.g. perturbations in temperature, nutrient availability, exposure to toxic agents, etc.). Global control of transcription under harsh conditions is mediated through mechanisms that alter the composition, activity, and target-DNA specificity of RNA polymerase (RNAP). The *B. subtilis* housekeeping RNAP holoenzyme is composed of β , β' , α_2 , σ^A , ω , δ , and ϵ . As part of the cellular response to environmental and metabolic changes, transcriptional regulators that serve as response effectors recruit or pre-recruit the RNAP holoenzyme complex to engage specific genes, whose products function in adaption and stress alleviation (Lee, Minchin et al. 2012).

B. subtilis Spx functions primarily to up-regulate expression of genes that function in preventing/alleviating thiol stress (Nakano, Kuster-Schock et al. 2003), and oxidative proteotoxic stress (Runde, Moliere et al. 2014). Protein-DNA crosslinking and promoter mutagenesis studies provided evidence that an AGCA cis-sequence upstream of an UP element-like region functions as a target for Spx/RNAP (Reyes and Zuber 2008, Nakano, Lin et al. 2010, Lin and Zuber 2012). Genome-wide ChIP-chip analyses confirmed that this regulatory promoter sequence is the

preferred target of the Spx/RNAP complex, and is moderately conserved among Spx-controlled genes (Rochat, Nicolas et al. 2012). A longstanding objective has been to reconcile the molecular interactions underlying the requirement of Spx to appropriate RNAP in order to bind to target promoters. Unlike many transcription factors that bind target promoter DNA prior to RNAP recruitment, Spx, devoid of intrinsic DNA-binding activity, must contact RNAP prior to engaging promoter DNA during transcriptional activation. Spx- α CTD interaction is essential for Spx-mediated transcription (Nakano, Kuster-Schock et al. 2003, Nakano, Lin et al. 2010). We hypothesize that not only is α CTD required for Spx-RNAP interaction, but Spx and α CTD both contribute amino acid residues that contact DNA when the Spx- α CTD complex interacts with promoter DNA. Thus, Spx and α CTD could form a heterodimeric DNA-binding complex, in which amino acids from both proteins constitute a DNA-binding surface within the Spx-RNAP complex. When complexed with RNAP, Spx residues R60 and K62 function in promoter DNA interaction. The spatial organization of the amino acid residues in the α CTD-Spx complex show an interesting symmetrical arrangement of large positively charged amino acid residues (SpxK62, R60; RpoAR261, K294, in Fig. 2.1b), which might function in DNA sequence recognition. The goal of this study is to uncover α CTD amino acid residues that are required for productive Spx-RNAP interaction with promoter DNA (Birch, Davis et al. 2017).

Initially, alanine-scanning mutagenesis of the *rpoA* gene encoding the C-terminal domain of α was conducted to uncover the amino acid residues functioning in Spx-RNAP interaction (Zhang, Nakano et al. 2006). All were constructed as markerless

alleles at the native *rpoA* locus (Zhang, Nakano et al. 2006, Geng, Zuber et al. 2007). While most mutations had no severe effects on *in vivo* activation of *trxB* transcription, Cys and Ala substitutions at Y263 (A267 in *E. coli*) almost completely abolished Spx-activated transcription. Furthermore, the Y263C and Y263A mutations render cells hypersensitive to diamide, a thiol-specific oxidizing agent (Nakano, Kuster-Schock et al. 2003). The tyrosine residue is highly conserved among Gram-positive species (Zuber 2004), of which many require Spx for virulence, viability, and/or survival during oxidative stress. As shown in a crystal structure of Spx- α CTD complex, the aromatic side chain of α Y263 participates in Van der Waals interactions with a leucine 56 and glycine 52 of Spx to form part of the Spx- α CTD interface (Lamour, Westblade et al. 2009). Indeed, an *rpoAY263C* mutation encodes an RpoA product defective in Spx-binding (Lin and Zuber 2012).

While Spx has no intrinsic DNA-binding activity, it could form part of a sequence-specific DNA interaction surface with residues of α CTD. We hypothesize that in addition to Spx-interaction, α CTD harbors residues that function in promoter-DNA interaction when bound to Spx. Alanine-scanning mutagenesis was conducted to replace the majority of the α CTD amino acid residues. We seek to conclude these studies by targeting the remaining α CTD amino acid residues (α K294, E255, E298, R261, R268A, and R289). Attempts to generate these α CTD Ala codon substitutions at the native locus (Fig. 2.1) were unsuccessful. RpoA K294 and R261 are highly conserved among bacterial species and participate in promoter recognition by RNAP in *E. coli*. Like Y263, the R268 (*E. coli* A272) and R289 (*E. coli* P293) residues are highly conserved among Gram-positive species, the R residues are likely

essential. Here we investigate the role of the putatively essential α CTD amino acid residues in Spx-dependent transcription and oxidative stress resistance. To complete the mutational analysis of α CTD, we generated ectopically expressed *rpoA* mutant alleles in merodiploid strains expressing the *rpoAY263C* allele from the native *rpoA* locus for complementation analysis. In summary, we found that the Spx-bound α CTD is not essential for productive Spx/RNAP-DNA interaction. Diminished *in vivo* activation of two Spx-activated promoters conferred by R261A, E255A and E298A mutants during high-salt stress suggests that these residues are required for optimal Spx/RNAP transcriptional activity. An alanine codon substitution at E255, a residue residing at the Spx- α CTD interface confers a defect in Spx-RNAP interaction. The E298 residue corresponds to E302 in *E. coli*, which has been shown to function in promoter DNA interactions along with R265 and K298 (R261 and K294 in *B. subtilis*). We present evidence that *rpoAE298A* mutation reduces the affinity of Spx/RNAP for promoter DNA. However, R261A and K294A, respectively, had less severe or stimulatory effects on Spx activated transcription, suggesting that DNA interaction by the Spx-bound α CTD is not essential within the Spx-RNAP-DNA complex.

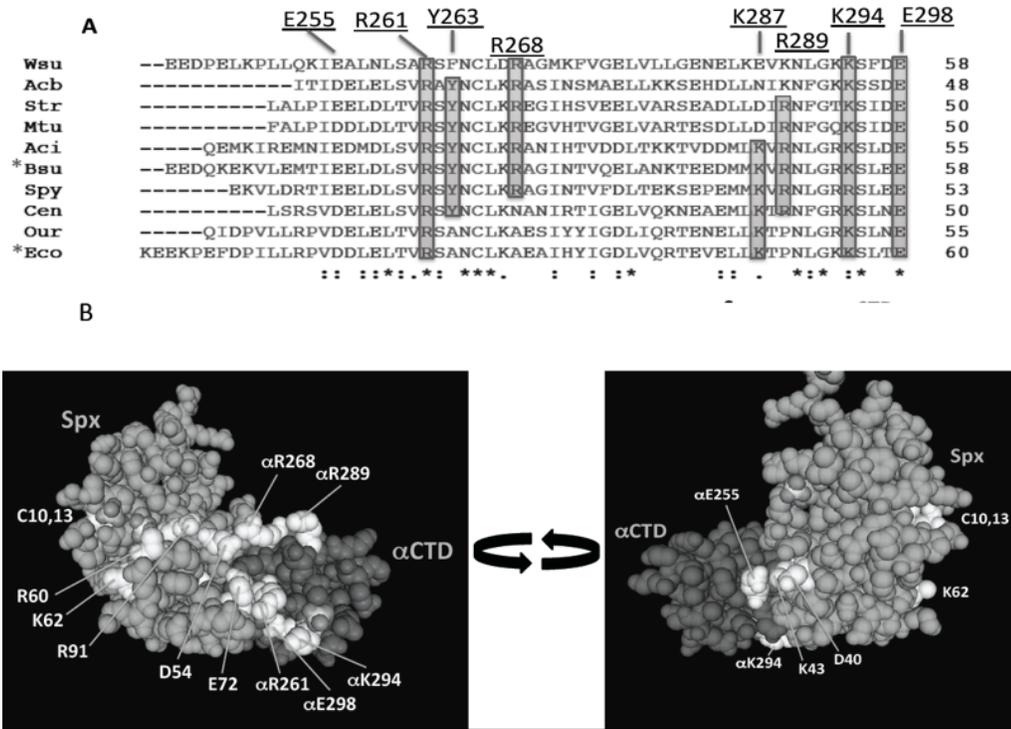


Figure 2.1. Alignment of Bsu α CTD with those of various bacterial species.

a. The C-terminal domains of α subunits from bacterial species of several major proteobacterial groups (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Epsilonproteobacteria*), plus *Mycobacterium tuberculosis* (Mtu), *Streptomyces coelicolor* (Str), *E. coli* (Eco), and *Streptococcus pyogenes* (Spy) are aligned with *B. subtilis* α CTD. Proteobacterial sequences were chosen for the alignment based on close similarity to *B. subtilis* α CTD primary structure. Other abbreviations: *Wolinella succinogenes* (Wsu), *Acinetobacter baumannii* (Acb), *Candidatus Entothionella* (Can), *Oligella ureolytica* (Our), *Aci Acidiphilium*. b. Spx- α CTD heterodimer complex crystal structure with relevant amino acid residues in both proteins highlighted (protein databank (pdb) file: 1z3e). The structure on the right is rotated 180° with respect to the structure on the left.

RESULTS

Alignment shows conserved positions of putatively essential residues in B. subtilis α CTD.

Previous alanine-scanning mutagenesis of the *B. subtilis* α CTD was conducted (Zhang, Nakano et al. 2006, Geng, Zuber et al. 2007) with the resulting mutant alleles integrated at the *rpoA* locus using a method described previously (Nakano, Zhu et al. 2000). These were tested for Spx-activated gene expression *in vivo* and confirmed the requirement for the Y263 residue for Spx-RNAP interaction, with L264 and E254 also required for optimal Spx-activated transcription. These residues likely function in establishing the interaction interface between Spx and α CTD (Newberry, Nakano et al. 2005, Lamour, Westblade et al. 2009). Several codon substitutions could not be created at the *rpoA* locus, and were assigned as putatively lethal mutations. In Fig. 2.1, we indicate the α CTD amino acid residues relevant to this study. The amino acid sequence of the *B. subtilis* α CTD was used to conduct a BLASTP search of homologs in alpha-, beta-, gamma-, delta-, and epsilon-proteobacteria. Those most closely homologous to *B. subtilis* α CTD were included in the alignment of Fig. 2.1a. Alignments of *B. subtilis* α CTD with *Streptomyces coelicolor*, *Mycobacterium tuberculosis*, *Streptococcus pyogenes* and *Escherichia coli* K12 were also included in Fig. 2.1 a. Several of the possible essential residue positions are conserved in all of the listed bacteria included in Fig. 2.1a (R261, K294, E298) and a conserved acidic residue corresponding to *B. subtilis* α CTD E255. Others (Y263, R268, R289) are not conserved in *E. coli*, but are detected in other classes of bacteria. The α Y263, which has been shown to be essential for Spx

interaction is conserved in Gram-positive species and in members of other classes of bacteria. While not conserved in the well-studied *E. coli* α CTD, these amino acid residues might play important roles in RNAP function in other species.

The locations of some of the residues are indicated in the Spx- α CTD structure (Newberry, Nakano et al. 2005, Lamour, Westblade et al. 2009, James, Hsieh et al. 2015) (Fig. 2.1 b). These are shown in relation to the positions of important residues of Spx (C10, C13, R60, K62, and R91) required for optimal Spx-activated transcription (Erwin, Nakano et al. 2005, Lin, Walthers et al. 2013). Several of the α CTD residues, some of which do not reside near the Spx- α CTD interface, potentially function in promoter interaction, or contact with other regulatory factors that might participate in Spx-dependent control. For example, residues in *E. coli* α CTD corresponding to R261, K294, and E298 (Fig. 2.1 a, *E. coli* residues R265, K298 and E302) are known to function in promoter DNA interaction (Benoff, Yang et al. 2002). The D259 residue of *E. coli* corresponds to E255 of *B. subtilis* α CTD, and has been shown to contact σ^{70} residue R603 (R362 in *B. subtilis* σ^A) during UP element- and activator-stimulated transcription (Chen, Tang et al. 2003, Ross, Schneider et al. 2003). α E255 of *B. subtilis* lies very near the Spx- α CTD contact interface, however. In summary, we provide a visualization of the spatial arrangement of the α CTD amino acid residues relevant to this study and show that they are widely conserved among bacterial species. We conducted experiments to examine the phenotypes of the *rpoAK294*, *E255*, *E298*, *R261*, *R268A*, *R289* Ala codon substitution mutations, as we felt the collected data would provide insight to their potential function during Spx-activated transcription and in transcriptional control in other bacterial species.

Complementation approach to uncover the role of putatively essential α CTD residues in Spx-activated transcription.

In order to alleviate oxidative stress, *B. subtilis* cells require protein-protein and protein-DNA interactions within a transcriptionally active Spx-RNAP-DNA complex. Mutations in genes encoding Spx or RNAP subunits that disrupt this complex will result in diminished Spx-RNAP transcriptional activity as well as impaired growth under oxidative stress conditions. Spx- α CTD binding is required for Spx-RNAP complex formation and only RNAP-bound Spx can contact promoter DNA (Nakano, Erwin et al. 2005). Another role potentially played by the amino acid residues within α CTD is in Spx- α CTD interaction with promoter DNA. We sought to determine whether RpoAK294, E255, E298, R261, R268A R289 residues function in concert with Spx amino acid residues to bind promoter DNA during transcriptional activation. The codons specify the remaining amino acid residues of the α CTD region that have been targeted for Ala codon substitution mutagenesis. Integrating these plasmid-borne mutations at the native *rpoA* was unsuccessful, suggesting that the codon substitutions severely compromised cell viability, and thus preventing us from obtaining the necessary mutant strains for this study. Integrating the plasmid-borne *rpoAY263C* mutation at the native *rpoA* gene locus (to construct “native *rpoAY263C*”) generates viable mutants, but which are hypersensitive to thiol stress, produce a form of RNAP that is largely inactive on Spx-activated promoters, and have severely impaired Spx- α CTD interaction. Our goal was to devise a strategy to generate viable strains for testing the activity of putatively lethal *rpoA* alleles (Fig. 2.2). The *B. subtilis rpoAY263C* mutant phenotype provides a non-lethal system

largely devoid of Spx-activated transcription and thiol stress resistance. By inducing expression of the mutant *rpoA* alleles in the *rpoAY263C* background, we presume that the Spx-RNAP complexes would contain primarily the α subunits encoded by the induced alleles. Therefore, the *rpoAY263C* mutant was used as a host for introduction of ectopically expressed *rpoA* mutant alleles to test the mutant alleles for complementation with respect to Spx-activated transcription and thiol stress resistance.

The *rpoA* alleles were fused to $P_{hyperspank}$ (Volkov, Mascarenhas et al. 2003), an IPTG-inducible promoter and the plasmids were integrated at the *amyE* locus. Ectopically integrating the subject mutant *rpoA* alleles in strains bearing *rpoAY263C* at the native *rpoA* gene locus does not disrupt viability, presumably due to low basal expression of these potentially lethal alleles in the absence of IPTG. Next, Spx-activated genes *trxA* and *yugJ* ((Nakano, Kuster-Schock et al. 2003), (Rochat, Nicolas et al. 2012)) transcriptionally fused to a promoter-less *lacZ* reporter gene) were introduced into the resultant merodiploid strains. The *rpoA* variants encode products bearing a His₆-tag fused at the C-terminus for affinity purification of RNAP and subsequent *in vitro* studies. Western blot analysis was performed using anti polyHis monoclonal antibody to determine if any of the *rpoA*-His₆ constructs affected mutant RpoA protein concentration. Western blot images showed that the *rpoA*-His₆ mutant variants did not compromise endogenous transcription of their respective RpoA-His₆ products (Birch, Davis et al. 2017).

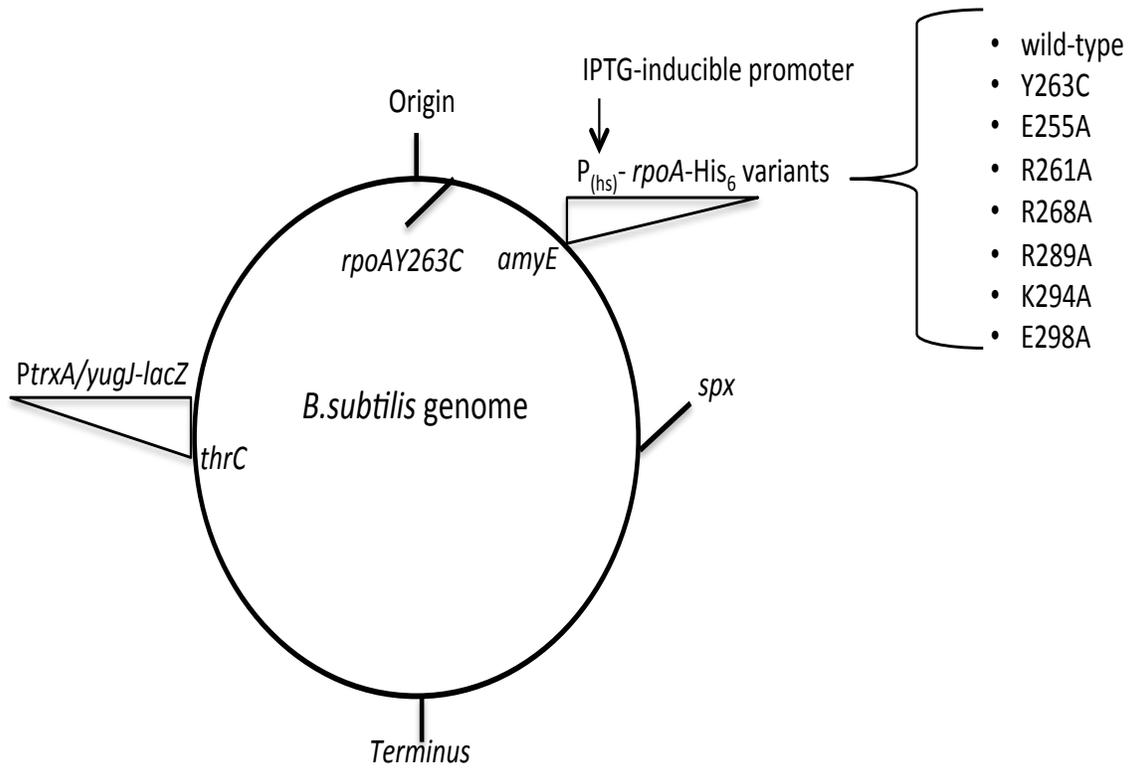


Figure 2.2. Schematic depicting the relevant genotype of subject strains. For complementation analysis, wild-type and mutant *rpoA* alleles will be ectopically expressed in diploid with the native *rpoAY263C*.

Testing complementation of the Y263C mutant is simply a proxy for the degree of transcriptional stimulation exerted by the *rpoA*-His₆ mutants from a very minimal state of Spx-dependent transcriptional activity and oxidative stress resistance (the Y263C phenotype). We are also able to generate viable strains bearing the ectopically expressed *rpoA*-His₆ variants in a WT *rpoA* background. However, the basal level of Spx-dependent transcriptional activity and oxidative stress resistance will be significantly higher, and may cause interference when interpreting the phenotypes of the ectopic *rpoA*-His₆ mutant alleles. If the residues undergoing substitution are not part of the Spx- α CTD interface, but are involved in Spx- α CTD promoter recognition, we anticipate that these mutant alleles will not complement Y263C, and thus could not mediate productive Spx-RNAP-promoter DNA complex formation because of a defect in Spx-RNAP-promoter DNA interaction that requires residues of Spx-bound α CTD. Previous reported studies have also utilized ectopically expressed alleles of *rpoA* in *E. coli* to uncover residue positions in α CTD functioning in interaction between RNAP and transcriptional activators (Tang, Severinov et al. 1994, Fritsch, Urbanowski et al. 2000).

Effect on disulfide stress sensitivity of ectopically expressed rpoA mutant alleles.

Due to the *rpoAY263C* mutant's defect in Spx-dependent control, this mutant exhibits a severe growth defect in the presence of the thiol specific oxidant diamide. Inducing the ectopic expression of wild-type *rpoA*-His₆ in the native *rpoAY263C* merodiploid fully complements *rpoAY263C* with respect to diamide sensitivity (Fig. 2.3). We sought to determine whether ectopically expressed *rpoAK294A*, *E255A*,

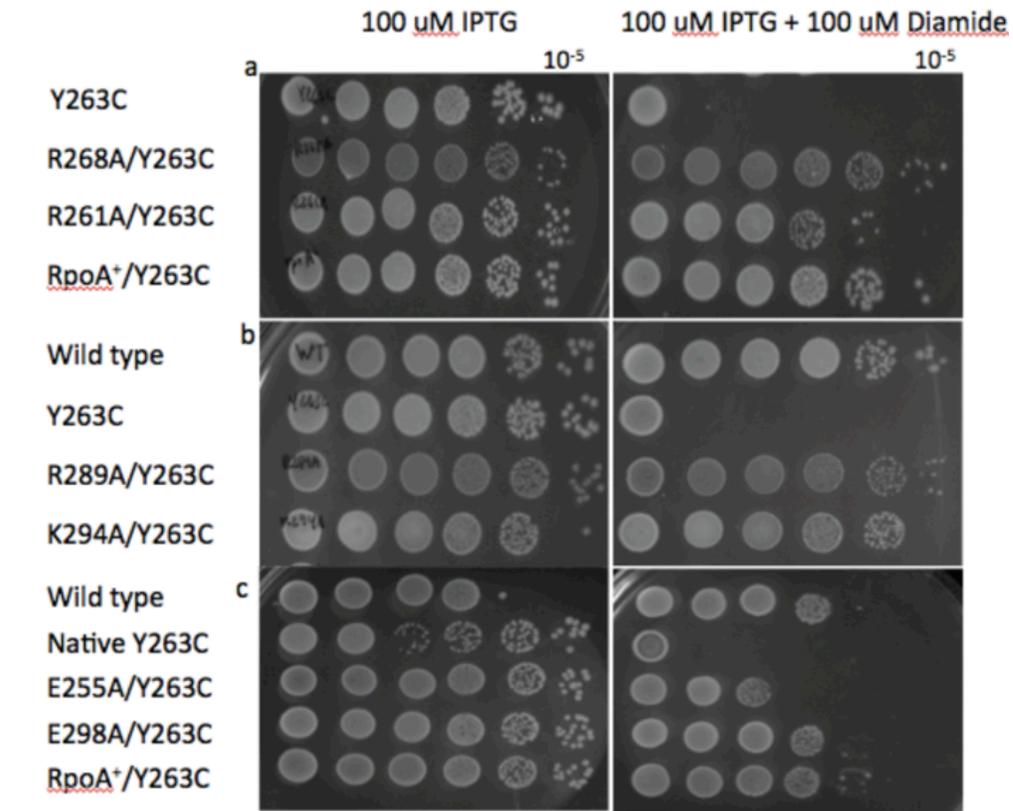


Figure 2.3. Complementation of *rpoAY263C* during exposure to disulfide stress. Effect of a. R268A and R261A, b. R289A and K294A, and c. E255A and E298A on disulfide stress resistance. Cultures were grown to mid-exponential phase in DSM and treated with 0.5 mM IPTG for one hour. Cultures were serially diluted and spotted onto sporulation agar containing either inducing agent IPTG (left) or IPTG and thiol-oxidizing agent diamide (right). The residue substitutions in the ectopically expressed *rpoA* alleles are indicated at the left of the spot plate images.

E298A, *R261A*, *R268A*, *R289A* conferred defects in oxidative stress resistance. If the mutated residues were required for Spx-mediated oxidative stress resistance, either by functioning in Spx-RNAP interaction or Spx-RNAP contact with promoter DNA, we hypothesize that they would be unable to rescue the severe growth defect of the Y263C mutant in the presence of diamide. Cultures were treated with IPTG to induce production of the RpoA-His₆ variants and dilutions were spotted on sporulation (DSM) agar containing IPTG with or without diamide. Induction of *E298A*, *R289A*, *R268A*, or the *K294A* mutants fully restored diamide resistance (Fig. 2.3). To a lesser extent, the *R261A* complemented the Y263C mutant as observed by a modest but reproducible decrease in cell viability (Fig. 2.3). The *E255A* exhibited the strongest defect in plating efficiency on diamide agar, which suggests that *E255* is required for optimal activation of thiol stress-response genes. Interestingly, while cells expressing ectopic *R268A* and *R289A* mutants rescued the hypersensitive Y263C mutant, they exhibited an IPTG-dependent small and slightly transparent colony phenotype, suggesting a defect in sporulation (Fig. 2.3 a and b, and see discussion). All together, the results indicate that most α residues are dispensable when conferring Spx-dependent thiol stress resistance in *B. subtilis*, but *E255* and to a minor extent, *R261* are necessary for optimal resistance to a toxic oxidant.

Effect of ectopically expressed rpoA alleles on Spx-activated transcription in vivo.

Though the complementation system was presumed to be useful, prior to implementation we were unsure if we would observe full complementation of the

Y263C mutant with the *RpoA*-His₆ variants during oxidative stress. Expressing some of the *RpoA*-His₆ variants restored viability of the Y263C mutant during growth in the presence of diamide, and thus confirmed the efficacy of the system. Next we determined whether we could examine restoration of Spx-activated transcription in the Y263C mutant by producing WT RpoA-His₆. As previously stated, the reduced oxidative stress response phenotype of the Y263C mutant is indicative of impaired Spx-activated transcription. The RNAP of an *rpoAY263C* strain shows severely impaired Spx-activated expression of *trxA* and *yugJ* promoter-*lacZ* fusions (Birch, Davis et al. 2017), as shown by measuring β -galactosidase activity ((Rochat, Nicolas et al. 2012), (Nakano, Kuster-Schock et al. 2003)). As expected, IPTG-induction of α Y263C-His₆ produced in the merodiploid with the native *rpoAY263C* (ectopic *rpoAY263C*-His₆/*rpoAY263C*) did not restore transcriptional activation of the promoter fusions. In contrast, IPTG-induction of *RpoA*⁺-His₆ resulted in significant stimulation of *trxA-lacZ*, and *yugJ-lacZ* (Birch, Davis et al. 2017). We sought to determine whether the *rpoAK294A*, *E255A*, *E298A*, *R261A*, *R268A*, *R289A* mutations affect the basal *in vivo* activation of Spx-controlled promoters. We hypothesize that if the mutants compromised Spx-activated transcription *in vivo*, they would not alleviate the severe transcriptional defect in the *rpoAY263C* mutant to the same extent as induced WT RpoA⁺-His₆ (*rpoA*⁺-His₆/*rpoAY263C*). The level of complementation will serve as a reporter as to whether the α CTD residues that have been targeted for mutagenesis are required for the assembly of active Spx-RNAP at these promoters. Induction of mutant α variants resulted in significant stimulation of *trxA-lacZ* and *yugJ-lacZ* activity upon addition of IPTG (Birch, Davis et al. 2017). It

is important to note that the *trxA* promoter has an elevated level of basal transcription likely due to the promoter architecture, which includes an extended -10 -TG- element. The induction of WT RpoA⁺-His₆ and the mutant derivatives showed significant stimulation of *trxA*- and *yugJ-lacZ*, however, the basal (non-stress) activity remained modest. This is likely due to low concentrations of active Spx, as cultures were incubated under unperturbed growth conditions. It is also possible that the His-tag on the α subunits impose unknown inhibitory effects on transcriptional activation, RNAP complex assembly or Spx- α CTD interaction. Nonetheless, the results indicate complementation of Y263C by most of the tested ectopically expressed mutant α subunits under normal growth conditions. In summary, during non-stress conditions, the *rpoAK294A*, *E255A*, *E298A*, *R261A*, *R268A*, *R289A* do not appear to confer severe defects in basal Spx-dependent activation *in vivo*.

Salt stress uncovers defects in Spx-activated expression in cells expressing rpoAR261A, rpoAE255A and rpoAE298A mutant alleles.

Toxic oxidants like diamide directly target *Bacillus* macromolecules and induce a rapid primary oxidative stress response. Additionally, high concentrations of toxic agents such as NaCl likely disrupt electron transport function, leading to the generation of reactive oxygen species (ROS) and thus induce a gradual secondary oxidative stress response (Tam le, Antelmann et al. 2006, Mols and Abee 2011). Salt stress was previously reported to induce the Spx regulon (Tam le, Antelmann et al. 2006). During oxidative stress, Spx rapidly accumulates in the cell, then pre-recruits

RNAP to target promoter DNA. The complex control of Spx, which includes ClpXP-mediated proteolysis, maintains the protein at a very low intracellular concentration if cells are not under stress. We previously demonstrated our ability to induce the expression of α CTD variants that could stimulate basal transcription of Spx-activated *lacZ* fusions in strains during unperturbed growth (Birch et al 2017, Fig S2). However, the study was conducted with cells that were not exposed to oxidative stress and thus harbored low intracellular levels of active Spx.

We sought to determine a way to examine the transcriptional activity of the RpoA-His₆ variants in a physiologically relevant condition: oxidative-stress induced elevation of intracellular Spx. However, in our lab, we observe that diamide impairs β -galactosidase activity. We sought to administer another stressor that induces the elevation of endogenous WT Spx without disrupting β -galactosidase activity. Preliminary growth assays show that *spx* null mutants are hypersensitive to elevated NaCl concentrations and western blot analysis shows a significant accumulation of Spx in cultures exposed to high NaCl in keeping with the previous observation of salt-induced expression of the Spx regulon (32). Salt stress likely results in oxidative stress as a secondary effect, which would lead to the gradual induction of Spx (Hoper, Bernhardt et al. 2006, Mols and Abee 2011). Indeed, we observe that β -galactosidase activity of Spx-controlled promoter-*lacZ* fusions showed a gradual and significant increase upon NaCl-stress. Cultures of the merodiploid strains were grown to mid-exponential phase. After IPTG addition, the cultures were split into two subcultures, to one of which 0.6 M NaCl was added. The addition of IPTG elevated the expression of the RpoA-His₆ mutant variants while the

higher NaCl concentration promoted induction of native *spx*. Induction of *yugJ-lacZ* was observed, but not in the *spx* deletion mutant (Fig. 2.4 a). The Spx-dependent increase in *yugJ-lacZ* and *trxA-lacZ* activity in high salt is gradual over 1.5-2.5 h.

Assays of Spx-directed β -galactosidase activity under high salt conditions uncover some differences among the *rpoA* merodiploid strains. Upon IPTG/NaCl-stress induction, *R268A*, *K294A*, *R289A* show levels of *yugJ-lacZ* activity similar to that of the control strain expressing *rpoA⁺-His₆* (Fig. 2.4 b). Upon IPTG/NaCl-stress induction the ectopic *rpoAR261A*, *rpoAE255A* and *rpoAE298* alleles in the *rpoAY263C* merodiploids resulted in a reduction of *yugJ-lacZ* expression (Fig. 2.4 b). The residue α E255 is located near the Spx- α CTD interface while α E298 (corresponding to *E. coli* α E302) resides between two helices that bear the α R261 and α K294 residues that normally function in α -DNA interaction, and is believed to serve as part of the DNA-binding region of the α subunit in *E. coli* (Gaal, Ross et al. 1996, Murakami, Fujita et al. 1996). The results suggest that during NaCl-stress induction of Spx, the R268, R289 are dispensable for transcriptional activation. To a moderate but significant degree, the R261A, E255A and E298A mutations impair transcriptional activity of the Spx-RNAP complex during NaCl stress. In summary, the RpoA-His₆ mutant variants do not confer severe defects in transcriptional activity or oxidative stress resistance, indicating that *rpoAK294*, E255, E298, R261, R268, R289 do not appear to comprise essential elements within the Spx-RNAP-DNA complex. Nonetheless, the remainder of this report will highlight our efforts to elucidate the causes of the R261A, E255A, and E298A phenotypes.

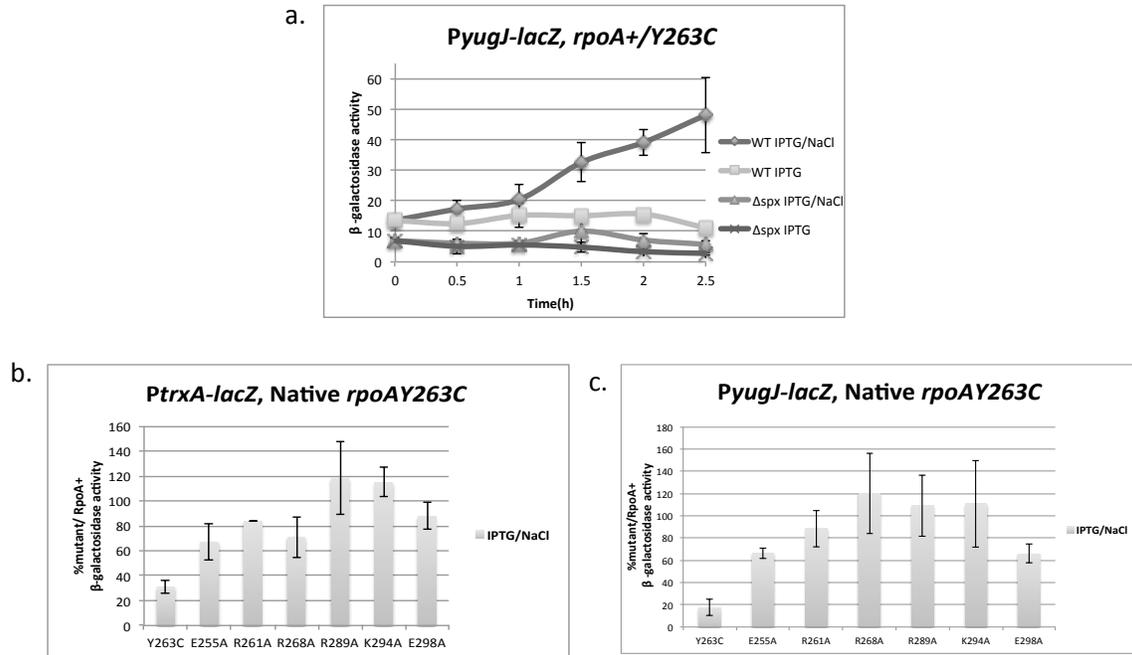


Figure 2.4. IPTG/NaCl-stress induced activation of *yugJ-lacZ*. a. Effect of *spx* deletion mutation on IPTG/NaCl-stress induction of *yugJ-lacZ* in *rpoA*⁺-*His*₆/*rpoA*Y263C strains. Graph is representative of three independent assays. b. Effect of α CTD mutations on *yugJ-lacZ* and c. *trxA-lacZ* expression during IPTG/NaCl induction. Strains are merodiploid having *rpoA*Y263C at the native locus (Native *rpoA*Y263C) and ectopically expressed *rpoA* mutant alleles with substitutions indicated at the bottom of each graph. Graph depicts β -galactosidase activity of samples collected 2 hours post IPTG/NaCl treatment. Graph is representative of three independent assays.

Evidence that rpoAE255 does not function in α CTD- σ^A interaction during Spx-activated transcription

E255 is the homologous position in *E. coli* α (D259), which makes electrostatic contact with the σ^{70} region 4.2 residue R603 (R362 in *B. subtilis* σ^A) in activator- or UP element-stimulated transcription. This suggests that an alternative role for RpoA residue E255 is to contact σ^A (*sigA*) C-terminal region 4 during Spx-stimulated transcription. Previous studies provided strong evidence that Spx activates transcription initiated by the σ^A form of RNAP (Lin and Zuber 2012), (Rochat, Nicolas et al. 2012), (Nakano, Zhu et al. 2000), (Nakano, Erwin et al. 2005), and the R362 residue of σ^A might participate in RNAP-Spx contact. We sought to compare the phenotype of the *rpoAE255A-His₆/rpoAY263C* and *sigAR362A* strains to that of the *rpoAE255A-His₆/rpoAY263C sigAR362A* strain and to determine whether the *rpoAE255* and *sigAR362* amino acid residues functioned in concert as binding partners, facilitating α CTD- σ^A interaction during Spx-mediated activation. We hypothesize that if the *rpoAE255* and *sigAR362* amino acid residues form a contact surface within the Spx-RNAP complex, the phenotype of the *rpoAE255A/sigAR362A* double mutant should not exceed the severity of the individual mutations.

Western blot analysis using anti-Spx antiserum was performed to determine if the *sigAR362A* mutation affected expression of *spx*, but little change was observed in the levels of Spx protein (Birch, Davis et al. 2017). The *rpoAE255/rpoAY263C* strains with and without the *sigAR362A* mutation were tested for *yugJ-lacZ* and *trxA-lacZ* expression. Relative to wild-type cells, the *rpoAE255A* and *sigAR362A* mutants each confer a 20-30% reduction in *yugJ-lacZ* expression in cells treated with IPTG and

NaCl (Fig 2.5). However, the level of expression is reduced further to *rpoAY263C* levels in the *rpoAE255A/sigAR362A* double mutant. Additionally, this strain confers a greater defect in disulfide stress resistance compared to strains bearing the individual mutations (Fig. 2.5 b, c). The additive severity in the phenotypes of the *rpoAE255A/sigAR362A* double mutant suggests that the region 4.2 of σ^A and $\alpha E255$ perform distinct roles in Spx-activated transcription, with $\alpha E255$ functioning primarily in Spx-RNAP interaction.

Purification of active RNAP from strains bearing ectopically expressed *rpoA-His₆* and mutant derivatives

Previous *B. subtilis* RNAP purifications were conducted using various forms of strains expressing C-terminal His-tagged β' subunit (*rpoC-His₁₀*) (Qi and Hulett 1998). By employing the same purification methods, we successfully purified active RNAP from *rpoAY263C* merodiploid cells expressing WT *rpoA⁺-His₆* or *rpoAR289A-His₆*. As shown on SDS-PAGE gels, the profile of the RNAP complexes resemble that of RNAP obtained from *rpoC-His₁₀* expressing strains (Birch, Davis et al. 2017). *In vitro* transcription assays demonstrated that RNAP from cells producing RNAP with RpoA⁺-His₆ or RpoAR289A-His₆ productively interacted with purified Spx to generate transcripts on a *trxB* DNA template (Birch, Davis et al. 2017). In summary, we have generated WT and mutant derivative α -His₆-producing strains, from which we can obtain active RNAP, using Ni chelate chromatography. This enables us to conduct the following *in vitro* studies aimed at elucidating the underlying causes of impaired transcriptional activity of the *rpoAR261A*, *E255A*, and *E298A* mutants.

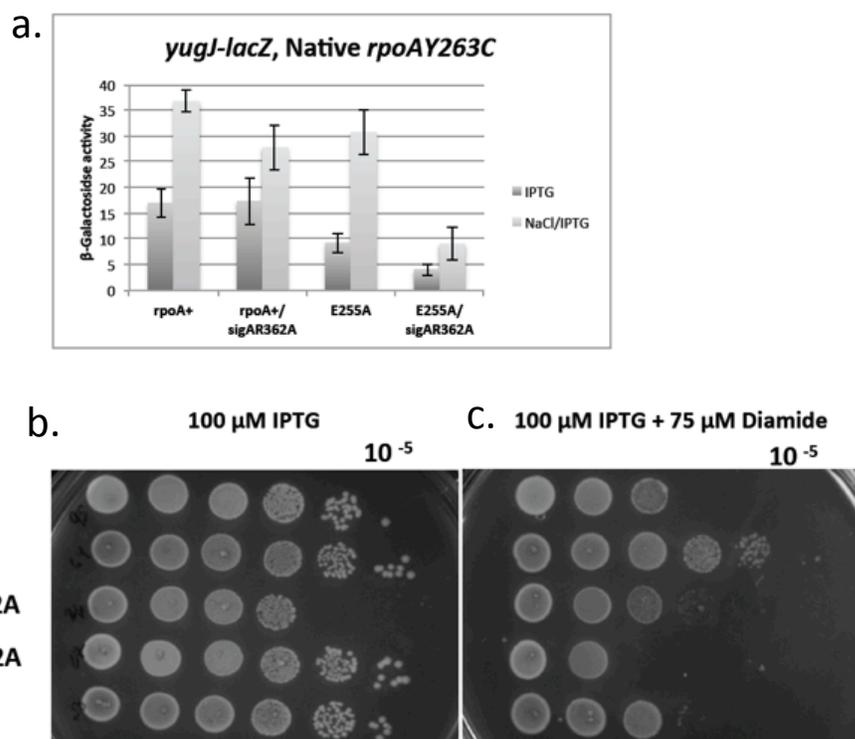


Figure 2.5. Additive effect of combined *rpoAE255A sigAR362A* mutations during activation of *yugJ-lacZ* and disulfide stress resistance. a. Effect of *rpoAE255A* and *sigAR362A* on activation of *yugJ-lacZ*. Cells were grown as described in Fig. 2.4. Graph depicts β -galactosidase activity of cells harvested 2 hours post IPTG and IPTG/NaCl treatment. Graph is representative of three independent assays. b. *rpoAY263C* merodiploids were grown as described in Fig. 2. 3 and spotted onto sporulation agar containing inducing agent or c. inducing agent and thiol oxidizing agent, diamide.

The rpoAE255A, rpoAE298A and rpoA261A mutations confer defects in Spx interaction with RNAP and/or α subunit.

We sought to determine if the *rpoAR261A*, *E255A* or *E298A* mutations conferred defects in Spx interaction with the α subunit and RNAP. This was accomplished using two approaches, Far-western blot (*rpoAE255A*, *rpoAE298A*) and size-exclusion chromatography (*rpoAE255A*, *rpoAR261A*, *rpoAE298A*). The E255 residue resides near the Spx- α interface, where it is believed to establish an electrostatic interaction with the lysine at position 43 of Spx (Fig. 2.1 b). The conserved R261 residue implicated in promoter DNA interaction is located near the Spx- α interface, where it could electrostatically interact with E72 of Spx (Fig. 2.1b). The E298 residue corresponds to E302 in *E. coli*, which has been shown to function with residues R265 and K298 (R261 and K294 in *B. subtilis*) in promoter DNA interaction.

Far-western blot served as the first *in vitro* Spx-RNAP binding affinity test in which purified RNAP subunits were electrophoretically resolved and immobilized on a nitrocellulose filter as bait protein, which was the target of purified Spx, the prey protein. We hypothesized that if E255A or E298A disrupt Spx-RNAP binding, the mutations will reduce the affinity of the Spx protein for the immobilized α subunit of RNAP. The immobilized proteins were treated with guanidine hydrochloride and subsequently with gradual reductions in guanidine hydrochloride concentrations to facilitate protein renaturation (Wu, Li et al. 2007). The filters were incubated in a solution containing purified Spx, and then probed with anti-Spx antibody to detect filter-bound Spx protein. As expected, there is no Spx signal corresponding to the immobilized α Y263C (Fig. 2.6 a, lane 1). Conversely,

Spx is found to interact with the immobilized α subunit in lanes in which the WT RpoA-His₆-containing RNAP preparation was applied (Fig. 2.6 a, lane 4). The α E255A protein shows reduced affinity for Spx, but Spx is observed to interact with α E298A protein (Fig. 2.6 a, lanes 2,3 respectively). Unexpectedly, interaction of Spx with bands corresponding to the RNAP large β and β' subunits was observed (see discussion). The results suggest that E255A impairs Spx- α CTD binding.

Reduced affinity of RNAP bearing α E255A, α E298A, and α R261A for Spx was observed by size-exclusion chromatography. We chose to use a P60 column, that resolves proteins between 3 kDa- 60 kDa and excludes proteins larger than 60kDa. The void volume (V_0), which is defined as the volume of buffer required to elute the proteins larger than 60 kDa was determined in preliminary experiments. RNAP of ectopically expressing WT *rpoA-His₆*, *Y263C-His₆*, *R261A-His₆*, *E255A-His₆* and *E298A-His₆* cells were separately combined with purified Spx protein and pre-incubated to facilitate *in vitro* Spx-RNAP complex formation. We hypothesize that because WT Spx (15 kDa) binds to WT α CTD in RNAP (~440 kDa), a significant amount of Spx will co-elute with RNAP in the void fractions. Incubating Spx with RNAP bearing mutant forms of α CTD that disrupt Spx-RNAP interaction will likely diminish co-elution of RNAP with Spx. The Spx/RNAP interactants were resolved on the column, in which the large RNAP complex along with any RNAP-bound Spx is expected to elute in the void fractions, before free Spx elutes (Fig. 2.7). The RpoA-His₆ variants were detected by western blot using anti-polyHis antibody, while Spx was detected using anti-Spx polyclonal antiserum. In the western blots, Spx is observed to co-

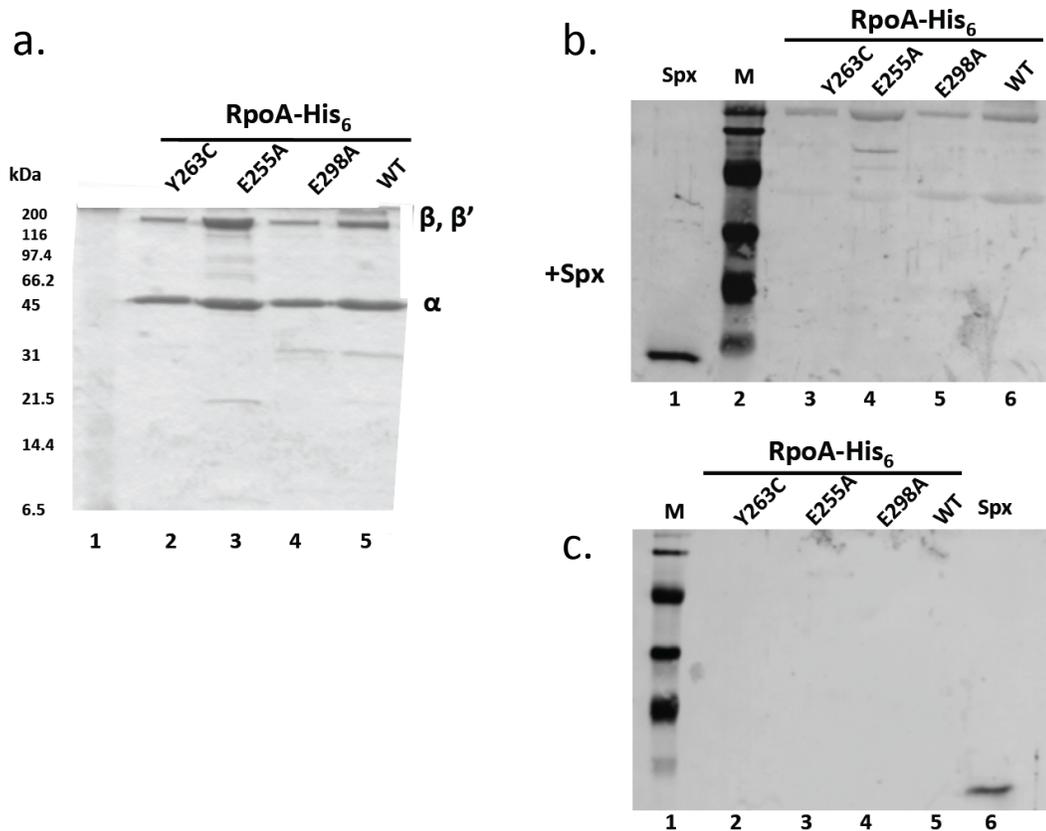


Figure 2.6. Effect of *rpoAE255A* and *rpoAE298A* mutations on *in vitro* Spx- α CTD interaction: Far-western blot. a. Coomassie-stained SDS-gel. Lane 1. RNAP extracted from ORB9575 (*rpoAY263C-His₆/rpoAY263C*). Lane 2. ORB9650 (*rpoAE255A-His₆/rpoAY263C*). Lane 3. ORB9651 (*rpoAE298A-His₆/rpoAY263C*). Lane 4. ORB9464 (*rpoA⁺-His₆/rpoAY263C*). RNAP variants were resolved by SDS-PAGE and immobilized on nitrocellulose filters as bait proteins in a far western. Filters were either incubated in Tris-buffered saline containing b. purified Spx or c. **not** containing purified Spx. Filters of b and c were subsequently treated with anti-Spx polyclonal antiserum. Numbers at the bottom of each gel denote the lane number.

elute with RNAP from *rpoA⁺-His₆/rpoAY263C* cells and later as free Spx protein (Fig. 2.7 a). As expected, very little Spx co-elutes with the RNAP from the *rpoAY263C-His₆/rpoAY263C* merodiploid in the void fractions, which confirms that the Y263C substitution in α CTD disrupts Spx-RNAP complex formation. Additionally, a reduced amount of Spx co-elutes *rpoAE255A* RNAP, most notably in fractions 21 and 22 (Fig. 2.7 b, c). Compared to *rpoA⁺-His₆/rpoAY263C*, *rpoAR261A/rpoAY263C* and *rpoAE298A/rpoAY263C* RNAP also showed reduced levels of co-eluting Spx (Fig. 2.7 d, e), with most of Spx eluting as unbound protein. In contrast to the far-western blot data, RNAP from the *rpoAE298-His₆/rpoAY263C* strain showed reduced affinity for Spx. While Spx can bind to denatured and renatured a subunit from this strain, interaction with purified *rpoAE298-His₆/rpoAY263C* RNAP is somewhat defective (Fig. 2.7 e). The requirement of E255, R261, and E298 for optimal Spx-RNAP interaction could account for the decreased Spx-dependent transcriptional activity in the mutant merodiploids during salt stress.

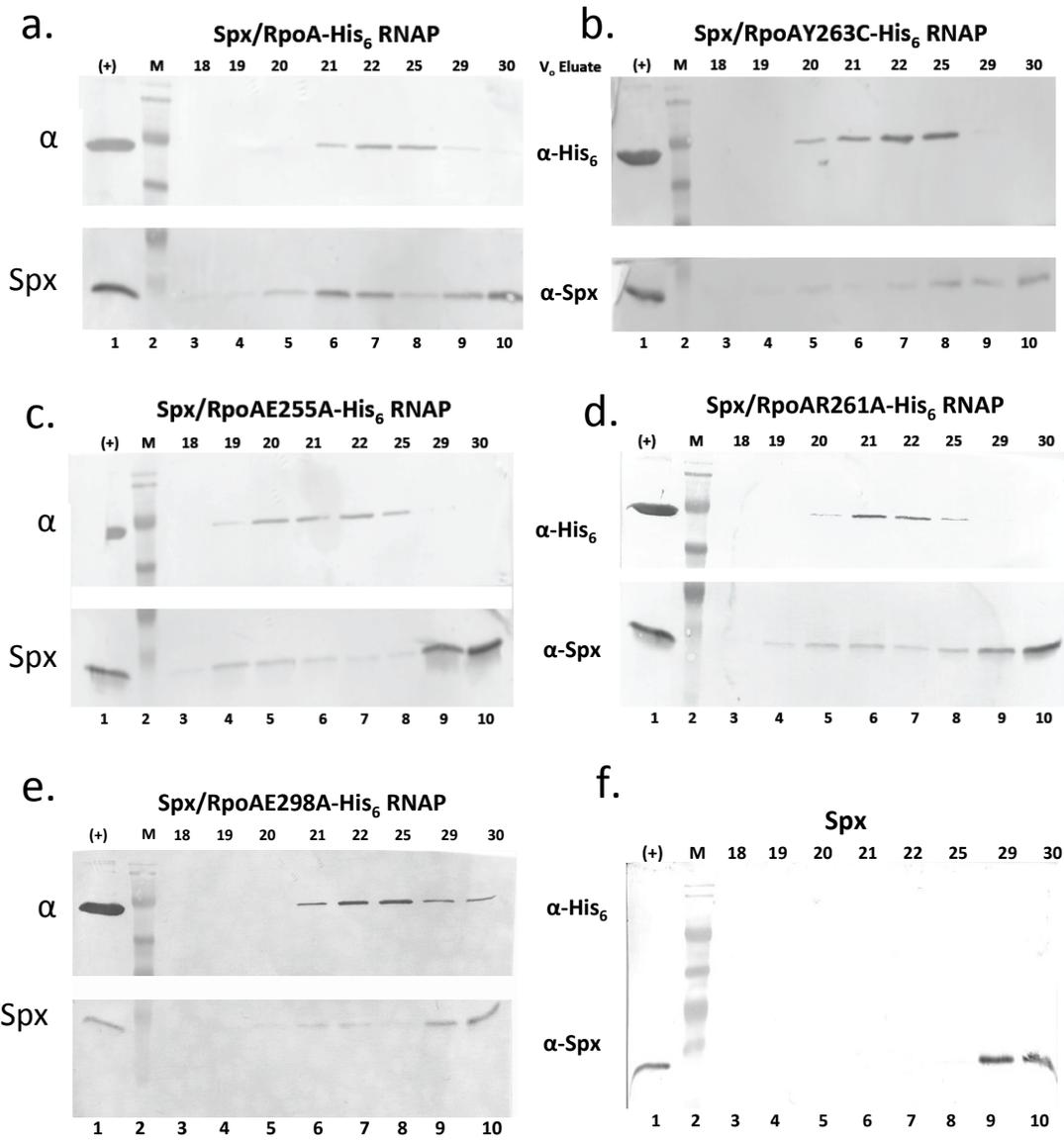


Figure 2.7. Effect of *rpoA* mutations on *in vitro* Spx-RNAP interaction: Size-exclusion chromatography. Spx was incubated with RNAP extracted from a. *rpoA*⁺-His₆/*rpoAY263C*, b. *rpoAY263C*-His₆/*rpoAY263C*, c. *rpoAE255A*-His₆/*rpoAY263C*, d. *rpoAE298A*-His₆/*rpoAY263C*, or e. *rpoAR261A*-His₆/*rpoAY263C* strains. Interactants were applied to a P60 gel filtration column and eluted using a Biologic LP chromatography system. Proteins in fractions encompassing the pre-determined void volume and subsequent fractions eluted from column were resolved on SDS-polyacrylamide gel and electrotransferred onto a nitrocellulose filter for western blotting. Numbers located at the top of each filter denote the elution fraction and numbers located at the bottom of each filter denote the gel lane number. The top half of each filter was treated with anti-polyHis antibody and each bottom half was treated with anti-Spx antibody. f. Elution profile of Spx protein. Western blot of Spx protein detected using anti-Spx antibody.

rpoAE255A and rpoAE298A mutations reduce Spx-enhanced affinity of RNAP for promoter DNA.

Electrophoretic mobility shift assays (EMSAs) were performed to determine if the *rpoA* mutations conferring defects in Spx-activated expression *in vivo* also reduced Spx-enhanced DNA binding by RNAP *in vitro*. Spx or BSA protein was combined with increasing concentrations of RNAP from the *rpoA* merodiploid strains in the presence of *PtrxA* promoter DNA. As was shown previously, Spx protein shows no affinity for promoter DNA, but when combined with *rpoA*⁺/*rpoAY263C* RNAP, a shift of *PtrxA* and *PyugJ* DNA to a higher molecular weight complex is observed at all RNAP concentrations (Fig. 2.8 a, (Birch, Davis et al. 2017)), as opposed to the reactions in which Spx is replaced by an equal concentration of BSA. RNAP from ectopically expressed *rpoAY263C*-His₆ showed much reduced affinity for *PtrxA* and *PyugJ* DNA in the presence of Spx (Birch, Davis et al. 2017). Reduced RNAP affinity for *PtrxA* DNA was observed in reactions containing RNAP from ectopically expressed *rpoAE255A*-His₆ and *rpoAE298A*-His₆ (Fig. 2.8 b, c). In summary, the results suggest that the E255A and E298A codon substitutions disrupt Spx-mediated RNAP affinity for promoter DNA. However, all together, the reduced severity of the E255A and E298A phenotypes suggests they are not essential, but are required for optimal Spx-RNAP-DNA complex formation.

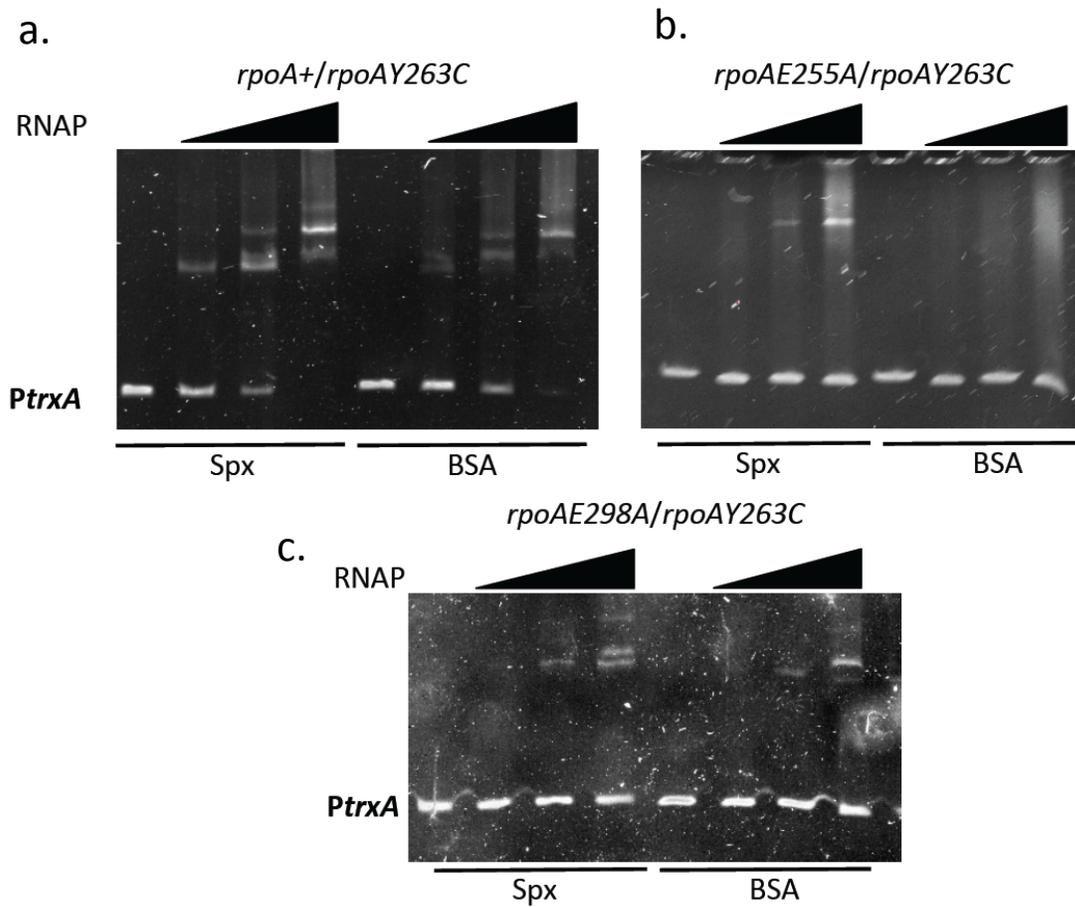


Figure 2.8. Electrophoretic mobility shift assays of RNAP affinity for *PtrxA* promoter DNA in the presence and absence of Spx protein. Promoter DNA of the *trxA* gene was combined with increasing concentrations (0, 0.025, 0.05, 0.1 μ M) RNAP of selected *rpoA* merodiploid strains. Either Spx protein or BSA was added (0.2 μ M final concentration) to each reaction. Band of unbound *PtrxA* DNA (3 nM reaction concentration) is indicated.

DISCUSSION

The α CTD that is bound to Spx potentially could play three roles; as a point of contact between Spx and RNAP, to facilitate positioning of Spx to contact other RNAP subunits, and/or to provide a component to the promoter DNA-binding surface of the Spx- α CTD complex. Previous alanine-scanning mutagenesis of the α CTD-encoding part of the *rpoA* gene identified only the Y263 residue as being essential for Spx-activated transcription. Several codons were not tested by alanine substitution due to our inability to generate the codon exchange at the native *rpoA* locus (E255, R261, R268, R289, K294, and E298). The residues tested in the *rpoAY263C* merodiploid strains were highly conserved residues that included those functioning in α -DNA interaction and α CTD- σ^A intersubunit contact in *E. coli*. To test the phenotype of the putatively lethal substitutions in *B. subtilis* α CTD, ectopically expressed *rpoA* alleles were generated by integration at the *amyE* locus as IPTG-inducible constructs.

Our merodiploid strains bear the *rpoAY263C* allele at the native *rpoA* locus, and its product could possibly function in the merodiploid strain by providing the α -DNA contact within the Spx/RNAP-promoter complex. However, our data show that this is not an essential function performed by Spx-bound α CTD, indicating that Spx is likely the major DNA-binding determinant in the Spx- α CTD complex. Some of the *rpoA* mutations (E255A, R261A, and E298A) conferred partial defects in Spx-activated transcription *in vivo* and/or during diamide sensitivity. The R261, E298 and K294 residues correspond to *E. coli* R265, E302, and K298, respectively, which form a DNA UP-element binding amino acid patch of α CTD. A greater reduction in

plating efficiency as well as reduced expression of *yugJ* supports a case for R261 playing a role in maintaining the Spx- α CTD-promoter DNA interface. However, this residue is very near the Spx- α CTD interface and could function in Spx-RNAP interaction. Further structural studies are necessary to further define the role of R261 in Spx-activated transcription. The E298A mutation confers a slight defect during NaCl-Stress induced activation of Spx-controlled promoters and impairs Spx-enhanced RNAP-DNA binding. However, the transcription defect along with the full complementation of the Y263C mutant during diamide exposure indicates that the E298A does not confer a severe phenotype.

In summary, our data and those of previous work (Zhang, Nakano et al. 2006) indicate that the α CTD residues normally associated with RNAP-DNA interaction are not required for productive interaction between Spx- α CTD and promoter DNA. However, it is possible that some α CTD amino acid residues, including E298, may function in non-nucleotide specific DNA interactions to optimize Spx-RNAP affinity for some Spx-activated promoters.

As shown in Fig. 2.1 a, several *B. subtilis* α CTD residues including R268 and R289 are not conserved in *E. coli* α , but are conserved in other classes of bacteria, in which they could function in essential processes. Ectopically expressing *rpoAR268A* and *R289A* mutant alleles in *rpoAY263C* merodiploid strains conferred a dominant negative effect on sporulation, as indicated by the translucent colony phenotype on sporulation agar plates (Fig. 2.3). This was tested further by plating heat-treated *rpoA⁺-His₆/rpoAY263C* and *rpoAR289A/rpoAY263C* cells from 24-48-hour cultures grown in sporulation medium and counting colony-forming units after plating on

nutrient agar plates. The *rpoAR289A-His₆* expressing cells showed sporulation frequency that was 2% of strains expressing WT *rpoA-His₆* after 24 hours, and 25% after 48 hours. The result shows that these residues could function in global transcriptional control during development, and perhaps in other complex regulatory networks that operate in other bacteria in which these a residue positions are conserved.

The α E255 residue is located at the Spx- α CTD interface. The homologous position in *E. coli* α CTD is an Asp residue that can interact with R603 of σ^{70} region 4.2 during CAP- and UP element-stimulated transcription initiation (Ross, Schneider et al. 2003). Our data from experiments examining Spx-dependent transcription and oxidative stress resistance in the *rpoAE255A-His₆/rpoAY263C sigAR362A* strain suggests that this interaction does not play a role in Spx-activated transcription. Nonetheless, the σ^A R362 residue (corresponding to *E. coli* σ^{70} R603) is necessary for optimal Spx-dependent *yugJ*- and *trxA-lacZ* expression, perhaps by serving as a contact point with RNAP-bound Spx protein.

Previous work provided evidence that a single monomer of Spx engages RNAP to activate transcription (Lin and Zuber 2012). It was proposed that Spx contacted other subunits besides α and that these additional interactions were required for transcriptional activation. The requirement for R362A of σ^A and the far-western blot (Fig. 2.6) results suggest that Spx may contact other RNAP subunits.

Additional contact sites in RNAP might explain why we find only a single monomer of Spx engaging holoenzyme despite the presence of two potential a contact surfaces. The role of α CTD in binding to Spx would be to position the

transcription factor to optimize interaction with other holoenzyme subunits and/or with promoter DNA. This in turn would place both Spx and RNAP in position to contact promoter elements required for Spx-activated transcription. Further structural analysis is required to uncover the functional contacts mediating productive engagement of Spx with RNAP. Mutational analysis of Spx to identify amino acid residues that may be required for Spx interactions with RNAP will be reported in the following sections.

CHAPTER 2, PART II:
MUTATIONAL ANALYSIS OF POSSIBLE σ^A -INTERACTING SPX AMINO ACID
RESIDUES

INTRODUCTION

Possible multi-subunit contact between Spx and multiple subunits of RNAP

In addition to α CTD, it is possible that Spx utilizes other contact sites within RNAP during activated transcription. During far-western blot analysis, Spx was shown to bind to a protein that co-migrates with the RNAP large subunits (β and β') (Ch. 2, Fig. 2.6), and preliminary yeast two-hybrid results (Nakano, S., M. M. Nakano, and PZ, unpublished) suggests that the site of Spx interaction is the region of the β subunit encompassing the flap domain previously implicated in σ factor interaction (Kuznedelov, Minakhin et al. 2002). Although rare, the T4 phage-encoded AsiA (Yuan, Nickels et al. 2009) and a recently characterized, phage-encoded transcription factor, gp39 of *Thermus thermophilus* phage P23-45 (Tagami, Sekine et al. 2014), were found to contact both β flap and region 4 of a major σ subunit.

The requirement for R362 of σ^A suggests that the σ^A is an additional Spx target (Ch. 2, Fig. 2.5). Previous work provided evidence that a single Spx monomer preferentially engages the σ^A form of RNAP holoenzyme (Lin and Zuber 2012). Moreover, the promoters of Spx-regulated genes are predominantly σ^A -dependent, and ChIP-chip analysis shows a genome-wide co-occurrence of Spx and SigA near transcription start sites of Spx-regulated genes (Rochat, Nicolas et al. 2012). The Spx paralog, MgsR activates transcription of genes that are members of the general

stress, σ^B regulon (Reder, Hoper et al. 2008, Reder, Pother et al. 2012). Evidence from studies of MgsR indicates that the regulator exerts control by interaction with the σ^B form of RNAP. Inspection of region 4.2 of σ^B reveals little homology with that of σ^A , and the notable absence of a conserved residue corresponding to σ^A R362. The divergent structures of σ^A and σ^B within region 4.2 could confer holoenzyme-specific contacts between the paralogous Spx proteins and RNAP.

Structural determinants of Spx activity

The first Spx- α CTD crystal structure was obtained by an *in vitro* Spx- α CTD complex assembly and provided insights regarding possible links between Spx structure and Spx function (Newberry, Nakano et al. 2005). The Spx protein structure is comprised of two main domains, of which the first contains the N-terminal CXXC redox center and the C-terminal tail required for ClpX recognition. Domain 2 encompasses the region of Spx oriented towards the Spx- α CTD interface. A glycine at position 52 in domain 2 is essential for Spx- α CTD interaction and participates in Van der Waals interactions with a tyrosine at position of α CTD. A short alpha helix, (“ $\alpha 4$ ”) is also located in domain 2 and contains residues R60 to N68 amino acid residues, which have been implicated in Spx-DNA binding (Nakano, Lin et al. 2010). Located distal of the α CTD binding surface of Spx, the active site Cys10 and Cys13 are prone to oxidized disulfide formation as demonstrated by electrospray ionization mass spectrometry (Nakano, Erwin et al. 2005). Spx shares high amino acid sequence similarity with its paralog MgsR, and its structural homolog arsenate reductase (ArsC), which contains an arsenate binding

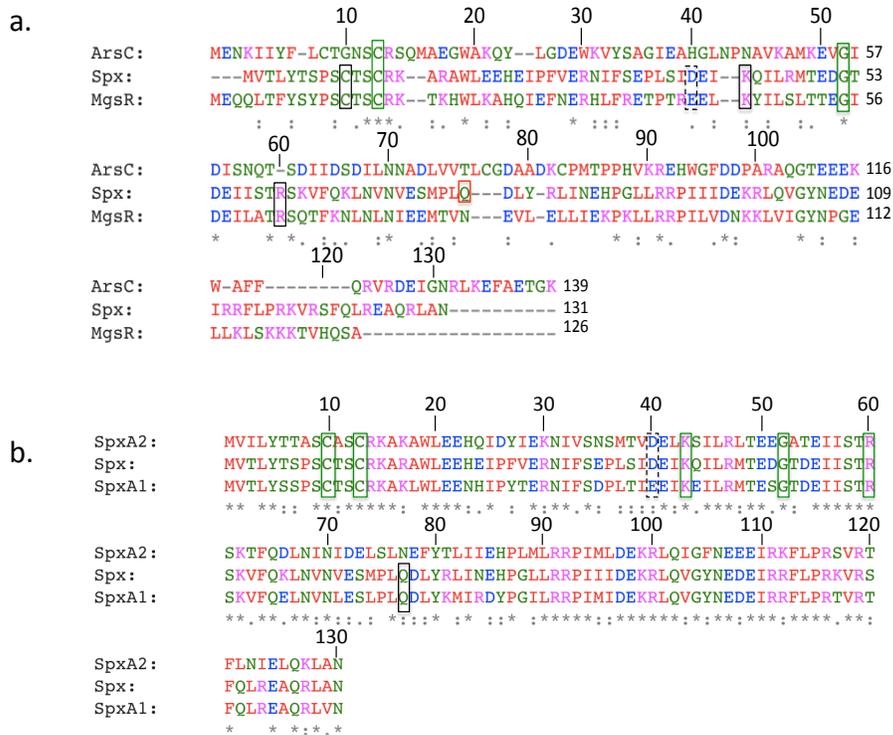


Figure 2.9. Amino acid sequence alignments of Spx and structural homologs

B. subtilis Spx bears homology to a. Arsenate Reductase (ArsC) and the general stress response regulatory MgsR, and b. the *B. anthracis* Spx paralogs SpxA1 and SpxA2.

Notable residues are highlighted and include the residues targeted for mutagenesis in this chapter (Spx K43, D40, and Q77).

Cys12 residue (Martin, DeMel et al.) corresponding to SpxC10 but does not contain a corresponding Spx C13 (Fig. 2.9 a.). Efficient release of arsenite at the ArsC Cys12 is facilitated by proximal side chains of an arginine triad composed of R60, R94, R104 (Martin, DeMel et al. , Shi, Mukhopadhyay et al. 2003). An arginine at position 92 of Spx corresponds to ArsCR94 and is poised to interact electrostatically with the C10 thiolate, however CXXC disulfide formation shifts the R92 side chain away from C10 and towards $\alpha 4$ (Nakano, Lin et al. 2010). Genetic epistasis studies of examining C10A and R92A implicate the arginine residue in redox control of Spx (Lin, Walthers et al. 2013). The crystal structure of SpxC10S, which mimics the reduced form, in complex with α CTD was obtained (Nakano, Lin et al. 2010). The structural analysis of the C10S mutant suggests that the overall conformation of Spx- α CTD is minimally perturbed upon reduction, with the exception of Spx α helix 4, which likely rotates and unfolds into a coiled structure. The redox-dependent repositioning of the side chains of residues within $\alpha 4$ may modulate Spx activity by altering Spx-RNAP interactions or Spx-promoter DNA interactions. EMSAs using purified α CTD and *trxB* promoter DNA formed a shifted complex on the gel, but incubation with Spx formed a supershift (Nakano, Lin et al. 2010).

An *spxG52R* mutant, which confers a severe defect in transcriptional activation and repression, failed to form a supershift, suggesting that the G52R mutation disrupts Spx- α CTD interaction (Zhang, Nakano et al. 2006, Nakano, Lin et al. 2010). SpxR60E also confers a severe defect in Spx-activated transcription but not in repression. EMSA results suggests that the R60, located in $\alpha 4$, is required to interact

with *cis*-acting elements of target promoters but does not function in Spx- α CTD complex stability (Nakano, Lin et al. 2010).

Spx amino acid residues K43, D40, and Q77 may function in Spx-RNAP interaction

The structure of the Spx- α CTD complex was examined to identify amino acid residues of Spx that may function in facilitating interactions between Spx and contact sites within RNAP. Spx amino acid residues K43, D40 and Q77 are surface exposed and located near RpoAE255 at the Spx- α CTD interface (Fig. 2.10). SpxK43 is conserved in MgsR and the *B. anthracis* Spx paralogs. SpxD40 is conserved in SpxA2 of *B. anthracis* and corresponds to ArsCH47, SpxA1E40, and MgsRE43. Finally, the SpxE77 is conserved in SpxA1 of *B. anthracis* and corresponds to MgsRN80, and SpxA2N77 (Fig. 2.9).

The *E. coli* D259 residue corresponding to *B. subtilis* RpoA α E255 residue has functions in α CTD- σ^{70} interaction during some cases of activator-stimulated transcription (Chen, Tang et al. 2003, Ross, Schneider et al. 2003). However, evidence suggests that RpoAE255 and σ^A R362 are likely not interacting partners (Ch. 2, Fig. 2.5) RpoA E255 likely interacts electrostatically with SpxK43 (Fig. 2.10). Instead of RpoA E255, SpxD40 is in close proximity to where the σ^{70} - α CTD contact surface would correspond, and thus may interact electrostatically with SpxK43 and also with σ^A R362. The side chain amino group of SpxQ77 and the backbone carbonyl of RpoA E255 are in close proximity and likely participate in hydrogen

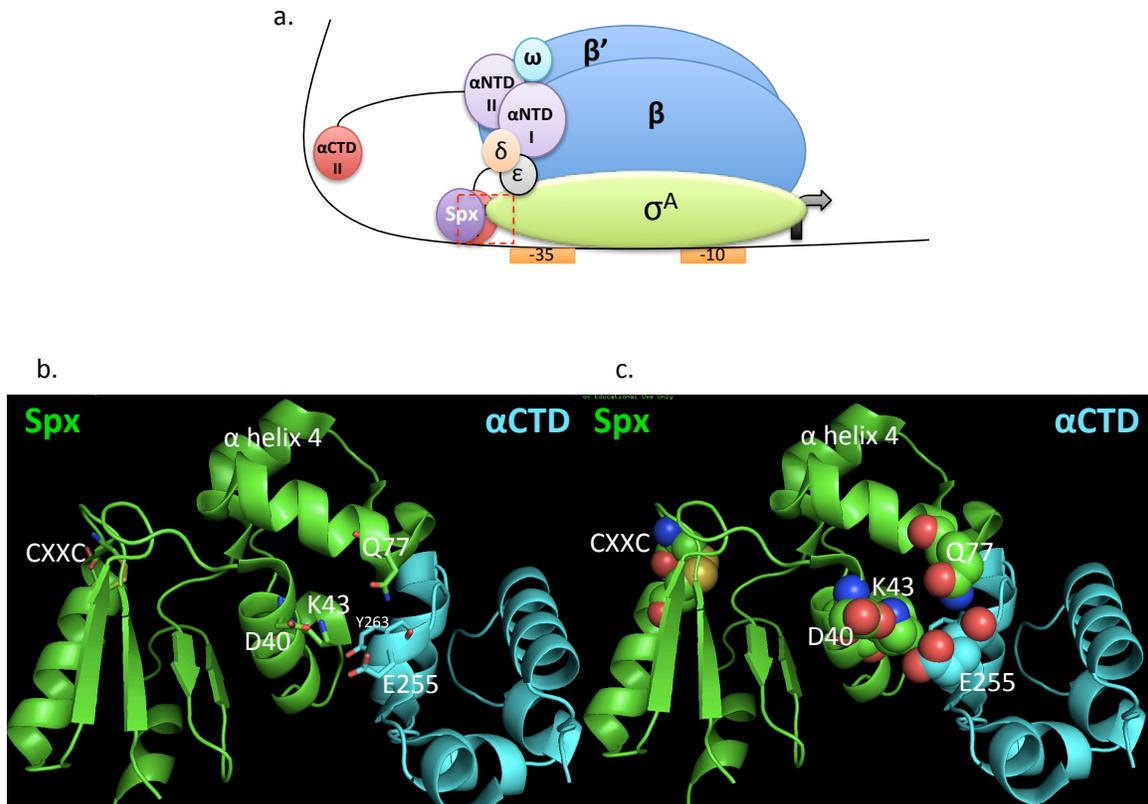


Figure 2.10. Spx amino acid residues K43, D40, and Q77 are located at the Spx-αCTD interface. a. Schematic depicting the hypothesis that sigA lies in proximity to the Spx-αCTD interface. Notable amino acid residues located near the Spx-αCTD interface are labeled and depicted in b. “stick” form, and c. “sphere” form. PyMOL graphics adapted from Spx-αCTD crystal (Newberry, Nakano et al. 2005). Pdb file: 1z3e.pdb. PyMol is a widely used biomolecular visualization tool.

bonding. Mutations in Spx or RNAP that impair Spx-RNAP interaction negatively affect Spx-activated transcription (Zhang, Nakano et al. 2006, Nakano, Lin et al. 2010, Birch, Davis et al. 2017). SpxK43, D40, and Q77 may be required for Spx-RNAP complex formation and thus were targeted for Ala codon substitution mutagenesis to determine whether these residues conferred defects in Spx-activated transcription.

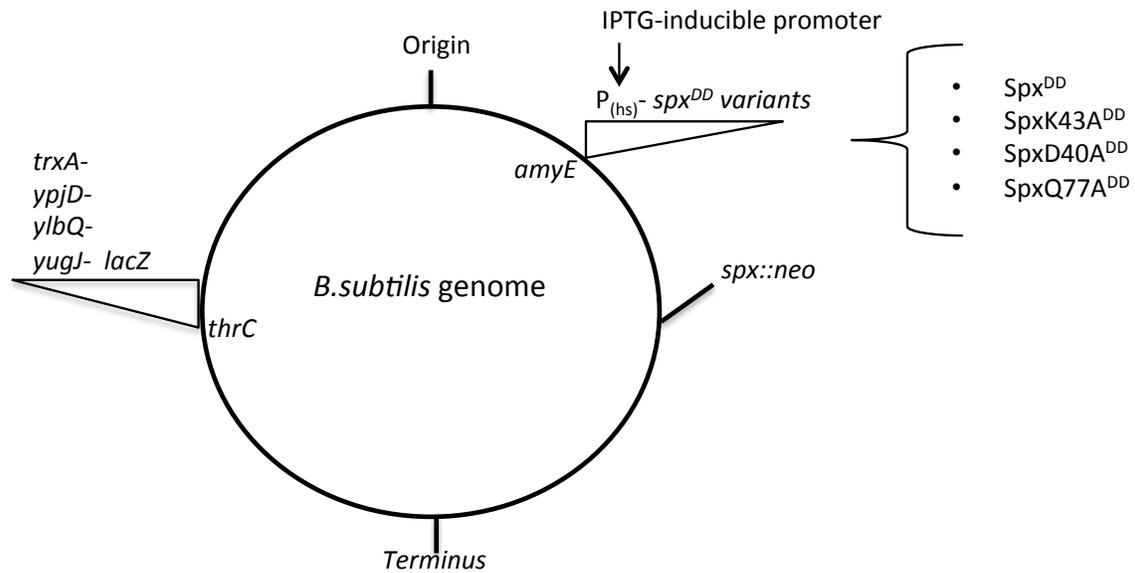


Figure 2.11. Relevant genotype of subject strains. The protease-resistant forms of the Spx variants are ectopically expressed under an IPTG inducible promoter. The constructs were introduced into strains bearing several promoter-*lacZ* fusions.

RESULTS

Differential structural requirements of Spx activity among members of the Spx regulon

The protease-resistant forms of the Spx mutants were generated and introduced to *B. subtilis* cells bearing transcriptional *-lacZ* fusions of Spx-activated genes (Fig. 2.11). To determine whether SpxD40A, K43A, or Q77A impair Spx-mediated activation, several promoters of Spx-activated genes were used to construct transcriptional *lacZ* fusions for *in vivo* expression assays. The Spx-activated genes used in this study include *trxB* (thioredoxin reductase), *yugJ* (butanol dehydrogenase), and the Bacillithiol biosynthesis gene containing operons *ypjD* (*bshBA*) and *ylbQ* (*bshC*), (Nakano, Lin et al. 2010, Gaballa, Antelmann et al. 2013, Birch, Davis et al. 2017).

The effects of SpxK43A, D40A and Q77A mutations on Spx activity vary considerably among the selected Spx-activated genes (Fig 2.12). SpxK43 and Q77 appear dispensable for *trxB* and *ylbQ* induction, while SpxK43A and Q77A mutants each lose approximately 50% activity at the *yugJ* and *ypjD* promoters. The D40 residue may perform a unique function at the *ypjD* promoter, as a D40A mutation confers a ~50% reduction in activation of this gene but does not impair transcription of the other three.

sigAR362A affects control of Phyperspank-expressed proteins

To conduct genetic epistasis studies, the aforementioned Spx mutants were ectopically expressed in the pDR111 vector in a σ^A R362A background similar to

experiments with RpoAE255A (Birch, Davis et al. 2017) . Constructs that are fused downstream of the Phyperspank promoter in the pDR111 vector are subject to constitutive repression by the LacI repressor, which is sensitive to the lactose analog IPTG (Britton, Eichenberger et al. 2002). Preliminary data showed that σ^A R362A mutation possibly impaired *lacI* transcription and increased the leaky expression of the *spx* and mutant derivatives (Fig. 2.13).

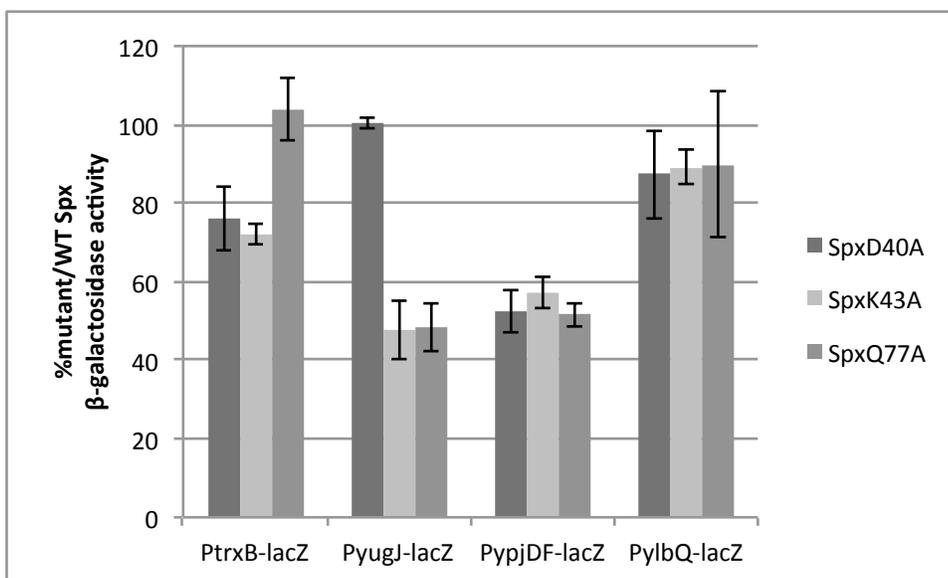


Figure 2.12. Differential Spx amino acid requirements for members of the Spx regulon. Cultures were grown to mid-exponential and split into two subcultures, to one of which 1mM IPTG was added. Cells were harvested between 0.5h to 2.5 h post induction and tested for β -galactosidase activity. The graph depicts the average percentage mutant activity over wild-type Spx at the *trxB*, *yugJ*, *yjD*, or *ylbQ* promoter.

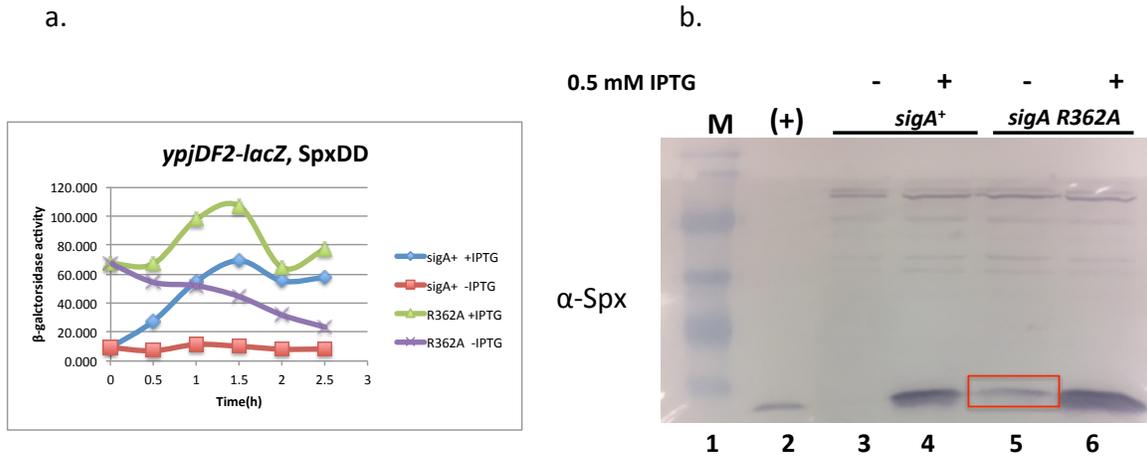


Figure 2.13. *sigAR362A* compromises control of Phyperspank-

expressed proteins. a. Cultures were grown, harvested, and tested for β -galactosidase as previously described. In the *sigAR362A* mutant, significant “leaky” expression likely caused elevated *ypjD-lacZ* expression in the absence of inducer. b. Bsu cells were grown to mid-exponential in DSM and treated with IPTG. Cells were harvested up to one hour post induction and lysates were prepared for western blotting to detect Spx in the lysates. Loaded 30 ug of total protein.

DISCUSSION

Structural analysis revealed that Spx amino acid residues D40, K43, and Q77 were located at the Spx- α CTD interface, which may also involve contact with region 4.2 of the σ^A subunit. We speculated that if the residues functioned in promoting Spx interaction with σ^A or possibly other surfaces within the RNAP holoenzyme, Ala codon substitutions might cause a general defect in Spx-activated transcription. However, expressing the D40A, K43A, and Q77A mutants exerted highly variable effects on Spx-stimulated expression among several Spx-controlled genes.

The promoter architecture of the subject Spx-activated genes differs and may reflect the differential requirements of Spx amino acid residues during transcription (Fig. 2.14). The results suggest that the conformation of Spx within the Spx-RNAP-DNA complex is specific to individual and/or potential classes of Spx-RNAP-utilized promoters. Therefore, Spx may function similarly to the the *E. coli* CAP/Crp regulator (catabolite activator protein/cAMP recptor protein) (Lawson, Swigon et al. 2004), wherein Spx might engage in different RNAP subunit contacts due to its position within the regulatory regions of Spx-activated promoters.

The results present herein underscore the importance of continuing structural analysis of the Spx-RNAP transcriptional complex. Examining the epistasis relationship with *sigAR362* may provide useful information but will require a different expression system such as the Xylose- inducible system of Schumann et. al (Kim, Mogk et al. 1996).

	-35	-10		
AAATAATTTGTAAGCA	TTAAAA	TAGCGT	GAACGAATGGGAGATGCTATACT	<i>trxA</i>
TTAATCGTGTTGAGCA	AAAAAA	TAGCGT	TACCCATGAGATTTATAGGAT	<i>trxB</i>
AACAGCAAACGTGCT	TAAACAT	TTGTCA	AACGTATCTGAATTAGCTAACAT	<i>ypjD</i>
TAAAGCACATGGGATCT	TTGAGA	AGTAA	TTTCTTCTTACTTCTGCTATGAT	<i>ylbQ</i>
GCTTATCAGCTTCTTTCC	AGCA	CC	TTGCAAATAAAACCATCTGGTATGCT	<i>yugJ</i>

Figure 2.14. Alignment of relevant promoter regions preceding subject

Spx-activated genes. Core promoter hexamers are highlighted in yellow.

Putative Spx-recognized “AGCA” cis-elements are highlighted in purple.

PART I: MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Plasmids and bacterial strains are listed in Table 2.1. *B. subtilis* strains were derived from JH642 (*trpC2 pheA1*). DNA fragments harboring the wild-type (WT) *rpoA* allele and mutant alleles with Ala codon substitutions were generated using oligonucleotides listed in Table 2.3. *B. subtilis* JH642 genomic DNA and a *rpoA* 5' oligonucleotide (*rpoA* F) and a *rpoA* 3' oligonucleotide (*rpoA* R) were used for generation of *rpoA*⁺-His₆ PCR fragment. The PCR product was digested with SphI and NheI and ligated with plasmid pDR111 (Britton, Eichenberger et al. 2002), which had been cleaved with the same enzymes. The resulting plasmid, pCB81, was used for *rpoA* mutant construction. The Ala codon substitutions in *rpoA* were generated by two-step PCR-based mutagenesis using a pair of complementary mutagenic oligonucleotides. Each oligonucleotide pair was used for the first PCR, together with either the *rpoA* 5' oligonucleotide (*rpoA* F) or the *rpoA* 3' oligonucleotide (*rpoA* R). The two PCR products were annealed and served as template for second-round PCR using *rpoA* F-R. The fragments were cleaved with NheI and SphI and inserted into pDR111 that was cleaved with the same enzymes. *E. coli* Top10 cells were used for propagation and plasmid DNA extraction. *E. coli* transformants were plated on lysogeny broth (LB) agar supplemented with 100 µg/mL ampicillin. The pDR111 derivatives were used to transform *B. subtilis* competent cells of strains bearing *lacZ* fusions integrated at the *thrC* locus. The *lacZ* fusions were made to the promoter of the *trxA* gene as previously described (Nakano, Kuster-Schock et al. 2003). The *yugJ-lacZ* fusion was constructed by amplifying the *yugJ* promoter region using oligonucleotides osn03-

80 and osn03-81 (Table 2.2) and JH642 chromosomal DNA as template for PCR. The fragment was cleaved with EcoR1 and BamH1 and inserted into plasmid pDG793 (Guerout-Fleury, Frandsen et al. 1996), which had been cleaved with the same enzymes. The *lacZ* fusion-bearing strains transformed with plasmid-borne *rpoA* DNA and transformants were selected on Difco sporulation medium (DSM) agar (Cutting and Vander Horn 1990) containing 75 µg/mL spectinomycin (Spc) or Spc in addition to 1 µg/mL erythromycin /25 µg/mL lincomycin (Erm/Ln). Final strains were confirmed by testing for Trp, Phe, Thr auxotrophy and Amy⁻ (amylase negative) phenotype (no zone of clearing on starch agar).

Assays of β -galactosidase activity. The effect of the α CTD Ala codon substitutions on the activity of Spx-controlled genes was determined by measuring β -galactosidase activity in cells carrying *trxA-lacZ* and *yugJ-lacZ* (ORB9464-9468, ORB9575, ORB9650-9651) fusions, incubated in the presence and absence of 0.5 mM IPTG. The strains were grown at 37 °C overnight in 2xYT (Fredrick, Caramori et al. 1995) supplemented with Spc and Erm/Ln. Overnight cultures were used to inoculate DSM at a starting optical density of 600 nm (OD₆₀₀) of 0.04. When the OD₆₀₀ of the cultures reached 0.35 to 0.5, the cultures were divided into two flasks and 0.5 mM IPTG was added to one of the flasks. Samples were taken at 0.5-hr intervals to assay β -galactosidase activity, which was expressed as Miller units (Miller 1972). The effect of the α CTD Ala codon substitutions on the activity of Spx-controlled genes during salt stress was determined as stated above. Cultures were split at an OD₆₀₀ of 0.35-0.5 and treated with 0.5 mM IPTG with or without 0.6 M

NaCl. Sterile water was added to the culture without NaCl addition to control for culture volume.

Spot dilution assay. Wild type *B. subtilis* *PyugJ-lacZ* bearing strain 9445 and the *rpoAY263C*, *PyugJ-lacZ* bearing strains were used as controls. *RpoAY263C* expressing WT *rpoA* and mutant *rpoA* derivatives at the *amyE* locus (Table 2.1) were grown for spot dilution. The strains were grown at 37 °C overnight in 2xYT supplemented with Spc or Erm/Ln. Overnight cultures were used to inoculate Difco sporulation medium (DSM) at a starting OD₆₀₀ of 0.04. When the OD₆₀₀ of the cultures reached 0.35 to 0.5, 0.1mM IPTG was added to each flask. Cells were grown further until OD₆₀₀ ~1.0, and serially diluted to 10⁻⁵. Cells were diluted in 1x T-base (Cutting and Vander Horn 1990) and 5 µL were spotted onto DSM agar containing 0.1 mM IPTG with or without 0.75 mM diamide. Plates were incubated at 37° C overnight.

Western blot analysis. The strains ORB9464-ORB9468, ORB9575, and ORB9650-9651 were tested for RpoA-His₆ production after induction with IPTG. Cells were cultured in DSM supplemented with spectinomycin with shaking at 37°C until an OD₆₀₀ ≈ 0.4-0.5. Samples of 15 mL were collected (t₀) prior to adding 0.5 mM IPTG. Incubation was continued and 15 mL samples were collected after 30 min and 60 min. Cells were harvested by centrifugation (5180xg, 4°C, 10 min) and pellets were re-suspended in 200 µl 1X T-base (Sonenshein, Cami et al. 1974). Cells were twice disrupted with glass beads during 5 min vortex- 2 min ice rest – 5 min vortex

intervals. Protein concentrations in the crude extract were determined using BioRad protein assay solution, and 30 μg of total protein was applied to a 15% SDS polyacrylamide gel. The proteins were electrotransferred to a nitrocellulose membrane and probed with anti-His₆ antibody followed by incubation with secondary anti-mouse antibody.

Sporulation assay. Selected *rpoA* merodiploid strains were used to inoculate 2.5 ml 2xYT medium with Spc for incubation at 37°C overnight with shaking in 10 ml sterile tubes. After incubation, 0.25 ml of the overnight culture was transferred to 25 ml DSM plus Spc and IPTG (100 μM) in 250 ml baffled flask. The DSM cultures were incubated at 37°C with shaking. Samples were taken every 30 min to measure OD₆₀₀, data from which were used to generate growth curves. When cultures reached the end of exponential growth, (T_0), incubation continued for 8 hr, when samples were collected for two series of 10-fold serial dilutions. One dilution series was heated 80°C for 30 min. 100 μl of each dilution, heated and unheated, were plated onto DSM agar plates, which were incubated overnight at 37°C. CFU (colony forming units) were counted on plates having between 30 to 300 colonies. Percent spores were calculated as heated CFU/unheated CFU x 100. The assay was performed in triplicate and percentages with standard deviation were calculated (Birch, Davis et al. 2017).

RNAP purification. Six 1 L cultures of *rpoAHis₆*-expressing *B. subtilis* strains were grown in LB broth at 37°C, 200 rpm to an OD₆₀₀ ~0.5 and 0.5 mM IPTG was added

1 hour prior to harvest. Cells were collected by centrifugation for (5180*g*, 4°C, 10 min.) and stored at -80°C until use. Cell pellets were thawed on ice and suspended in low salt lysis buffer (100 mM NaCl, 50 mM Tris 7.8, 5mM MgCl₂, 20% glycerol, 5 mM β-mercaptoethanol) with protease inhibitor cocktail tablets (Roche, cOmplete tablets, Mini) and disrupted by three passages through an Avestin Emulsiflux C3 emulsifier. Cell debris was removed by two centrifugation cycles for 30 min (15k rpm (Sorvall), 4°C). Supernatant was applied to 5 mL Ni-NTA bed and incubated at 4°C with gentle agitation for 1 hour. Supernatant was drained and column was washed with 30-column volume (CV) of 30 mM imidazole in low salt lysis buffer. Five elution fractions were collected in 6 CV of 200 mM imidazole in low salt lysis buffer and stored at 4°C overnight. Heparin column chromatography was performed at 4°C with a 1 mL Hitrap HP Heparin column and BioRad LP Biologic chromatography system. Ni-column elution fractions containing RNAP were pooled and applied to the equilibrated heparin column at 1 mL/min. Fractions were collected using a 90 minute 100 mM-1 M [NaCl] lysis buffer gradient at 1 ml/min and 1 mL/tube. NaCl concentration reached maximum between 45 and 60 min. Nine fractions encompassing the A₂₈₀ peak were examined by SDS-PAGE for RNAP subunits. Heparin column elution fractions were pooled and diluted to 100 mM NaCl in low salt lysis buffer containing no NaCl. HiQ column chromatography was performed at 4 °C with a Macro MiniPrep HiQ column and LP Biologic chromatography system. Diluted fractions were collected using the same gradient program as previously described (Lin and Zuber 2012). Fractions were pooled, concentrated to 0.5 mL using a 15 ml Centricon filter (10-kDa cutoff; Amicon), and

dialyzed against storage buffer (10 mM Tris-HCl, 100 mM KCl, 50% glycerol, pH 8.0). The final concentration of the solution containing RNAP was determined by the a protein assay (Bio-Rad) with bovine serum albumin (BSA) as the standard. The final RNAP preparation was stored at -20°C.

Electrophoretic mobility shift assay (EMSA). EMSA was performed using a previously described procedure (Barendt, Birch et al. 2016) to test RNAP mutants for Spx-enhanced promoter affinity. A DNA fragment of 171 bp containing the promoter region of *trxA* was amplified from JH642 genomic DNA using the primers *osn76* and *osn77* (Nakano, Erwin et al. 2005). The *PyugJ* fragment was the same used for construction of the *yugJ-lacZ* fusion presented above. Spx and σ^A protein were purified from intein-chitin-binding domain fusion proteins as described previously (Nakano, Zheng et al. 2002, Nakano, Geng et al. 2006).

Far-western blot. RNAP solutions purified from ORB9464, ORB9575, ORB9650, and ORB9651 were resolved by SDS gel electrophoresis followed by nitrocellulose blotting. Blotted proteins were denatured and renatured by 4 rounds of guanidine hydrochloride (GuHCl) treatment. The nitrocellulose filters were treated with 6 M, 3M, then 1M GuHCl in AC buffer (100mM NaCl, 20 mM Tris-HCl (pH7.5), 0.5mM EDTA, 10% glycerol, 1mM DTT, 0.1% Tween) for 30-minute intervals of rocking at room temperature. The filters were then incubated in AC buffer with 0.1M GuHCl and no GuHCl for 1-hour and overnight, respectively, at 4°C. The filters were next blocked with 5% milk followed by a 3-hour room temperature incubation with or without 5 μ g of purified Spx in AC buffer. Following three 5-min washing steps in

TBST, western blot with Spx anti-serum was conducted as described above. Protocol was adapted from Wu et al (Wu, Li et al. 2007).

Size-exclusion chromatography. Purified Spx together with RNAP solutions purified from ORB9464, ORB9575 or ORB9650 were diluted in running buffer (10 mM Tris 7.8, 150 mM KCl, 5 mM MgCl₂, 5 mM β-mercaptoethanol, 20% glycerol) to a final concentration of 6 μM and 650 nM, respectively. Each 200 μL Spx-RNAP mixture was pre-incubated for binding at room temperature for 20 minutes prior to loading onto a 1.5 x 44 cm column packed with Biogel P-60 gel polyacrylamide resin (medium, 3-60kDa MWCO, Bio-rad). Using a BioRad LP Biologic Chromatography system, ninety 1 mL fractions were collected at 0.16 mL/min. Fractions encompassing the observed UV-vis peak, which centered around the pre-determined void volume of ~20 mL were collected for each run. The fractions were precipitated in 4X volume of acetone at -20C for at least one hour prior to SDS-PAGE and western blotting using Spx antiserum or polyHis monoclonal antibody.

Table 2.1. Bacterial Strains and Plasmids (Chapter 2: Part I)

Strain	Relevant Genotype	Source
JH642	<i>Wild-type Bacillus subtilis JH642 trpC2 pheA1</i>	This study
ORB3621	<i>JH642 rpoAY263C</i>	This study
ORB4350	<i>JH642 SPβC2del2::Tn::SpoIIIG-lacZ</i>	Satola 1991
ORB4592	<i>JH642 thrC::PtrxA-lacZ</i>	Nakano 2003
ORB4594	<i>JH642 rpoAY263C thrC::PtrxA-lacZ</i>	Nakano 2003
ORB9445	<i>JH642 thrC::PyugJ-lacZ</i>	Nakano 2003
ORB9162	<i>JH642 rpoAY263C thrC::PtrxA-lacZ amyE::Phs-rpoA-His6</i>	This Study
ORB9256	<i>JH642 rpoAY263C thrC::PtrxA-lacZ amyE::Phs-rpoAR268A-His6</i>	This Study
ORB9257	<i>JH642 rpoAY263C thrC::PtrxA-lacZ amyE::Phs-rpoAR289A-His6</i>	This Study
ORB9325	<i>JH642 rpoAY263C thrC::PtrxA-lacZ amyE::Phs-rpoAK294A-His6</i>	This Study
ORB9354	<i>JH642 rpoAY263C thrC::PtrxA-lacZ amyE::Phs-rpoAR261A-His6</i>	This Study
ORB9446	<i>JH642 rpoAY263C thrC::PyugJ-lacZ</i>	This Study
ORB9464	<i>JH642 rpoAY263C thrC::PyugJ-lacZ amyE::Phs-rpoA-His6</i>	This Study
ORB9465	<i>JH642 rpoAY263C thrC::PyugJ-lacZ amyE::Phs-rpoAR261A-His6</i>	This Study
ORB9466	<i>JH642 rpoAY263C thrC::PyugJ-lacZ amyE::Phs-rpoAR268A-His6</i>	This Study
ORB9467	<i>JH642 rpoAY263C thrC::PyugJ-lacZ amyE::Phs-rpoAR289A-His6</i>	This Study
ORB9468	<i>JH642 rpoAY263C thrC::PyugJ-lacZ amyE::Phs-rpoAK294A-His6</i>	This Study
ORB9575	<i>JH642 rpoAY263C thrC::PyugJ-lacZ amyE::Phs-rpoAY263C-His6</i>	This Study
ORB9594	<i>JH642 amyE::Phs-rpoAR268A-His6</i>	This Study
ORB9596	<i>JH642 amyE::Phs-rpoAR289A-His6</i>	This Study
ORB9611	<i>JH642 amyE::Phs-rpoA-His6</i>	This Study
ORB9612	<i>JH642 rpoAY263C amyE::Phs-rpoA-His</i>	This Study
ORB9612	<i>JH642 rpoAY263C amyE::Phs-rpoAR289A-His</i>	This Study
ORB9616	<i>JH642 rpoAY263C thrC::PtrxA-lacZ amyE::Phs-rpoAY263C-His6</i>	This Study
ORB9643	<i>JH642 amyE::Phs-rpoA-His6 SPβC2del2::Tn::SpoIIIG-lacZ</i>	This Study
ORB9644	<i>JH642 amyE::Phs-rpoAR268A-His6 SPβC2del2::Tn::SpoIIIG-lacZ</i>	This Study
ORB9645	<i>JH642 amyE::Phs-rpoAR289A-His6 SPβC2del2::Tn::SpoIIIG-lacZ</i>	This Study
ORB9650	<i>JH642 rpoAY263C thrC::PyugJ-lacZ amyE::Phs-rpoAE255A-His6</i>	This Study
ORB9651	<i>JH642 rpoAY263C thrC::PyugJ-lacZ amyE::Phs-rpoAE298A-His6</i>	This Study
ORB9654	<i>JH642 rpoAY263C thrC::PtrxA-lacZ amyE::Phs-rpoAE255A-His6</i>	This Study
ORB9655	<i>JH642 rpoAY263C thrC::PtrxA-lacZ amyE::Phs-rpoAE298A-His6</i>	This Study
ORB9826	<i>JH642 rpoAY263C thrC::PyugJ-lacZ amyE::Phs-rpoA-His6 sigAR362A</i>	This Study
ORB9827	<i>JH642 rpoAY263C thrC::PyugJ-lacZ amyE::Phs-rpoAE255A-His6 sigAR362A</i>	This Study
ORB9835	<i>JH642 rpoAY263C thrC::PyugJ-lacZ amyE::Phs-rpoA-His6 spx::neo</i>	This Study
ORB9956	<i>JH642 rpoAY263C thrC::PtrxA-lacZ amyE::Phs-rpoA-His6 sigAR362A</i>	This Study
ORB9957	<i>JH642 rpoAY263C thrC::PtrxA-lacZ amyE::Phs-rpoAE255A-His6 sigAR362A</i>	This Study

Plasmids	Description	Source
pDR111	cloning vector, IPTG inducible Phyperspank(Phs), Amp ^R , Spc ^R	Ben-Yehuda 2003
pCB81	pDR111:: Phs- <i>rpoA</i> -His ₆ (0.9 kb DNA fragment), Spc ^R	This study
pJD1	pDR111:: Phs- <i>rpoAK294A</i> -His ₆ (0.9 kb DNA fragment), Spc ^R	This study
pMD14	pDR111:: Phs- <i>rpoAY263C</i> -His ₆ (0.9 kb DNA fragment), Spc ^R	This study
pMD7	pDR111:: Phs- <i>rpoAR261A</i> -His ₆ (0.9 kb DNA fragment), Spc ^R	This study
CB110	pDR111:: Phs- <i>rpoAR268A</i> -His ₆ (0.9 kb DNA fragment), Spc ^R	This study
CB112	pDR111:: Phs- <i>rpoAR289A</i> -His ₆ (0.9 kb DNA fragment), Spc ^R	This study
CB121	pDR111:: Phs- <i>rpoAE255A</i> -His ₆ (0.9 kb DNA fragment), Spc ^R	This study
CB123	pDR111:: Phs- <i>rpoAE298A</i> -His ₆ (0.9 kb DNA fragment), Spc ^R	This study
pDG793	cloning vector, Amp ^R , Erm ^R	Guerout-Fleury 1996
pSN79	pDG793:: <i>PtrxA-lacZ</i> , Erm ^R	Nakano 2003
pSN94	pDG793:: <i>PyugJ-lacZ</i> , Erm ^R	Nakano 2003

**Ampicillin (Amp), Erythromycin/Lincomycin (Erm), Spectinomycin (Spc)

Table 2.2 Oligonucleotides (Chapter 2: Part I)

Name	Sequence (5' --> 3')	Description	Restriction Site
orpoA F	ACCTGCTAGCTTTTGAAGGAGGGTTTTAAGTAATGATCGAGATTGAAA	Flanking	NheI
orpoA 2R	ACCTGCATGCTCAATGATGATGATGATGATGATGATCGTCTTTGCGAAGTCCGAGTCCAAGT	Flanking	SphI
orpoA261f	GGA TCT TTC TGT TGC TTC TTA CAA CTG CTT	Nested/Mutagenic	-
orpoA261r	AAG CAG TTG TAA GAA GCA ACA GAA AGA TCC	Nested/Mutagenic	-
E255A IF	CTT GAA ATG ACA ATT GAA GCA TTG GAT CTT TCT GTT	Nested/Mutagenic	-
E255A IR	AAC AGA AAG ATC CAA TGC TTC AAT TGT CAT TTC AAG	Nested/Mutagenic	-
orpoA268f2	ACA ACT GCT TAA AGG CTG CGG GTA TTA ACA	Nested/Mutagenic	-
orpoA268r2	TGT TAA TAC CCG CAG CCT TTA AGC AGT TGT	Nested/Mutagenic	-
orpoA2892f	ATG ATG AAA GTT GCA AAT CTA GGA CGC	Nested/Mutagenic	-
rpoA2892r	GCG TCC TAG ATT TGC AAC TTT CAT CAT	Nested/Mutagenic	-
E298A F	CGC AAA TCA CTT GCA GAA GTG AAA GCG	Nested/Mutagenic	-
E298A R	CGC TTT CAC TTC TGC AAG TGA TTT GCG	Nested/Mutagenic	-
orpoA294f	GAT GAA AGT TCG AAA TCT AGG ACG CGC ATC ACT TGA AGA AGT GAA AGC G	Nested/Mutagenic	-
orpoA294r	CGC TTT CAC TTC TTC AAG TGA TGC GCG TCC TAG ATT TCG AAC TTT CAT C	Nested/Mutagenic	-

Part II: MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table 2.3. *B. subtilis* strains were derived from JH642 (*trpC2 pheA1*).

Construction of K43A, D40A and Q77A mutant *spx^{DD}* alleles. Codon substitution mutations of the protease-resistant Spx form (*Spx^{DD}*) (Nakano, Kuster-Schock et al. 2003) were constructed by two-step PCR using complementary mutagenic primer pairs (Table 3.2) in a procedure similar to that used for the amino acid substitutions of the *rpoA* variants. The pSN56 plasmid, bearing pDR111::*spx^{DD}* (Nakano, Kuster-Schock et al. 2003) was used as template. The PCR product was digested with HindIII and EcoRI and ligated with plasmid pDR111, which had been cleaved with the same enzymes. The *lacZ* fusion-bearing strains transformed with plasmid-borne mutant *spx^{DD}* alleles and final strain construction was performed as described in part I: materials and methods.

Assays of β -galactosidase activity. The effect of the Spx-Ala codon substitutions on the activity of Spx-controlled genes was determined by measuring β -galactosidase activity in cells carrying *trxB*-, *yugJ*-, *ypjD*-, and *ylbQ*- fusions, incubated in the presence and absence of 1 mM IPTG. Details are as previously described in Part I: materials and methods.

Table 2.3. Strains and plasmids (Chapter 2: Part II)

Strain	Relevant Genotype	Source
JH642	<i>Wild-type Bacillus subtilis JH642 trpC2 pheA1</i>	This study
ORB7276	<i>JH642 thrC::PtrxB-lacZ amyE::Phs-spxDD</i>	Satola 1991
ORB9169	<i>JH642 thrC::PypjDF2-lacZ amyE::Phs-spxDD</i>	Nakano 2003
ORB9622	<i>JH642 thrC::PylbQ-lacZ amyE::Phs-spxDD</i>	Nakano 2003
ORB9902	<i>JH642 thrC::PyugJ-lacZ amyE::Phs-spxDD</i>	Nakano 2003
ORB9904	<i>JH642 thrC::PByugJ-lacZ amyE::Phs-spxQ77ADD</i>	This Study
ORB9905	<i>JH642 thrC::yugJ-lacZ amyE::Phs-spxK43ADD</i>	This Study
ORB9906	<i>JH642 thrC::yugJ-lacZ amyE::Phs-spxD40ADD</i>	This Study
ORB9907	<i>JH642 thrC::PypjDF2-lacZ amyE::Phs-spxQ77ADD</i>	This Study
ORB9908	<i>JH642 thrC::PypjDF2-lacZ amyE::Phs-spxK43ADD</i>	This Study
ORB9909	<i>JH642 thrC::PypjDF2-lacZ amyE::Phs-spxD40ADD</i>	This Study
ORB9949	<i>JH642 thrC::PtrxB-lacZ amyE::Phs-spxK43ADD</i>	This Study
ORB9950	<i>JH642 thrC::PtrxB-lacZ amyE::Phs-spxD40ADD</i>	This Study
ORB9951	<i>JH642 thrC::PtrxB-lacZ amyE::Phs-spxQ77ADD</i>	This Study
ORB9952	<i>JH642 thrC::PylbQ-lacZ amyE::Phs-spxQ77ADD</i>	This Study
ORB9953	<i>JH642 thrC::PylbQ-lacZ amyE::Phs-spxK43ADD</i>	This Study
ORB9954	<i>JH642 thrC::PylbQ-lacZ amyE::Phs-spxD40ADD</i>	This Study

Plasmids	Description	Source
pDR111	cloning vector, IPTG inducible Phyperspank(Phs), Amp ^R , Spc ^R	Ben-Yehuda 2003
pDG793	cloning vector, Amp ^R , Erm ^R	Guerout-Fleury 1996
pCB130	<i>pdr111::Phs-spxQ77ADD</i>	This Study
pCB132	<i>pdr111::Phs-spxK43ADD</i>	This Study
pCB134	<i>pdr111::Phs-spxD40ADD</i>	This Study
pSN56	<i>pdr111::Phs-spxDD</i>	Nakano 2003

**Ampicillin (Amp), Erythromycin/Lincomycin (Erm), Spectinomycin (Spc)

Table 2.4 Oligonucleotides (Chapter 2: Part II)

Name	Sequence (5' --> 3')	Description	Restriction Sites
K43A F	ATC AAT TGA TGA AAT GCA ACA AAT TCT TCG AAT G	Nested/Mutagenic	-
K43A R	R CAT TCG AAG AAT TTG TTG CAT TTC ATC AAT TGA T	Nested/Mutagenic	-
D40A F	TTC TCT GAA CCT TTA TCA ATT GCT GAA ATA AAA C	Nested/Mutagenic	-
D40A R	GTT TTA TTT CAG CAA TTG ATA AAG GTT CAG AGA A	Nested/Mutagenic	-
Q77A F	ATG CCG CTG GCA GAT TTG TAC	Nested/Mutagenic	-
Q77A R	GTA CAA ATC TGC CAG CGG CAT	Nested/Mutagenic	-
oMMN173	CGAGGAAGCTTAGATGTTTCATCCTACTA	Flanking	HindIII
oMMN174	TACCAGCAGGTCGACAAATAAAAGAAGG	Flanking	SalI

CHAPTER 3:
REDOX CONTROL AND SPX-DEPENDENT REGULATION OF THIOL
HOMEOSTASIS GENES

INTRODUCTION

Thiol stress and redox buffering

Maintenance of prokaryotic and eukaryotic physiological and global regulatory networks relies partially on an unperturbed intracellular redox status. The predominant pool of intracellular cysteine persists in the reduced state due to cytoplasmic mechanisms that maintain thiol homeostasis (Bardwell 1994). Reactive cysteine thiols are subject to a variety of oxidative modifications and, therefore, comprise among the most abundant vulnerable targets to toxic oxidants. At physiological pH, the pKa of cysteine thiols within proteins can be lowered by neighboring amino acid residues to induce deprotonation and thus a shift from Cys-SH to the much more reactive and unstable Cys S⁻ thiolate form (Ezraty, Gennaris et al. 2017). In the presence reactive oxygen (ROS), reactive nitrogen species (RNS), and reactive electrophilic species (RES) the resultant thiolate undergoes rapid reversible oxidation to highly unstable intermediates including Cys-SOH, Cys-SNO, and Cys-S-R (e.g. alkylated) moieties, respectively. Sodium hypochlorite (NaOCl), a common disinfectant and endogenous product of the mammalian innate immune system can trigger Cys-SH oxidation to Cys-Cl intermediates. The reversibility of such oxidative modifications provides a window of opportunity for cells to prevent

further oxidation of these intermediates (i.e. redox buffering zone). As previously stated, exposure to oxidizing agents during insufficient or compromised redox buffering can lead to the irreversible modification of Cys-SOH intermediates to sulfinic (Cys-SO₂H) and then further to sulfonic (Cys-SO₃H) (Van Laer, Hamilton et al. 2013). Additionally, compounds such as diamide trigger reversible non-native disulfide bond formation and depletion of cysteine and LMW thiols (Kosower, Kosower et al. 1969, Gaballa, Newton et al. 2010), while iodoacetamide and related compounds alkylate cysteine thiols (Smythe 1936). The wide range of thiol modifications can induce proteotoxic effects including thiol depletion, protein misfolding and aggregation. Toxic oxidants induce formation of intra- or intermolecular disulfides or S-thiolation (mixed disulfide) of the cysteinyl intermediates with non-protein redox buffering compounds. Such compounds will deplete the LMW thiol pool by triggering formation of RSSR or RS-toxin complexes, which may lead to induction of cognate reductases and biosynthesis enzymes to replenish the reduced LMW thiol pool.

LMW thiols can function in concert with ROS scavenging/ detoxifying enzymes, the TrxA/TrxB/NADPH system, and protein and DNA repair factors to alleviate oxidative stress. In *B. subtilis*, Spx positively regulates the *trxA/trxB*-encoded Thioredoxin/Thioredoxin reductase system in response to disulfide stress (Nakano, Erwin et al. 2005). The essentiality of *trxA* in *B. subtilis* is at least partially attributable to the lack of a functionally overlapping glutaredoxin system, like that which exists in *E. coli* (Prinz, Aslund et al. 1997). Free cysteine and small thiol containing molecules function primarily as non-protein redox buffers. The most

extensively studied LMW thiol glutathione (GSH) performs essential physiological and antioxidant functions in many Gram-negative bacteria and eukaryotes but is not present in Gram-positive bacteria (Van Laer, Hamilton et al. 2013). In Actinomycetes, mycothiol (MSH) prevails as the predominant LMW thiol that is functionally analogous to glutathione (Jothivasan and Hamilton 2008). Bacillithiol (BSH), a major LMW thiol in Gram-positive bacteria lacking GSH and MSH was discovered in *B. anthracis* extracts and in a mixed disulfide with *B. subtilis* peroxide sensing repressor OhrR (Lee, Soonsanga et al. 2007, Nicely, Parsonage et al. 2007).

Primary Functions of BSH

The physiological functions of bacillithiol and S-bacillithiolation of target proteins include maintenance of redox homeostasis, stress resistance, detoxification of toxic agents and metal homeostasis (Gaballa, Newton et al. 2010, Fang and Dos Santos 2015).

BSH may play a role in oxidative stress resistance. BSH-deficient mutants exhibit heightened sensitivity to paraquat in *B. subtilis* and altered Fe-S metabolism (Fang and Dos Santos 2015). In addition to protecting vulnerable proteins from over-oxidation, LMW thiol mediated disulfide exchange can exert redox-regulatory control of some proteins. Exposure to the strong oxidant sodium hypochlorite (NaOCl) induces protective S-bacillithiolation of many essential or conserved proteins including the methionine synthase MetE, a peroxiredoxin (YkuU), the ferredoxin-NADP⁺ oxidoreductase YumC, and many metabolic enzymes and proteins that function in the biosynthesis of cysteine, thiamine and guanine

monophosphate (GMP) (Chi, Gronau et al. 2011, Chi, Roberts et al. 2013). Methionine synthase (MetE) and OhrR are the most highly S-bacillithiolated proteins during NaOCl treatment. S-bacillithiolation of MetE is protective but also inhibitory, a measure that likely postpones protein synthesis during oxidative stress by creating a condition of transient methionine auxotrophy. In *E.coli*, MetE is glutathionylated during oxidative stress (Gaballa, Newton et al. 2010, Fang and Dos Santos 2015) and also induces a transient methionine auxotrophy (Hondorp and Matthews 2004). As mentioned previously, OhrR is a peroxide-sensing transcriptional repressor of *ohrA*, which encodes an organic hydroperoxide detoxifying peroxiredoxin (Lee, Soonsanga et al. 2007). S-bacillithiolation disrupts DNA binding of the OhrR repressor, leading to higher concentrations of the OhrA peroxiredoxin that confers specific protection against NaOCl together with BSH (Chi, Gronau et al. 2011).

Unexpectedly, treating BSH deficient mutants with the thiol specific oxidant diamide induces intracellular thiol depletion but does not significantly perturb growth or cytosolic redox status (Gaballa, Newton et al. 2010). This suggests compensatory functions of cysteine and/or CoASH during disulfide stress. However, BSH deficient strains confer growth defects under low pH and excess NaCl implicating the LMW thiol in other stress responses.

Additional functions of BSH

The scope of BSH function extends beyond S-thiolation since nucleophilic and metallophilic sulfhydryls rapidly form adducts with an assortment of potentially

toxic compounds. BSH functions in maintaining metal homeostasis (Ma, Chandrangsu et al. 2014) and as a cofactor for enzymes essential for detoxification of reactive electrophilic species (RES), such as methylglyoxal and antimicrobial compounds like Fosfomycin (Chandrangsu, Dusi et al. 2014), (Gaballa, Newton et al. 2010) .

Methylglyoxal (MG) and glyoxal are alpha-oxoaldehydes that are mainly produced as natural byproducts of glycolysis. During carbon overflow or phosphate limitation, methylglyoxal synthase (MgsA) catalyzes MG synthesis to prevent toxic accumulation of glycolytic intermediates and facilitate phosphate turnover (Chandrangsu, Dusi et al. 2014). Initiation of this methylglyoxal shunt is a physiological response to glucose oxidation conserved among many bacteria and humans (Allaman, Belanger et al. 2015, Kosmachevskaya, Shumaev et al. 2015). MG is highly toxic, however, as the reactive nucleophile promotes DNA damage, protein modifications and disruption of protein synthesis (Ferguson, Totemeyer et al. 1998). The most extensively characterized bacterial MG detoxification pathway is the glutathione-dependent GlxI/II glyoxalase system (MacLean, Ness et al. 1998). Accumulated MG binds GSH to form MG-GSH conjugates, which trigger the GlxI/II-mediated multi-step conversion to lactate. Subsequent accumulation of S-D-lactoyl-GSH intermediates stimulates the GSH-gated KefBC K⁺ efflux system that acidifies the cytoplasm during RES stress response (MacLean, Ness et al. 1998). A functionally homologous MG detoxification system in *B. subtilis* involves bacillithiol-dependent GlxAB glyoxalase system, an S-lactoyl-BSH intermediate and a KhtSTU K⁺ efflux pump system (Chandrangsu, Dusi et al. 2014). Indeed, BSH-deficient *B. subtilis*

confer heightened sensitivity to methylglyoxal (Gaballa, Newton et al. 2010). It is worth noting that in addition to *bsh* biosynthesis genes, other members of the Spx regulon contribute to methylglyoxal resistance including the largely uncharacterized *ytpQ* gene and *yraA*, which encodes a general stress response protein functioning in protein quality control and also BSH-independent methylglyoxal detoxification (Zuber, Chauhan et al. 2011, Chandrangsou, Dusi et al. 2014).

In addition to MG-detoxifying glyoxalases, fosfomycin-resistance S-transferases require cognate LMW thiols as cofactors. Fosfomycin is a broad-spectrum antibiotic that disrupts cell wall biosynthesis. Opening of the fosfomycin epoxide ring is an essential mode of inactivation, which requires FosA-mediated glutathionylation in *E. coli* and FosB-mediated S-bacillithiolation in *B. subtilis* (Allocati, Federici et al. 2009, Gaballa, Newton et al. 2010). *B. subtilis* cells deficient in BSH exhibit over a 50% reduction in fosfomycin resistance (Gaballa, Newton et al. 2010).

Bacillithiol and other LMW thiols have been shown to contribute to metal homeostasis. The malate-derived carboxyl moiety and sulfhydryl group coordinate Zn^{2+} to form a $BSH_2::Zn^{2+}$ complex *in vitro*, suggesting a role for BSH in Zn^{2+} specific buffering (Ma, Chandrangsou et al. 2014). A *czd cadA* double mutant lacking two functionally redundant Zn^{2+} Cd^{2+} efflux systems showed heightened sensitivity Zn^{2+} and Cd^{2+} , when also bearing a *bshC* mutation (Ma, Chandrangsou et al. 2014). Also, BSH deficient mutants exhibit modest sensitivity to Cd^{2+} in minimal medium which was suggested to be due to BSH deficiency-dependent drop in the intracellular Zinc pool and subsequent repression of the CadA efflux system (Fang and Dos Santos 2015). Finally, maintaining the BSH pool is important for resistance against

alkylating agents as suggested by heightened sensitivity of BSH deficient mutants to iodoacetamide and monobromobimane (Gaballa, Newton et al. 2010).

Role of bacillithiol in other bacteria

Bacillithiol has been recently identified in other members of *Firmicutes* including *S. aureus*, *S. carnosus*, and also members of the genus *Bacillus*: *B. anthracis*, *B. megaterium*, *B. pumilus* and *B. amyloliquefaciens* (Chi, Roberts et al. 2013). Upon NaOCl treatment, many orthologous S-bacillithiolated proteins observed in the *B. subtilis* proteome were S-bacillithiolated in *B. megaterium*, *B. pumilus*, *B. amyloliquefaciens*, and *S. aureus*.

In some bacillithiol producing *S. aureus* isolates (Newman), BSH functions in detoxification of monobromobimane and rifamycin, the parent compound of the widely used thiol-reactive anti-tuberculosis drug, rifampicin (Newton, Fahey et al. 2012). *S. aureus* BSH mutants confer heightened sensitivity to alkylating agent iodoacetamide, the antibiotic fosfomycin, Cu^{2+} and Cd^{2+} , and oxidants hydrogen peroxide, cumene hydroperoxide, and diamide (Rajkarnikar, Strankman et al. 2013). *S. aureus* BSH also promotes Fe-S cluster biosynthesis and maintains metal homeostasis, which contribute to virulence and survival during host infection (Rosario-Cruz, Chahal et al. 2015, Rosario-Cruz and Boyd 2016). BSH depleted *S. aureus* showed a severe drop in survival after a 48-hour incubation in murine macrophages (Pother, Gierok et al. 2013).

Biosynthesis of Bacillithiol

Bacillithiol (Cys-GlcN-Mal) is a 398 Da alpha-anomeric glycoside of L-cysteinyl-D-glucosamine with L-malic acid. Bioinformatics, phylogenomic profiling, and examination of mycothiol biosynthesis were conducted to predict the stepwise biosynthesis of BSH and identify the genes encoding BSH biosynthetic enzymes (Gaballa, Newton et al. 2010), (Fig. 3.1). BSH biosynthesis genes *bshA*, *bshB1/bshB2*, and *bshC* encode a malic acid glycosyltransferase, functionally redundant deacetylases, and a cysteine ligase, respectively. BshA first catalyzes glycosidic linkage formation between L-malate and UDP-N-acetylglucosamine (UDP-GlcNAc) to generate a GlcNAc-Mal intermediate. Next, GlcN-mal is formed by BshB1/B2-mediated hydrolysis of the acetyl group of GlcNAc-mal. Finally, BshC catalyzes the addition of cysteine to GlcNAc-mal to form bacillithiol. BshA and BshC are essential for BSH production while *B. subtilis* cells bearing either a *bshB1* or *bshB2* null mutation maintain partial, or wild-type levels of BSH, respectively (Gaballa, Newton et al. 2010).

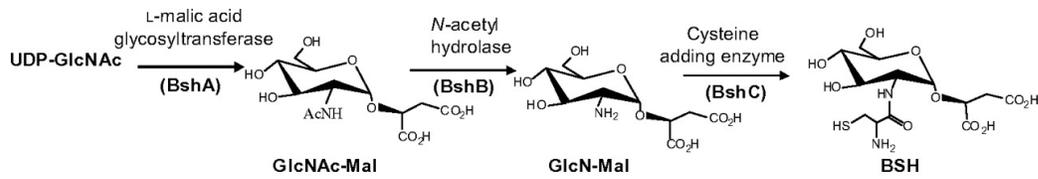


Figure 3.1. Predicted Bacillithiol biosynthesis pathway. Schematic obtained from (Gaballa, Newton et al. 2010). The BshA (encoded by *bshA* gene) glycosylase, two functionally redundant hydrolases BshB1 and B2 ("*bshB*" herein, *bshB2*), and the BshC (*bshC*) Cysteine-adding enzyme are required for BSH biosynthesis.

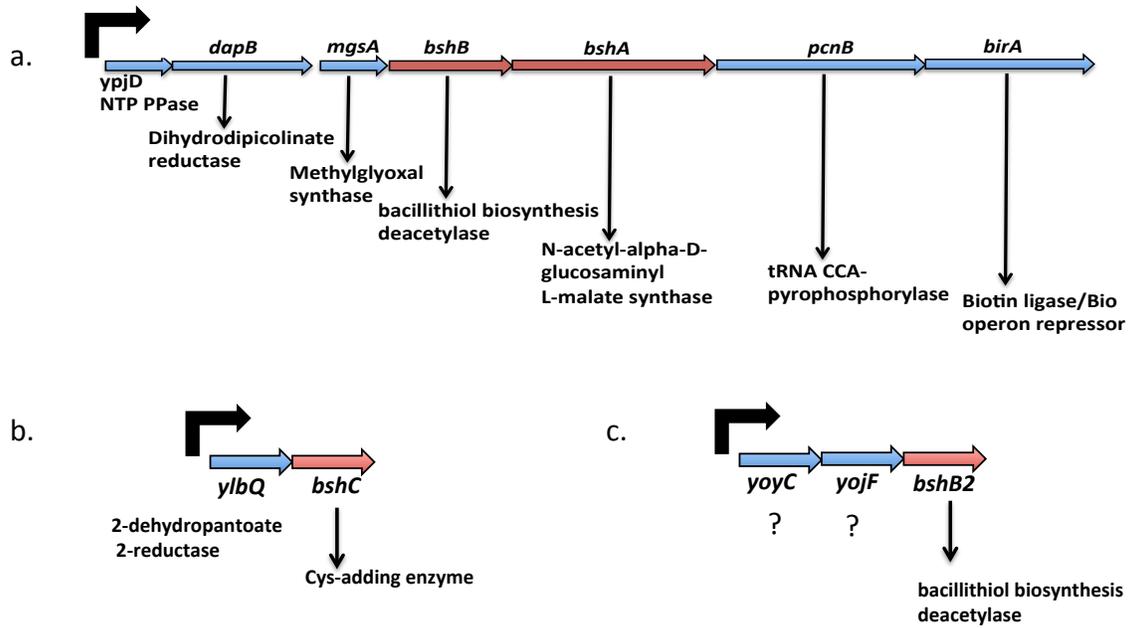


Figure 3.2. Genomic organization of relevant *bsh* biosynthesis genes. a. *bshB1* (*bshB*) and *bshA* genes reside in a 7-gene operon. b. *bshC* resides in a dicistron with *ylbQ* (*panE*). Black arrows indicate promoter regions. c. *bshB2* resides in an operon with two uncharacterized genes *yoyC* and *yjoF*.

Genomic organization of B. subtilis BSH biosynthetic genes

The *ypjD* promoter precedes a 7-gene operon containing genes *ypjD*, *dapB*, *mgsA*, *bshB1*, *bshA*, *cca*, *birA* (Fig. 3.2 a), (Gaballa, Antelmann et al. 2013). The *ypjD* gene encodes a *Bacillus* conserved MazG-like nucleotide pyrophospho-hydrolase (NTP). The *B. anthracis* YpjD ortholog BA1554 preferentially hydrolyzes ATP to AMP and PP_i, a specificity potentially conserved in *B. subtilis* (Kim and Hong 2016). In *E. coli* MazG has been implicated in modulating (p)ppGpp alarmone levels during nutritional stress, while in *Mycobacterium smegmatis*, it functions as a housekeeping enzyme for degradation of damaged NTPs during oxidative stress (Lyu, Tang et al. 2013). The *dapB* gene encodes a dihydropicolinate reductase, which uses NADP⁺ as a cofactor to catalyze the fourth step in an alternative pathway of lysine biosynthesis involving acetylated intermediates. Downstream of *dapB* lies *mgsA*, which encodes a methylglyoxal synthase essential for initiating the methylglyoxal shunt, a glycolytic bypass during carbon overflow or phosphate limitation. A sudden shift in glucose uptake disrupts metabolic flux, leading to the accumulation of potentially toxic triose-phosphates. To prevent the toxic accumulation of triose-phosphates, MgsA converts the glycolytic intermediate dihydroxy acetone phosphate (DHAP) to phosphate and methylglyoxal, a cytotoxic electrophile (Landmann, Busse et al. 2011). Methylglyoxal is then excreted, eventually converted to pyruvate or detoxified by BSH-dependent or independent pathways as previously discussed. The product of the essential *cca* gene is an ATP-dependent CCA tRNA nucleotidyl transferase, which appends CCA residues to the 3' end of tRNAs during maturation or recycling (Campos Guillen, Jones et al. 2017). Finally, *birA* is another

essential gene whose product responds to intracellular levels of biotin and functions as either a transcriptional repressor or a biotin-protein ligase. Upon binding biotinoyl-5'-AMP as a co-factor, HoloBirA is posed to dimerize and become a holo-repressor of transcription, or form a complex with biotin carboxyl carrier proteins (BCCPs) to catalyze biotinylation at target Lysine residues (Satiaputra, Shearwin et al. 2016). Specifically, in biotin-replete conditions, HoloBirA repressor binds to the *bioO* operator in the promoter region of the *bioWKFDB-ytbQ* operon (Bower, Perkins et al. 1995). Alternatively, accumulation of essential biotin-dependent BCCPs like acetyl-coA carboxylase, the initiating enzyme in fatty acid synthesis shifts the functional equilibrium of Holo-BirA to protein-ligase activity (Cronan 2002).

The *bshC* gene resides in a dicistronic operon with *ylbQ* from which transcription may initiate at either the *ylbQ* (*panE*) or downstream at the *bshC* promoter (Fig. 3.2 b). The, *ylbQ* gene encodes a putative 2-dehydropantoate 2-reductase which is a component of the pantothenate biosynthesis machinery alongside the products of the *panBCD* genes downstream of the *ypjD* operon. The *bshB2* gene is located in a polycistronic unit downstream of two uncharacterized genes *yofF* and *yoyC* from which transcription initiates at the *yoyC* promoter (Fig. c).

B. subtilis* *ypjD* operon organization is conserved in *B. anthracis

The organization of 7-gene *ypjD* operon containing *bshB1* and *bshA* is conserved in *B. anthracis* from which transcription initiates at the promoter of the *ypjD* ortholog *BA1554*. Expression of all genes of the *B. subtilis* *bshBA* operon requires Sp_x

and in *B. anthracis*, both SpxA1 and SpxA2 can activate *bsh* operon transcription (Barendt, Lee et al. 2013).

Cis-acting elements of Spx-activated promoters

The concerted modulation of gene expression governed by cis-regulatory elements and trans-acting factors comprises a fundamental mode of transcriptional regulation in bacteria. In an effort to characterize the mechanism of Spx-activated transcription, earlier studies examined whether Spx affected RNAP-DNA complex formation upon Spx- α CTD binding (Nakano, Erwin et al. 2005). DNase I footprinting experiments with purified Spx, *PtrxA* and *PtrxB* templates, and RNAP showed that addition of oxidized Spx induces a shift in the RNAP-dependent protection pattern of *trxA* and *trxB*. This *in vitro* Spx-RNAP interaction causes an α CTD-dependent protection pattern spanning -62 to -40 of *PtrxA* and -53 to -35 of *PtrxB*. Follow up studies conducting 5' promoter truncation analysis showed that a reduction in Spx-dependent activation consistently increased in severity beginning at -50 and -48 of *PtrxB* and *PtrxA*, respectively (Reyes and Zuber 2008). In further studies, *trxB* promoter from -114 to -36 was fused 5' to the -36 of the *srfA* promoter, which precedes a gene subject to Spx-mediated activator interference, and is thus repressed upon Spx- α CTD-RNAP interaction (Nakano, Nakano et al. 2003, Reyes and Zuber 2008). Significant *in vivo* de-repression of the *trxB-srfA*₋₃₆ hybrid was observed upon induction of Spx as shown by β -galactosidase assays. DNA-protein crosslinking indicated that Spx promotes RNAP-DNA interactions at the *PtrxB-srfA* hybrid and not the wild type *srfA* promoter (Reyes and Zuber 2008). Taken

together, compounding evidence suggested that the sequence spanning -49-N₄AGCAN₆TAGCGT-30 of *P_{trxB}* contained cis-acting sites crucial for Spx-dependent transcription. Indeed, subsequent promoter mutational analysis showed that *P_{trxB}* C-43A and G-44T transversions virtually abolished *in vivo* activation of *trxB*, indicating that the GC dinucleotide within -45-AGCA-42 is essential for transcription. Additionally, two base pair substitutions within a poly-A-tract centered at -46- -36 moderately reduced *trxB* transcription, and this region was proposed as a target for Spx-bound α CTD. Nucleotide-specific-protein-DNA-crosslinking assays showed that when complexed with RNAP holoenzyme, Spx binds to G-44 of the -45-AGCA-42 motif of the *trxB* promoter, confirming that this acting site is the preferred target of Spx.

Using ChIP-chip analysis, genome wide identification of Spx-RNAP target genes revealed 144 transcription units comprising hundreds of putative members of the Spx-regulon (Rochat, Nicolas et al. 2012). Subsequent transcriptomic analysis and promoter sequence alignments suggested that the relevant putative regulatory region of Spx-activated promoters is not highly conserved, but the promoters display extended -35 and -10 elements recognized by the σ^A -form of RNAP. Within the promoters of genes exhibiting high Spx-dependent activation a cytidine at position -43 correlated with Spx-activation and was often preceded by a guanidine and to a lesser extent, thymidine at position -44. The authors noted, however, that the -45-AGCA-42 motif like those in the promoters of *trxB* and *trxA* is only conserved in about 10% of Spx-dependent promoters, and that while the transcriptional effect of this motif is high, its functional relevance does not extend

broadly across the Spx-regulon. Conversely, either an A or T at position -43 of the extended -35 element correlated with Spx-mediated repression.

Architecture of the bsh operon promoters

Recent evidence indicates that unlike *trxB* and *trxA*, an SpxC10A mutant which mimics reduced Spx, can activate the *bshBA* biosynthetic gene operon (*ypjD*) *in vivo* (Gaballa, Antelmann et al. 2013). This suggests that at least one member of the Spx regulon (*ypjD* operon containing *bshBA*) does not require Spx in its oxidized disulfide form but the underlying mechanism of activation is not yet known. In efforts to uncover the mechanism of C10A-activated transcription of *ypjD*, we first examined the *ypjD* promoter region.

In the *B. subtilis* *ypjD* promoter region (*PypjD*), the σ^A -utilized core -35 and -10 hexamers (Gaballa, Antelmann et al. 2013) are mostly conserved and also are identical to those in the promoter of *B. anthracis* *ypjD* ortholog *BA1554* (*PBA1554*) (Fig. 3.3). However, the upstream sequences expected to direct Spx-activated transcription are not homologous. The -35 element of *B. subtilis* *PypjD* is preceded by an AT-rich region, similar to promoters of other Spx-activated genes. Interestingly, where the -45-AGCA-42 motif of *PtrxA* and *PtrxB* are conserved, *PypjD* harbors a palindrome of AGCA's (-54-AGCA-N₄-TGCT-43) in which the complement (TGCT) resides almost precisely where the AGCA often resides in some Spx-activated promoters. The *ylbQ* promoter also bears an AGCA significantly upstream of where the conserved motif often resides, but it lacks the complement detected in *PypjD* (Fig. 3.3).

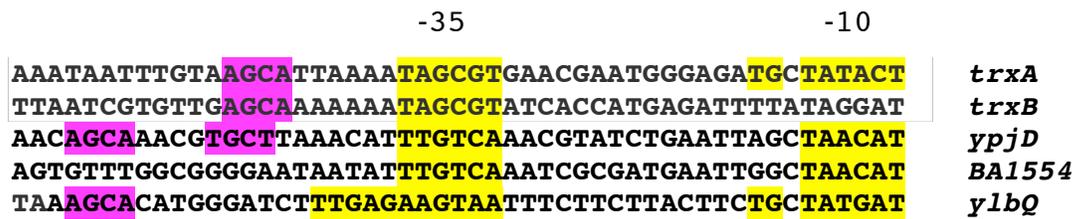


Figure 3.3. Alignment of relevant promoter regions of several Spx-activated genes. The promoters of thioredoxin (*trxA*), thioredoxin reductase (*trxB*), bacillithiol biosynthesis operons in *B. subtilis* (*ypjD*, and *ylbQ*), and that of *B. anthracis* (*BA1554*) are shown. The core promoter hexamers are highlighted in yellow and AGCA motifs are highlighted in purple.

Even more striking is a tetrad of alternating pseudo-tandem repeats spanning from -58 to -29, overlapping the -35 of *PypjD* (Fig. 3.4). These tandem repeats are reminiscent of those residing in the promoters of PhoP-regulated genes. The PhoPR two-component signal transduction system controls one of three responses activated by *B. subtilis* to adapt to phosphate-limiting conditions (PHO response). Under low phosphate conditions, PhoP is phosphorylated by PhoR and then, as dimers and/or oligomers, will contact NNAACA-like tandem repeats to positively or negatively regulate target genes (Liu, Eder et al. 1998, Eder, Liu et al. 1999, Salzberg, Botella et al. 2015).

The unique promoter architecture of *PypjD* may provide insights as to the cause of SpxC10A-stimulated transcriptional activity. The aim of this study is to compare the redox requirements Spx for *in vivo* activation of *trxB* with that of *ypjD* and *ylbQ* and also identify possible *PypjD* cis-acting elements that are required for optimal Spx-activated transcription. In addition, using *B. subtilis* as a host, we aim to reconstruct *in vivo* activation of *B. anthracis bshBA* operon genes (*ypjD/BA1554*) by SpxA1 and SpxA2, as well as *B. subtilis* Spx and SpxC10A using the *B. subtilis* expression system to test Spx redox requirement for *B. anthracis bsh* gene transcription.

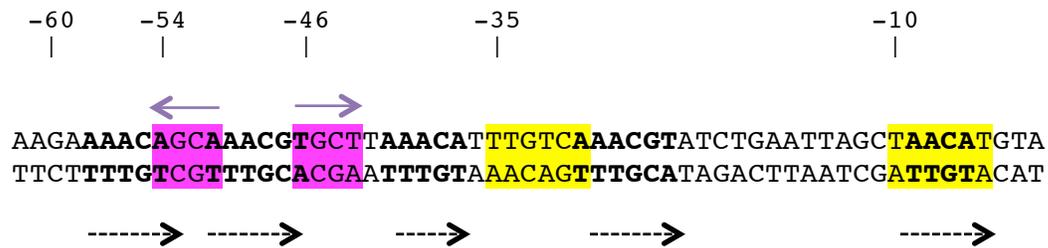


Figure. 3.4. Unique promoter architecture of *ypjD*. The relevant promoter region of *ypjD* is depicted on both DNA strands. The core promoter elements and AGCAs are highlighted as in Fig. 3.3. The palindrome of AGCAs is denoted by solid purple arrows and alternating tandem repeats are in bold and denoted by dashed arrows.

RESULTS

Effects of PypjD truncations on Spx-dependent activation

To locate the region of the promoter required for Spx-activated transcription of *ypjD*, 5' truncation analysis was conducted. Promoter fragments encompassing upstream DNA to -62, the -49, and the -35 were fused to the promoterless *-lacZ* reporter gene integrated at the *thrC* (threonine biosynthesis) locus. The fusions were then introduced into an *spx* null mutant. The resultant strains were transformed with chromosomal DNA isolated from strains carrying the wild-type *spx^{DD}* at the *amyE* locus. The *spx^{DD}* allele, which is under control of an IPTG-inducible promoter, bears a C-terminal double aspartate codon substitution that renders Spx protease-resistant (Nakano, Kuster-Schock et al. 2003). One hour of IPTG-induction of *Spx^{DD}* resulted in significant activation of the *PypjD-lacZ* fragment encompassing the -62, nicknamed "F2" (Fig. 3.5). Minimal activity of *ypjDF2-lacZ* was observed in the absence of IPTG, confirming that *in vivo* expression of *ypjD* is Spx-dependent. The promoter truncations encompassing the -49 ("*PypjDΔ1*") and the -35 ("*PypjD-35*"), resulted in an approximately 80% and over 90% decrease in Spx-dependent induction, respectively. The results suggest that the promoter region encompassing the -62, which includes the palindrome of AGCAs and the tetrad of alternating tandem repeats, is required for optimal Spx-dependent transcription of *ypjD*.

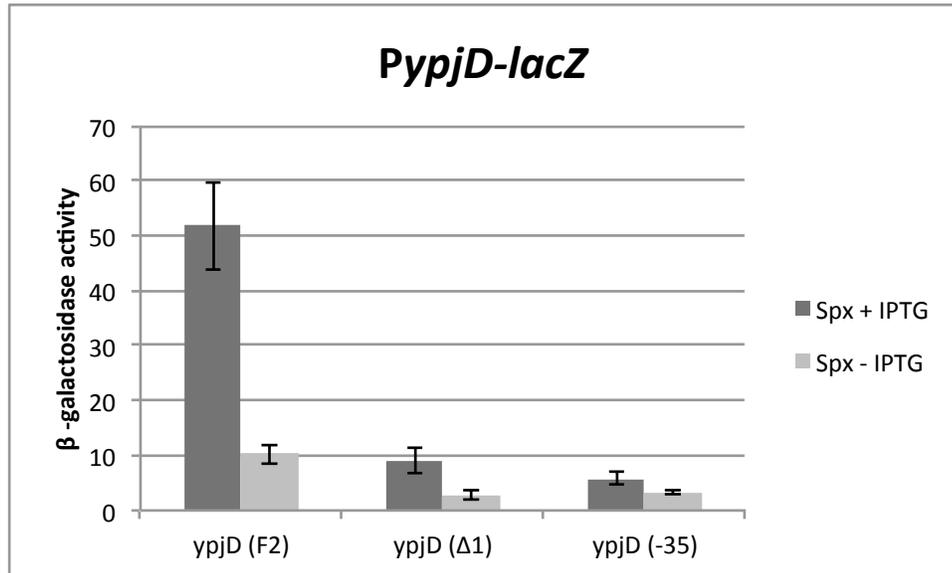


Figure 3.5. 5' truncation analysis of *PypjD*. Cultures were grown to mid-exponential and split to two subcultures, to one of which 1 mM IPTG was added. Cells were harvested every 0.5 h and tested for β -galactosidase activity. Assays are average of three independent assays.

Differential redox requirements of Spx activity among the Spx regulon

To corroborate results obtained by Gaballa et. al, the effect of mutations at the redox center (C10XXC13) of Spx on *trxB*- and *ypjDF2-lacZ* expression were compared. Serine or alanine codon substitutions of Spx C10 or C13 generate mutants that are pseudo-mimetic of reduced Spx and retain one free thiol. Double serine or alanine codon substitutions of Spx generate a mutant form of Spx devoid of free thiols. The activity of Spx^{DD}, SpxC10S^{DD}, and SpxC10C13S^{DD} on *trxB* and *ypjD* was examined. As previously observed, C10S^{DD} is severely defective in activation of *trxB-lacZ* (Fig. 3.6 a). However, a C10C13 double serine substitution partially restores activity at the *trxB* promoter. Shunji Nakano, a previous member of the Zuber-Nakano lab has previously observed this phenotype (Nakano and Zuber, unpublished work). Conversely, the redox state of Spx is inconsequential during *in vivo* expression of *ypjD-lacZ*, as shown by the comparable activity among wild-type Spx^{DD}, C10S^{DD}, and C10C13S^{DD} (Fig. 3.6 b).

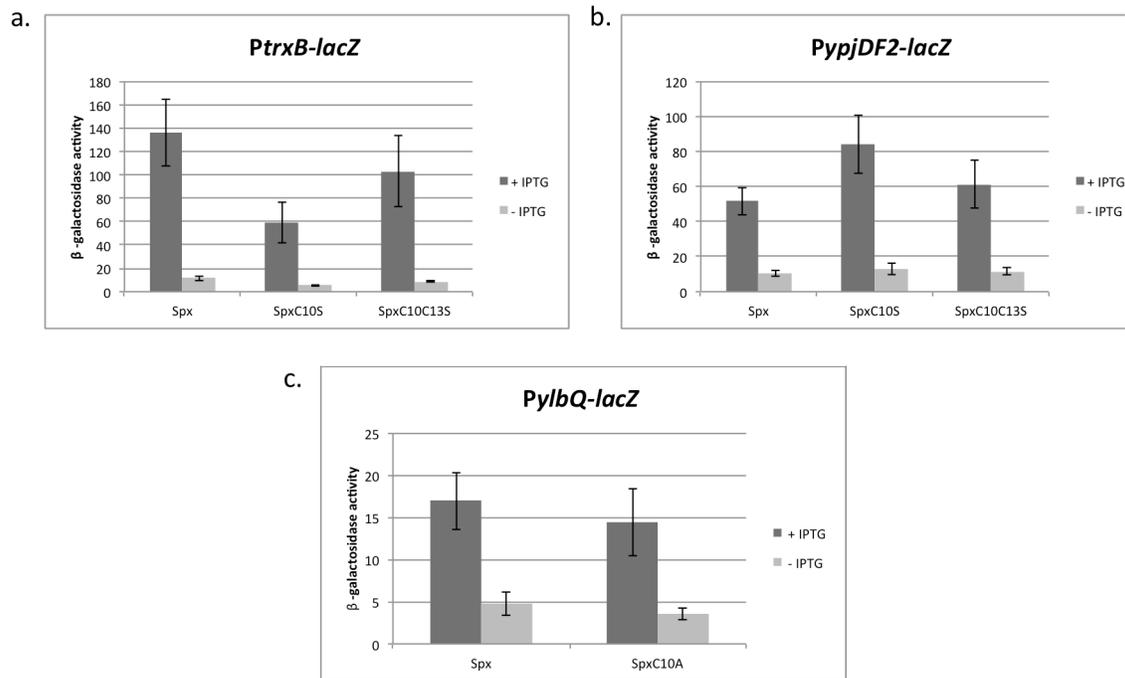


Fig. 3.6. Differential redox requirements for Spx activity among genes of the Spx regulon. a. Examining the effect of C10S and C10C13S mutations on *trxB-lacZ* and *ypjD-lacZ* expression. b. Examining the effect of C10A mutation on *ylbQ-lacZ* expression. Cultures were grown as described in the materials and methods of this chapter. Cells were harvested every 0.5 h and tested for β -galactosidase activity.

In line with previous reports, an SpxC10A mutant retains activity comparable to wild-type Spx at the *ylbQ* promoter (Fig. 3.5 c), (Gaballa, Antelmann et al. 2013)

In summary, at the *trxB* promoter, a free thiol inhibits Spx activity (C10S), suggesting that a disulfide is required to eliminate the inhibitory thiol (C10C13S). Finally, *in vivo* expression of *bsh* biosynthetic operons does not require Spx in its oxidized disulfide form.

Differential redox requirements of B. subtilis Spx during activation of B. subtilis ypjD and B. anthracis ypjD (BA1554).

The operon organization and the core promoter elements of *B. subtilis ypjD* and *B. anthracis ypjD* (denoted herein as *BA1554*) are conserved (Fig. 3.3). However, the upstream promoter regions share little homology and the *BA1554* gene promoter shows no obvious potential cis-acting sites that could serve as an Spx-responsive element. The transcriptional start sites have been determined, for *B. subtilis ypjD* in a previous report (Gaballa, Newton et al. 2010), and in our lab for the *B. anthracis BA1554* gene by 5' RACE (rapid amplification of cDNA ends). Strains bearing SpxA1^{DD} were grown to mid-log, treated with IPTG and harvested for subsequent RNA extraction. An antisense gene-specific primer (GSP1) and SuperScript II reverse transcriptase were then used for first strand cDNA synthesis from the 5' end. Next, a polymeric tail was added to the 3' end of the purified cDNA template using dCTP and terminal deoxynucleotidyl transferase (TdT). The tailed cDNA template was then PCR amplified using a nested GSP (GSP2). Selected amplicons were cloned into *E. coli* vectors and sent for sequencing.

Microarray analysis revealed significant induction of *bsh* biosynthetic genes upon overexpression of *B. anthracis* SpxA1^{DD} and SpxA2^{DD} proteins (Barendt, Lee et al. 2013). *B. subtilis* was used as a heterologous host to reconstruct SpxA1- and SpxA2- dependent activation of *PBA1554 in vivo*. The *PBA1554* spanning -340 to +8 was fused to *lacZ* in an SpxA1^{DD} or SpxA2^{DD} background. Induction of either SpxA1^{DD} or SpxA2^{DD} stimulated expression of *PBA1554-lacZ* (Fig. 3.6). The low basal activity of *PBA1554-lacZ* in the absence of IPTG confirmed that expression was dependent on either SpxA1 or SpxA2. *B. subtilis* Spx^{DD} can also stimulate *in vivo* expression of *BA1554* largely to the same level as that achieved by the *B. anthracis* Spx paralogs. This is not entirely surprising, as *B. subtilis* Spx bears considerable homology with the SpxA1 and SpxA2 proteins (Barendt, Lee et al. 2013). Moreover, SpxA2 and *B. subtilis* Spx enhance the *in vitro* RNAP holoenzyme affinity for *B. anthracis* *PBA1554* and *B. subtilis* *PypjD*, respectively (Fig. 3.8). However, in stark contrast with *B. subtilis* *ypjDF2-lacZ*, *B. subtilis* SpxC10A is less than 50% as active as wild-type *B. subtilis* Spx at the *BA1554* promoter (Fig. 3.7). This suggests that the promoter architecture requirements of Spx activity and/or mechanism of Spx-activated transcription differ between *B. subtilis* and *B. anthracis* *ypjD*.

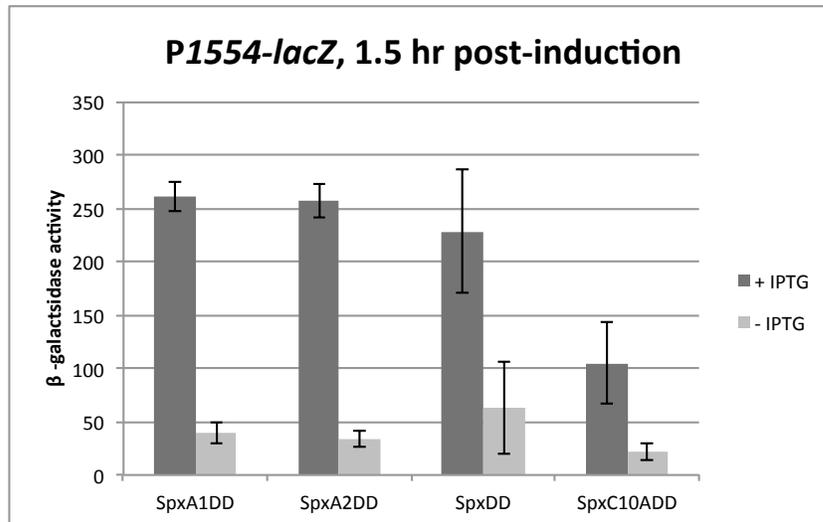


Figure. 3.7. In vivo activation of *B. anthracis ypjD* (BA1554) by *B. anthracis* SpxA1 and SpxA2 and *B. subtilis* Spx and SpxC10A. Cultures were grown as described in the materials and methods of this chapter. Cells were harvested after 1.5h and tested for β -galactosidase activity.

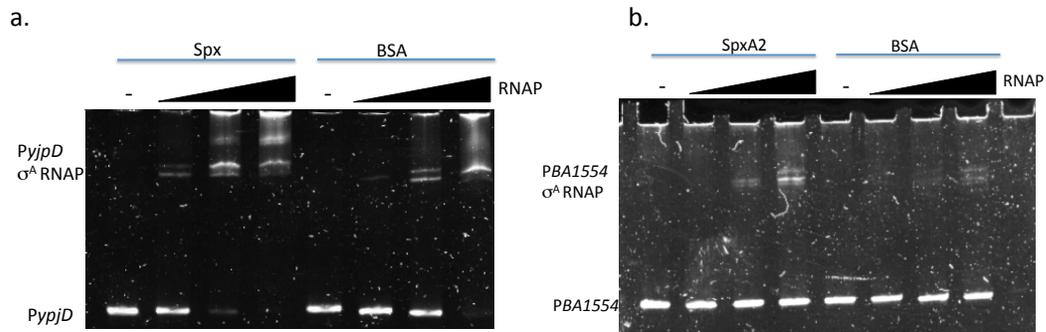


Figure 3.8. Spx-enhanced affinity of RNAP for *yjD* promoter DNA:

Electrophoretic mobility shift assays (EMSAs). Examining a. *B.*

anthracis SpxA2-enhanced RNAP affinity for *PBA1554* DNA and b. *B.*

subtilis Spx-enhanced RNAP affinity for *PypjD*.

Effects of ypjD promoter mutations on Spx-dependent activation

The non-conforming redox-independent activity of Spx on *ypjD* may be at least partially attributable to *cis*-acting elements located within the *PypjD* region encompassing upstream DNA to the -62. Several base substitution or deletion mutations were introduced at the *ypjDF2* and the *ypjDΔ1* promoter variants to determine what nucleotides were required for Spx^{DD}- and also SpxC10A^{DD}-dependent activation (Fig. 3.9). The *PypjDF2* construct served as the wild-type full-length *ypjD* promoter fragment to which the Spx and SpxC10A-dependent activation of each mutant promoter derivative was normalized. As expected, SpxC10A retained wild-type activity on *ypjDF2* and the *PypjDΔ1* truncation mutant (Fig. 3.9). The notable decrease in Spx and C10A activity at the *PypjDΔ1* truncation suggests that the region upstream of -49 may include *cis*-acting sites required for Spx and/or C10A activity at *PypjD*. G-44T (*ypjDF2A*) and C-43A (*ypjDF2T*) transversions located in the -54-AGCA--51 motif ("-54 box") of *ypjDF2* each resulted in approximately 40-45% reduction in Spx and C10 activity. The -56-AC--55 dinucleotide, which is centered at the intervening sequence of the palindrome of AGCAs, and within the AACGT tandem repeat was next targeted for mutagenesis. Unexpectedly, a -56-AC--55 deletion (*ypjDΔAC*) or an A-56G, C-55T double base substitution (*ypjDGT*) reduced Spx and C10A-activated transcription by 60-70% (Fig. 3.9). This suggests that sequence between the palindrome half sites, and to a much lesser extent, nucleotides centered at the -54AGCA-51 box are required for optimal Spx-dependent activation.

Base substitutions in the nucleotides comprising the -46-TGCT--43 motif (“-46 box”) were generated in both the full-length *ypjD* (*ypjDF2*), and the *ypjDΔ1* truncation derivative. Puzzling results were obtained upon examining the effects of the following mutations. The phenotypes of each substitution were dependent on whether the sequence upstream of the -46 box was present. A C-44A transversion decreased Spx and C10A-stimulated expression of full-length *PypjD* by approximately 60% (*ypjDF3A*) (Fig. 3.9). Unexpectedly, the same substitution in the truncated *ypjD derivative*, which lacks the upstream -61-A₂GA₃C-AGCAA--50 region, exerted a stimulatory effect on expression (*ypjDΔ1* vs *ypjDFA*). A similar trend is observed in a G-45T mutation, albeit to a more dramatic extent. A G-45T mutation in full-length *ypjD* (*ypjDF3T*) almost abolishes Spx- and SpxC10A-dependent expression, to the same extent as the truncation mutant (*ypjDFA1*). Remarkably, generating the G-45T substitution at the truncated promoter (*ypjDFT*) almost fully restores expression. The differential effects of the C-44A and G-45T mutations suggests that *ypjD* is under positive control, however, the substitutions in combination with deleting the upstream region suggests that it is also under negative control. Furthermore, this negative control appears to be masked when the upstream sequence, encompassing the AGCA, is present (*ypjDFT* vs *ypjDF3T* and *ypjDFA* vs. *ypjDF3A*).

Next, in an effort to generate both a full length and truncated *ypjD* promoter that more closely resembles that of a canonical Spx-activated gene (i.e. *trxA*, *trxB*), a double substitution within the -46-TGCT--44 box was generated to yield a -46-AGCA--44 mutant. The double substitution in the full-length *ypjD* (*PypjDF2*) resulted

in about a 45% reduction in Spx activity and interestingly, C10A retained less than 50% of wild-type Spx activity at this promoter. This is the first *PypjD* derivative in which a significant defect in SpxC10A activity was observed. Surprisingly, the double substitution in the truncation mutant that lacked the upstream sequence (*ypjDΔ1'*) completely abolished both Spx- and C10A-dependent expression.

In summary, it appears that the entire region encompassing the -62 of *PypjD* contains cis-acting sequences required for Spx and SpxC10A activity. The regulation of *ypjD* is clearly more complex than other members of the Spx regulon. The results suggest that *ypjD* is subject to both positive and negative control, and this control likely involves Spx and also at least one other factor.

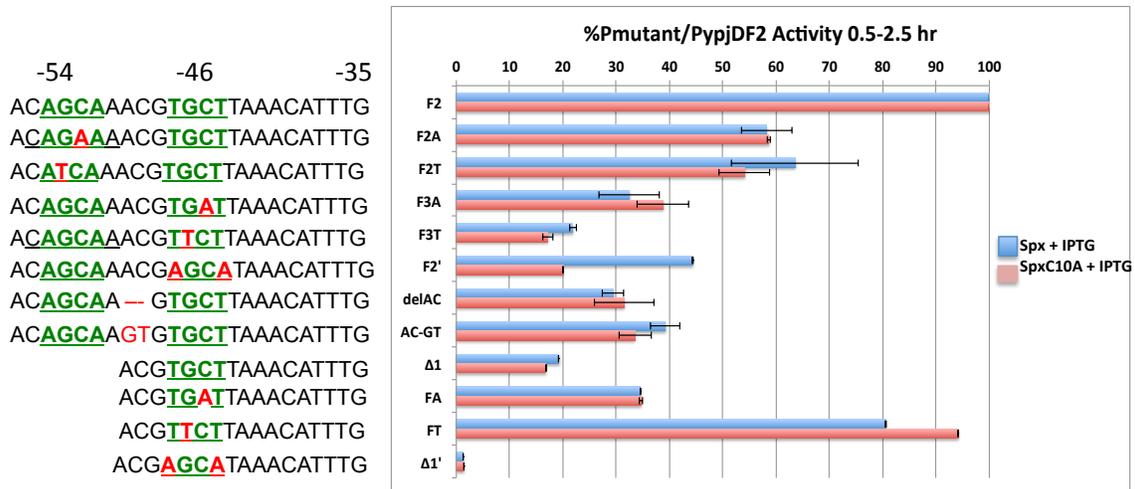


Figure 3.9. Examining effects of *ypjD* base substitution mutations on Spx- and SpxC10A- dependent activation. Cultures were grown as described in the materials and methods of this chapter. Cells were harvested after every 0.5h and tested for β -galactosidase activity. Red font denotes a site of promoter mutagenesis and the palindrome of AGCAs is highlighted in green. Graph depicts %activity of wild-type Spx (blue lines) or SpxC10A (red lines) on mutant *ypjD* derivatives, normalized to *ypjDF2*.

DISCUSSION

We have examined the promoter requirements of Spx-dependent activation of *B. subtilis* and *B. anthracis bsh* biosynthetic operons *in vivo*. Induction of *B. subtilis* Spx is sufficient for expression of *BA1554-lacZ*, however, unlike *B. subtilis ypjD*, an SpxC10A mutant is significantly defective. Like *ylbQ*, the *ypjD* promoter does not require Spx in its oxidized disulfide form, distinguishing it from the promoter of its *B. anthracis* counterpart, as well as other members of the *B. subtilis* Spx regulon.

A possible explanation for SpxC10A activity on *ypjD* and *ylbQ* may involve Spx functioning as the sole regulator by interacting with *PypjD* and *PylbQ* DNA in a manner distinct from canonical Spx-activated genes. Perhaps the redox state of Spx during *ypjD* and *ylbQ* activation is irrelevant because the structural requirements of Spx at these promoters are much less stringent than at the *trxA* and *trxB* promoters. Structural analysis of an SpxC10S- α CTD complex suggests that upon reduction, the alpha helix 4 of Spx (" α 4"), which contains large basic residues, likely unravels and unfolds (Nakano, Lin et al. 2010). This may lead to local electrostatic repulsion or steric hindrance at the Spx-DNA interface. The promoter topology of *ypjD* and *ylbQ* may require Spx oriented in a distinct conformation to form an Spx-DNA binding surface that is insensitive to changes in helix 4 region conformation. It is tempting to speculate that the -46-TGCT-43 motif of *PypjD* is at least one target of Spx, because a -46-AGCA-43 double mutant (*ypjDF2'*) renders SpxC10A approximately half as active as wild-type Spx.

Results obtained by promoter mutational analysis suggest that the gene is subject to complex regulation. The *B. subtilis ypjD* promoter harbors unique features including regions of dyad symmetry and alternating tandem repeats that overlap the -35. The occurrence of these elements overlapping a core promoter is often functionally significant, as they are prominent targets of dimeric or oligomeric activators and repressors (Harlocker, Bergstrom et al. 1995, Zhang, Banerjee et al. 2009, Gemayel, Cho et al. 2012, Barta, Hickey et al. 2014).

Promoter truncation analyses indicate that the *PypjD* bears cis-acting sites required for optimal Spx-activated transcription between the -35 and the -62. All mutations generated spanning the -54 to -43 moderately to severely inhibited Spx and SpxC10A activity. Considering the scenario wherein Spx is the sole trans acting factor, it is challenging to envision the DNA binding surface of this small monomeric regulator spanning such an extensive regulatory sequence. Small regulators can span large promoter regions by imparting curvature in promoter DNA, but this usually requires dimeric or multimeric complexes (Dhavan, Crothers et al. 2002). Intriguingly, the effects of downstream base substitutions in the vicinity of the TGCT motif were either stimulatory or inhibitory depending on whether the upstream sequence was present. Compensatory effects of the upstream cis-acting elements, multiple trans-acting factors, or both may partly explain this phenomenon.

In the coactivator hypothesis, Spx may associate with RNAP and function cooperatively with another *ypjD* activator. It is possible that the coactivator is pre-bound to *PypjD*, and preferentially recruits RNAP holoenzyme containing bound

Spx. Spx could facilitate recruitment by mediating coactivator and RNAP interaction, a function presumably retained by SpxC10A. A C10A mutation impairs Spx interaction with promoter DNA, but not with RNAP (Erwin, Nakano et al. 2005, Lin and Zuber 2012). However, it is important to note that Spx very likely, in some capacity, still functions directly at the *ypjD* promoter. Unlike the other mutant derivatives of *PypjD*, SpxC10A is significantly defective in activating the T-46A, T-43A double mutant of full-length *ypjD* (*ypjDF2'*). If the Spx-dependent activation of *ypjD* was solely indirect, and thus allowed C10A-mediated induction, then the C10A mutant should not confer a defect on any mutant form of *PypjD*. It is also possible that sequential and/or cooperative binding of Spx-RNAP and a co-activator is required to promote the optimal formation of a closed transcriptional complex. Their synergistic promoter DNA interaction activities may involve the *ypjD* binding affinity of one regulator being dependent on the other. In *B. subtilis*, it was proposed that global regulators CodY and CcpA dimers form complexes with the α subunit of RNAP, bind to consecutive cis-acting sites, and stimulate transcription at the *ackA* (acetate kinase) promoter to a greater extent than each independent regulator (Wunsche, Hammer et al. 2012). Alternatively, Spx may function as an anti-repressor where Spx-RNAP can compete for binding sites at the *ypjD* promoter to preclude repressor binding, displace a previously bound repressor by physical interaction, or modulate the topology of *ypjD* to reduce repressor binding.

Speculations on the functional significance of the ypjD operon organization.

Considering the functions of the all genes within the *ypjD* operon may be useful, as identifying a co-regulator of this operon is still underway. The products of the *ypjD* operon include an enzyme that likely hydrolyzes ATP to AMP+PP_i (YpjD), an NADPH generating lysine biosynthesis enzyme (DapB), an enzyme that converts DHAP to methylglyoxal and P_i (MgsA), BshB, BshA, an ATP-dependent enzyme that catalyzes the formation of mature tRNA^{Cys}-CCA and PP_i (CCA), and an ATP-dependent bifunctional biotin ligase/ repressor of biotin biosynthesis operons (BirA).

The *ylbQ* (*panE*) gene of the *ylbQ-bshC* operon encodes a pantothenate biosynthesis enzyme and some Spx-activated transcription initiation at *ypjD* reads through *birA* into the downstream *panBCD* operon encoding pantothenate biosynthesis enzymes (Gaballa, Newton et al. 2010). Based on genomic co-occurrence of these operons, it was suggested that BSH and CoASH biosynthesis pathways might be composed of coordinating enzymes (Gaballa, Newton et al. 2010). Moreover, Coenzyme A (CoASH) is an essential biotin precursor (Lin and Cronan 2011). Also, an apparent commonality among these enzymes is the presence of phosphate-related cofactors, substrates, or byproducts. Therefore, there may be a physiological link between pantothenate-, Coenzyme A-, or phosphate- metabolism and the regulation of this operon.

A functional link between CCA and BSH has been speculated (Campos Guillen, Jones et al. 2017). *B. subtilis* cells accumulated CCA-less tRNA^{Cys} during mercury-induced oxidative stress in the absence of tRNA quality control nucleases (Cruz

Hernandez, Millan et al. 2013). The results suggested that the oxidative stress-induced depletion of cysteine induces RNase-dependent degradation of uncharged tRNA^{Cys} molecules, leading to the accumulation of CCA-less tRNA^{Cys} species. Thus, it is hypothesized that such conditions could require up-regulation of *cca* to preserve the cysteine pool, which is essential for protein quality control, synthesis of BSH, and alleviating disulfide stress (Campos Guillen, Jones et al. 2017).

Finally, co-transcription of *mgsA* and *bsh* biosynthesis genes confers the most obvious physiological advantage. Glyceraldehyde-3-phosphate (GAP) is an integral glycolytic intermediate and the substrate for the GAP dehydrogenase GapA (White and Garcin 2017). GAP can be isomerized to DHAP, the substrate for MgsA. Conditions of excess glucose (carbon overflow) lead to phosphate and GAP depletion, causing an accumulation of DHAP, which triggers the MgsA-catalyzed methylglyoxal (MG) shunt (Landmann, Busse et al. 2011). MG must then be detoxified by various mechanisms including S-bacillitiation and subsequent cytoplasmic acidification, a multi-step pathway initiated by BSH-dependent enzymes (Chandrangsu, Dusi et al. 2014). During oxidative stress, GapA is inactivated upon oxidative of its active site Cys thiolate in *B. subtilis* (Chi, Roberts et al. 2013), or by S-bacillithiolation in *S. aureus* (Imber, Huyen et al. 2017). GapA inactivation is important for shifting metabolic flux from glycolysis to the pentose phosphate pathway, the pathway that provides the reducing power for components of antioxidant defense like the TrxA/TrxB/NADPH system (Grant 2008). Furthermore, *gapA* and other catabolic genes are down-regulated, while the *zwf* encoding the enzyme that initiates the pentose phosphate pathway is up-regulated

during diamide-induced accumulation of Spx (Rochat, Nicolas et al. 2012). Since inactivation of GapA may lead to the accumulation of DHAP, thus mimicking carbon overflow, it is tempting to implicate MgsA and BSH in stress-induced metabolic flux.

In summary, intracellular redox status prevails as the major determinant of *ypjD* induction. However, physiological linkages to the apparently complex regulation of *bsh* biosynthetic gene operons may extend to intracellular carbon-, phosphate-metabolism, and/or the complex interplay between the three.

MATERIALS AND METHODS

Bacterial strains and plasmids. Plasmids and bacterial strains are listed in Table 3.1. All *B. subtilis* strains were derived from JH642 (*trpC2 pheA1*).

Construction of strains bearing promoter-lacZ fusions. *B. subtilis* JH642 genomic DNA was used as a template to amplify the promoter regions of *ylbQ*, and *B. subtilis ypjD* and mutant derivatives. *B. anthracis* Sterne 7702 genomic DNA was used as a template to amplify the *B. anthracis ypjD/BA1554* promoter. Base substitutions in the *ypjD* promoter were generated using mutagenic forward oligonucleotides (Table 3.2). The *ylbQ* and *B. anthracis ypjD* promoters were amplified with respective flanking forward and reverse primer pairs. The *PypjD* forward primer and each *PypjD* mutagenic primers were paired with a common reverse primer to amplify the *ypjD* promoter and each mutant derivative, respectively. Each PCR product was digested with BamHI and EcoRI and ligated with plasmid pDG793 (Guerout-Fleury, Frandsen et al. 1996) which had been cleaved with the same enzymes. pDG793 is a *thrC* integration plasmid and double-crossover recombination is facilitated by selection for erythromycin resistance followed by screening for Thr- auxotrophy. Each plasmid was used to transform ORB3834 (*spx::neo*), and erythromycin-resistant (Erm^R) Thr- transformants were then transformed with chromosomal DNA isolated from strains carrying the wild-type and mutant *spx^{DD}* derivatives at the *amyE* locus.

***B. anthracis* RNAP purification.** RNAP was prepared from *B. anthracis* Sterne strain ORB8216 (*rpoC-His6*) using a protocol similar to *B. subtilis* RNAP purification. The β' subunit of *B. anthracis* RNAP in ORB8216 bears a His₆ affinity tag at the C terminus. Cultures of ORB8216 were grown at 37°C overnight in 2xYT and 5 ml were used to inoculate 1 liter of 2xYT (4 to 5 liters total). The 1-liter cultures were grown at 37°C with shaking until reaching an OD₆₀₀ of 1.0. Cells were collected by centrifugation using a ST-H760 rotor in a Sorvall Super T21 Hi-Speed centrifuge at 4,200 rpm for 15 min. The cells were suspended in 30 ml of 1x T-base buffer (Harwood and Cutting 1990). The cells were pelleted by centrifugation at 6,000 rpm for 10 min in a SL-50T rotor and a Sorvall Super T21 centrifuge. The pellets were frozen at -80°C until RNAP purification. Cells were suspended in 15 ml/liter culture of 50 mM Tris-HCl (pH 7.8), 100 mM NaCl, 5 mM MgCl₂, 20% glycerol, and 5 mM β -mercaptoethanol. Cells were lysed by three passages through an Avestin Emulsiflex-C3 emulsifier. RNAP was purified using a three-column procedure described previously using Ni-NTA, heparin, and HiQ anion-exchange chromatography (Reyes and Zuber 2008). RNAP fractions were concentrated using Millipore 10,000-molecular-weight cutoff centrifugal filter units, dialyzed in 10 mM Tris-HCl (pH 7.8)-10 mM MgCl₂-0.1 mM EDTA-50% glycerol, and then stored at -20°C.

Purification of B. anthracis SpxA2. Used for EMSA studies with *B. anthracis* PBA1554 promoter DNA and *B. anthracis* RNAP, SpxA2 obtained from Dr. Barendt, a previous Zuber-Nakano lab member. The purification protocol of SpxA2 has been previously described (Barendt, Birch et al. 2016)

Electrophoretic mobility shift assay (EMSA). EMSA was performed using a previously described procedure (Barendt, Birch et al. 2016) to test Spx mutants for Spx-enhanced promoter affinity. The *PypjDF2* fragment was the same used for construction of the *ypjDF2-lacZ* fusion presented above. Spx and σ^A protein were purified from intein-chitin-binding domain fusion proteins as described previously (Nakano, Zheng et al. 2002, Nakano, Geng et al. 2006).

Assays of β -galactosidase activity. The effect of the Spx-Ser or Spx-Ala codon substitutions in Spx on activity of Spx-controlled genes the effect of base substitutions in the *ypjD* promoter on the activity of *ypjD* was determined by measuring β -galactosidase activity. Assays were conducted with selected strains listed in table 3.1, incubated in the presence and absence of 1 mM IPTG. The strains were grown at 37 °C overnight in 2xYT (Fredrick, Caramori et al. 1995) supplemented with Spc and Erm/Ln. Overnight cultures were used to inoculate DSM at a starting optical density of 600 nm (OD_{600}) of 0.04. When the OD_{600} of the cultures reached 0.35 to 0.5, the cultures were divided into two flasks and 1 mM IPTG was added to one of the flasks. Samples were taken at 0.5-hr intervals to assay β -galactosidase activity, which was expressed as Miller units (Miller 1972).

Table 3.1 Strains and plasmids (Chapter 3)

Strain	Relevant Genotype	Source
JH642	<i>Wild-type Bacillus subtilis JH642 trpC2 pheA1</i>	This study
ORB7276	<i>JH642 thrC::PtrxB-lacZ amyE::Phs-spxDD</i>	Satola 1991
ORB8904	<i>JH642 thrC::PBA1554-lacZ amyE::Phs-spxDD</i>	Nakano 2003
ORB8907	<i>JH642 thrC::PBA1554-lacZ amyE::Phs-spxA1DD</i>	Nakano 2003
ORB8912	<i>JH642 thrC::PBA1554-lacZ amyE::Phs-spxC10ADD</i>	Nakano 2003
ORB8962	<i>JH642 thrC::PBA1554-lacZ amyE::Phs-spxA1DD</i>	This Study
ORB9103	<i>JH642 thrC::ypjDΔ1-lacZ amyE::Phs-spxDD</i>	This Study
ORB9105	<i>JH642 thrC::ypjDΔ1-lacZ amyE::Phs-spxC10ADD</i>	This Study
ORB9167	<i>JH642 thrC::PypjDFA-lacZ amyE::Phs-spxDD</i>	This Study
ORB9168	<i>JH642 thrC::PypjDFT-lacZ amyE::Phs-spxDD</i>	This Study
ORB9169	<i>JH642 thrC::PypjDF2-lacZ amyE::Phs-spxDD</i>	This Study
ORB9170	<i>JH642 thrC::PypjDF2A-lacZ amyE::Phs-spxDD</i>	This Study
ORB9171	<i>JH642 thrC::PypjDF2T-lacZ amyE::Phs-spxDD</i>	This Study
ORB9173	<i>JH642 thrC::PypjDFA-lacZ amyE::Phs-spxC10ADD</i>	This Study
ORB9174	<i>JH642 thrC::PypjDFT-lacZ amyE::Phs-spxC10ADD</i>	This Study
ORB9175	<i>JH642 thrC::PypjDF2-lacZ amyE::Phs-spxC10ADD</i>	This Study
ORB9176	<i>JH642 thrC::PypjDF2A-lacZ amyE::Phs-spxC10ADD</i>	This Study
ORB9177	<i>JH642 thrC::PypjDF2T-lacZ amyE::Phs-spxC10ADD</i>	This Study
ORB9243	<i>JH642 thrC::PypjDF3A-lacZ amyE::Phs-spxDD</i>	This Study
ORB9244	<i>JH642 thrC::PypjDF3T-lacZ amyE::Phs-spxDD</i>	This Study
ORB9245	<i>JH642 thrC::PypjDF3GT-lacZ amyE::Phs-spxDD</i>	This Study
ORB9246	<i>JH642 thrC::PypjDF3ΔAC-lacZ amyE::Phs-spxDD</i>	This Study
ORB9247	<i>JH642 thrC::PypjDF3A-lacZ amyE::Phs-spxC10ADD</i>	This Study
ORB9248	<i>JH642 thrC::PypjDF3T-lacZ amyE::Phs-spxC10ADD</i>	This Study
ORB9249	<i>JH642 thrC::PypjDF3GT-lacZ amyE::Phs-spxC10ADD</i>	This Study
ORB9250	<i>JH642 thrC::PypjDF3ΔAC-lacZ amyE::Phs-spxC10ADD</i>	This Study
ORB9299	<i>JH642 thrC::PypjD-35-lacZ amyE::Phs-spxDD</i>	This Study
ORB9370	<i>JH642 thrC::PtrxB-lacZ amyE::Phs-spxC10SDD</i>	This Study
ORB9372	<i>JH642 thrC::PtrxB-lacZ amyE::Phs-spxC10C13SDD</i>	This Study
ORB9622	<i>JH642 thrC::ylbQ-lacZ amyE::Phs-spxDD</i>	This Study
ORB9624	<i>JH642 thrC::ylbQ-lacZ amyE::Phs-spxC10ADD</i>	This Study

Plasmids	Description	Source
pDR111	cloning vector, IPTG inducible Phyperspank(Phs), Amp ^R , Spc ^R	Ben-Yehuda 2003
pDG793	cloning vector, Amp ^R , Erm ^R	Guerout-Fleury 1996
pCB36	<i>pDG793::PBA1554-lacZ</i>	This Study
pCB87	<i>pDG793::PypjDΔ1-lacZ</i>	This Study
pCB91	<i>pDG793::PypjDFA-lacZ</i>	This Study
pCB92	<i>pDG793::PypjDFT-lacZ</i>	This Study
pCB93	<i>pDG793::PypjDF2-lacZ</i>	This Study
pCB94	<i>pDG793::PypjDF2A-lacZ</i>	This Study
pCB96	<i>pDG793::PypjDF2T-lacZ</i>	This Study
pCB98	<i>pDG793::PypjDF3A-lacZ</i>	This Study
pCB100	<i>pDG793::PypjDF3T-lacZ</i>	This Study
pCB104	<i>pDG793::PypjDF3ΔAC-lacZ</i>	This Study
pCB108	<i>pDG793::PypjDF3GT-lacZ</i>	This Study
pCB113	<i>pDG793::PypjD -35-lacZ</i>	This Study
pCB117	<i>pDG793::PylbQ-lacZ</i>	This Study
pCB125	<i>pDG793::PypjDF2'-lacZ</i>	This Study
pCB127	<i>pDG793::PypjDΔ1'-lacZ</i>	This Study
pMMN895	<i>pDR111::Phs-spxC10SDD</i>	This Study
pMMN896	<i>pDR111::Phs-spxC10C13SDD</i>	This Study
pDYR9	<i>pDG793::PtrxB-lacZ</i>	Reyes 2008

**Ampicillin (Amp), Erythromycin/Lincomycin (Erm), Spectinomycin (Spc)

Table 3.2 Oligonucleotides (Chapter 3)

Name	Sequence (5' --> 3')	Description	Restriction Sites
ocb51	ATGTAAATGGCATTGCTCCGCTGA	Flanking	BamHI
ocb54	TCC TTC CTC TTT CAT GTT AGC CAA TTC ATC G	Flanking	EcoRI
oypjDr	TCCTTCTTTAAATTGGCCTAT	Flanking	BamHI
oypjDF2	AAGAAAACAGCAAACGTGCTTAAACATTTGTCAAACGTAT	Flanking	BamHI
oypjDF2A	AAGAAAACAGAAAACGTGCTTAAACATTTGTCAAACGTAT	Flanking/Mutagenic	BamHI
oypjDF2T	AAGAAAACATCAAACGTGCTTAAACATTTGTCAAACGTAT	Flanking/Mutagenic	BamHI
oypjDFA	ACGTGATTAACATTTGTCAAACGTA	Flanking/Mutagenic	BamHI
oypjDF3T	ACGTTCCTAAACATTTGTCAAACGTA	Flanking/Mutagenic	BamHI
oypjDFT	AAGAAAACAGCAAACGTtCTTAAACATTTGTCAAACGTATCTGAAT	Flanking/Mutagenic	BamHI
oypjDF3A	AAGAAAACAGCAAACGTGaTTAAACATTTGTCAAACGTATCTGAAT	Flanking/Mutagenic	BamHI
oypjDFdel	AAGAAAACAGCAAGTGCTTAAACATTTGTCAAACGTATCTGAAT	Flanking/Mutagenic	BamHI
oypjDF3G	AAGAAAACAGCAAGTGTGCTTAAACATTTGTCAAACGTATCTGAAT	Flanking/Mutagenic	BamHI
ocb83b	AAGAAAACAGCAAACGAGCATAAACATTTGTCAAACGTAT	Flanking/Mutagenic	BamHI
ocb84b	ACGAGCATAAACATTTGTCAAACGTAT	Flanking/Mutagenic	BamHI

CHAPTER 4:
SUMMARIES, FUTURE DIRECTIONS, AND FINAL REMARKS

Exploring the role of the RNAP α subunit in Spx-activated transcription

The study presented in part I of chapter 2 sought to determine if Spx-bound α CTD plays a role in productive Spx/RNAP-promoter interaction, aside from its function as a site for Spx-RNAP contact. Attempts to generate Ala codon exchange for α E255, α R261, α R268, α R289, α K294, and α E298 at the native locus were unsuccessful, suggesting that these residues are essential for cell viability. Residues α R261, α K294, and α E298 correspond to *E. coli* α R265, α K298, and α E302 residues which have been shown to function in promoter DNA contact. To determine whether these RpoA residues were required for Spx-RNAP-DNA binding, we ectopically expressed the putatively lethal *rpoA* mutant variants in diploid with the *rpoAY263C* (Y263A equivalent) mutant. The *rpoAE298A* and *E255A* mutants confer a partial defect in NaCl-stress induced activation of Spx-controlled genes. Additionally, strains expressing *rpoAE255A* are defective in disulfide stress resistance and harbor a mutant form of RNAP, which has reduced affinity for Spx. The α E255 residue corresponds to *E. coli* α D259, which has been proposed to interact with a σ^{70} R603 residue (Bsu σ^A R362). However, the *rpoAE255A* and *sigAR362A* mutant phenotypes increase in severity when combined, suggesting differing roles of these residues in Spx-activated transcription.

By complementation analysis, we show that Spx-bound α CTD amino acid residues do not likely function in Spx-RNAP-DNA contact *in vivo*, except indirectly by

serving as a contact site for Spx-RNAP binding. α CTD and σ^A function is essential to Spx-activated transcription, however, our findings suggest that Spx fulfills the critical sequence-specific DNA-binding requirements largely independent of these RNAP subunits at Spx-activated promoters. Further structural analysis is required to uncover the functional contacts mediating productive engagement of Spx with RNAP.

Mutational analysis of possible σ^A -interacting Spx amino acid residues

SpxK43, D40, and Q77 are located near α E255 at the Spx- α CTD interface, which may form a contact surface for σ^A . Mutations in Spx or RNAP that interfere with Spx-RNAP interaction can diminish Spx activity at target promoters. SpxK43A, D40A, and Q77A mutations were tested for defects in expression of several Spx-activated genes (*trxB*, *yugJ*, *ylbQ*, *ypjD*). Unexpectedly, the amino acid requirements of Spx varied widely among the four genes. The D40 residue, which was hypothesized to interact with SigAR362, appears to be required only for activation of *ypjD*. Spx K43A and Q77A mutants retained wild-type activity at the *trxB* and *ylbQ* promoters but only about 50% activity at the *ypjD* and *yugJ* promoters.

The results support the postulation that the structural orientation of Spx within the Spx-RNAP-DNA complex at the promoters of Spx-controlled genes varies more than previously stated. It is possible that the Spx residues function at the interface between Spx and other RNAP components during activation of a subset of Spx-activated genes or they perform other gene-specific functions.

Future directions

Further studies are required to determine the role of Spx D40, K43 and Q77 in Spx-RNAP interaction and/or Spx-dependent regulation. Since Spx-mediated interference of activator-stimulated transcription requires productive Spx- α CTD complex formation (Nakano, Nakano et al. 2003), the D40A, K43A, and Q77A mutants will be tested for *in vivo* repression of *srfA-lacZ* expression. Alternative approaches to the epistasis study must be employed since the *sigAR362A* mutant affects the expression of the IPTG-inducible *spx* constructs used in our study. Recent work showed that NaCl stress could induce native Spx accumulation (Birch, Davis et al. 2017). Therefore, it is now feasible to conduct site-directed mutagenesis of *spx* at its native locus, and subsequent NaCl-stress could be administered to stimulate native Spx mutant derivatives. Next, the mutant constructs generated at the native *spx* locus in conjunction with the σ^A R362A mutant may circumvent the previously encountered technical challenges. The pX-xylose-inducible system (You, Sekowska et al. 2008) may also serve as an alternative to the pDR111-IPTG-inducible system (Nakano, Kuster-Schock et al. 2003), provided that the regulatory system remains unperturbed in a σ^A R362A mutant background. It might be possible to generate mutant *spx* constructs for SpxK43A, Q77A, and D40A protein purification. The purified proteins can be used for Far-western blot analysis and size-exclusion chromatography to examine whether the mutations disrupt Spx-RNAP and/or Spx- α subunit binding. A structural study involving high-resolution cryo transmission electron microscopy has been initiated to examine in detail the Spx-RNAP contact sites.

Redox control and Spx-dependent regulation of thiol homeostasis genes

Oxidation of the Spx redox disulfide center is required for activation of many thiol homeostasis genes but likely not for activation of bacillithiol biosynthetic operons *ypjD* and *ylbQ* of *B. subtilis*. Using *in vivo* gene expression assays, we reconstructed the redox independent activation of *ypjD* and *ylbQ* by Spx and SpxC10A. In sharp contrast, *B. subtilis* Spx can stimulate transcription of *B. anthracis* *ypjD* (BA1554) to the same extent as observed by the *B. anthracis* Spx paralogs but an SpxC10A mutant is significantly defective. To determine whether the *ypjD* harbored intrinsic determinants (s) of SpxC10A-activation transcription, the promoter was targeted for site-directed mutagenesis and 5' promoter truncation studies. The *ypjD* promoter contains regions of dyad symmetry and alternating tandem repeats. The upstream promoter sequence spanning -50 to -62 determined whether downstream base substitutions promoted or impaired *ypjD-lacZ* expression, a phenomenon that could be observed in a gene under both positive and negative control. The complex nature of *ypjD* (and perhaps *ylbQ*) regulation may be explained by the existence of a co-activator of Spx and/or a repressor exerting transcriptional control of the operons functioning in bacillithiol biosynthesis.

The transcriptional activation of *bsh* biosynthetic gene operons is insensitive to the redox state of the Spx CXXC motif, however, many Spx-controlled genes require Spx in its oxidized disulfide state. Since a C10C13S mutation partially rescues the C10S-specific defect in expression of *trxB*, it is likely that oxidation of the Spx redox

center is required to alleviate the transcription inhibition exerted by a free thiol in Spx.

Future directions

Several experiments were previously conducted to further elucidate the mechanism of Spx-regulation of the *bsh* operons. Attempts at *in vitro* transcription and electrophoretic mobility shift assays were made to detect direct Spx-DNA binding or transcriptional activation at *bsh* operon promoters. However, ambiguous results and reproducibility issues proved a challenge. Results obtained from these experiments upon more optimization and troubleshooting could prove invaluable, and hence, these assays should be revisited. Alternatively, the *in vitro* results may suggest that an additional factor is required or active Spx-RNAP-DNA complex formation.

Strains bearing *ypjD*- and *ylbQ-lacZ* fusions have been transformed with gene-disruption libraries that contained plasmids with gene insertions stochastically integrated into sequences regions within the *B. subtilis* genome. The resultant strains were examined for a Lac^{+/-} phenotype of X-gal containing agar. We sought to isolate mutants that lead to heightened or reduced expression of the genes and may function as regulatory factors. Some candidates were isolated, however, many conferred pleiotropic effects that suggest indirect effects. RelA was identified as a potential indirect positive regulator of *ypjD*, since *ypjD-lacZ* bearing cells exhibited a Lac⁻ phenotype in a *relA* insertion mutant background. During nutritional stress, RelA is required for the synthesis and degradation of the (p)ppGpp alarmone,

triggering the stringent response (Wendrich and Marahiel 1997). RelP and RelQ encode redundant ppGpp synthetases but only RelA can degrade ppGpp (Natori, Tagami et al. 2009). Accumulation of ppGpp is inversely correlated with GTP levels, which is the primary initiating nucleotide of *B. subtilis* transcripts, including *ypjD* (Natori, Tagami et al. 2009, Gaballa, Antelmann et al. 2013). Therefore, it is likely that the lack of RelA hydrolase activity in the *relA* mutant causes an accumulation of ppGpp and decrease in the initiating GTP required for *ypjD* transcription.

Protein-DNA crosslinking, and DNase I footprinting studies could provide insights into the RNAP occupancy at *PypjD* and *PylbQ* prior to and upon binding Spx. Also, promoter deletion analysis and nucleotide mutagenesis of *ylbQ* should comprise future studies. The promoter regions of the two *bsh* operons are not highly homologous in sequence, however, it is possible that they are subject to the same form of control, given the high level of activity conferred by production of the SpxC10A mutant protein. Also, examining *ypjD* expression during conditions of phosphate limitation, or growth on alternative sulfur sources may prove useful.

As for *B.anthraxis*, phenotypic analysis of BSH depleted mutants should be pursued since BSH has been implicated in the virulence and stress resistance of *S. aureus* (Newton, Fahey et al. 2012, Pother, Gierok et al. 2013).

The search for the additional regulator of *ypjD* remains a priority. The search for homologous promoter sequences continues. Using a biotinylated *ypjD* promoter in a streptavidin pull down assay, we can add *B. subtilis* lysate before or after adding purified Spx. Perhaps a complex of Spx and a co-activator or repressor can

be extracted and identified by gel electrophoresis, with candidates chosen for identification through tandem mass spectrometry.

FINAL REMARKS

B. subtilis and other low GC-content Gram-positive bacterial species have evolved to employ members of the Spx family of proteins as crucial contributors to stress management and virulence. It has yet to be determined why Spx often exists as multiple paralogs in some bacteria. One reason may stem from the need for a more finely tuned mobilization of genes required during response to a various stress stimuli. In at least some species, Spx paralogs are induced during different phases of growth, have only partially overlapping regulons and fulfill specific roles under various stress conditions (Barendt, Lee et al. 2013, Zheng, Xu et al. 2014, Galvao, Rosalen et al. 2017).

B. subtilis Spx complexes with RNAP to prioritize activation of Spx-dependent genes over those under control of other activators and housekeeping genes (Nakano, Kuster-Schock et al. 2003, Zuber 2009) during proteotoxic stress. It is possible that by engaging in pre-recruitment, Spx and RNAP holoenzyme form a DNA-binding complex more discerning of target promoter DNA than the two alone. Binding to α CTD, and potentially other subunits of RNAP, may be essential to induce a DNA-binding conformational change that would otherwise not occur in free Spx. The nucleotide-specific DNA binding residues of RNAP may be limited to the regions of sigma that bind the core promoter region.

Results from the Spx mutational analysis (chapter 2, Part II) suggest that the Spx may engage RNAP and/or promoter DNA in multiple conformations. The molecular topology of Spx-RNAP-recognized promoters likely varies considerably. While the -44-GC--43 dinucleotide was present in 71% of promoters preceding Spx-activated genes, the presence of the -45- AGCA--42 motif dropped to 10% (Rochat, Nicolas et al. 2012). Since Spx-controlled genes do not bear strongly conserved cis-elements within their promoters, the question remains how exactly Spx/RNAP attains promoter specificity.

Though the transcriptional activity of the Spx/RNAP complex was not measured, ChiP-chip analysis showed that many Spx/RNAP binding sites were detected in cells treated with or without diamide (Rochat, Nicolas et al. 2012). One must question the physiological consequences of reduced Spx maintaining activity at *ypjD* and *ylbQ* promoters (i.e in the absence of oxidative stress). During non-stress conditions or growth with the preferred carbon source, the up-regulation of *mgsA* may lead to the deleterious production of methylglyoxal. Also, aberrant *bsh* production may titrate UDP-Glc-Nac, an important precursor for cell wall biosynthesis and also free cysteine, which is required for incorporation into proteins, cofactor biosynthesis and as an antioxidant. An additional regulator targeting *PypjD* (maybe *PylbQ*) may be required to ensure that the regulation of these operons is coordinated with the physiological needs of the cell, which may extend beyond maintenance of thiol homeostasis.

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