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Purification of Global Regulator, Spx, and RNA Polymerase from Staphylococcus aureus for Use in In Vitro Transcription of Redox Genes

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Purification of Global Regulator, Spx, and RNA Polymerase from
Staphylococcus aureus for Use in *In Vitro* Transcription of Redox Genes

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Abstract

Spx is a global regulator discovered in *Bacillus subtilis* to suppress basal growth and development processes and activate transcription of genes involved in thiol homeostasis when a cell encounters oxidative stress. Its activity relies on reversible thiol-disulfide bond formation and binding RNA polymerase rather than DNA. The discovery that *Staphylococcus aureus* global virulence regulator, SarA, is more active upon cysteine reduction suggests that redox response could mediate virulence in this important human pathogen. We describe the cloning of *spx* from *S. aureus* strain RN6390, overexpression in *Escherichia coli,* and purification of native protein. Antibodies against Spx were raised for western analysis. Spx from *S. aureus* was highly active in a *B. subtilis in vitro* transcription system, stimulating expression of *trxB*, the gene encoding thioredoxin reductase, without reducing agents. RNA polymerase was partially purified from *S. aureus*, and the enzyme was active, catalyzing transcription of *rpsD*, but not *trxB*.

Introduction

The Gram-positive coccus, *Staphylococcus aureus*, is an important human pathogen, causing a wide variety of diseases ranging in severity from superficial boils and abscesses to life-threatening septicemia. Depending on the tissue colonized, an *S. aureus* infection can result in bacterial meningitis, endocarditis, osteomyelitis, endophthalmitis, pneumonia, septic arthritis, toxic shock syndrome, and food poisoning. However, the organism is also part of the normal human flora, existing innocuously on the skin or in the nasal passages of approximately one-third of the population. Infection can result when a break in the skin or mucous membranes grants the commensal microbe access to the bloodstream or other tissues, defining *S. aureus* as an opportunistic pathogen, the potency of which can be attributed to its broad range of virulence factors, ability to withstand many environmental stresses and persist outside the host, and rapid evolution into multiple-drug resistant strains.

S. aureus is a prominent source of nosocomial infections. This is due in large part to its ability to colonize medical apparatuses such as catheters, prosthetic implants and valves, and orthopedic devices. Growth within prostheses allows the bacteria to enter musculoskeletal tissue, rendering it the leading cause of osteomyelitis (Frederiksen *et al*., 1993). More important is the prevalence of multiple-drug resistant isolates within the hospital setting. Treatment options for *S. aureus* infections are rapidly becoming obsolete as the number of antibiotic-resistant strains skyrockets. Methicillin-resistant *S. aureus* (MRSA) currently constitutes over half of clinical isolates (Oliveira *et al*., 2002). The evolution of MRSA strains resistant to vancomycin (Boyle-Vavra *et al*., 2001; Weigel *et al*., 2003), a drug of "last resort," constitutes an immediate threat to human health worldwide. The ease with which the organism gains genetic elements to nullify traditional antimicrobials suggests that researchers must employ innovative strategies in

approaching the design of therapeutic agents. To this end, the regulatory pathways by which *S. aureus* controls virulence factor production must be fully understood.

S. aureus produces two major classes of temporally-regulated virulence factors: cell wall-associated proteins and secreted exoproteins. The former class includes adhesins such as the collagen-, elastin-, and fibronectin-binding proteins (*cna*, *ebpS*, *fnbA* and *fnbB),* intracellular adhesin (*ica*), and clumping factor (*clfA* and *clfB*) (reviewed in Bronner *et al*., 2004). Staphylocoagulase (*coa*), although it is actually an extracellular protein, is also preferentially expressed with the surface-associated factors (Lebeau *et al*., 1994). Expressed in the early stage of infection or at the onset of exponential growth phase in culture, these proteins allow the bacteria to colonize host tissue and navigate the bloodstream. Protein A (*spa*) binds the Fc portion of circulating antibodies, thus rendering the burgeoning colonies invisible to the host's immune system (King and Wilkinson, 1981). A global phenotypic switch occurs later in infection or during the late exponential phase in culture, whereby the expression of surface proteins is suppressed in favor of the latter class of virulence factors. The secreted proteins include exoenzymes, such as proteases (*eta, etb, sspA, sspB,* and *spl*), lipase (*geh*), and nuclease (*nuc*), and toxins, including classes of hemolysins (*hla, hlb, hld*), leukotoxins (*luk*), enterotoxins, exotoxins (*set*), and toxic-shock syndrome toxin (*tst*) (reviewed in Bronner *et al*., 2004). *In vitro,* this is likened to cells scavenging for nutrients as the requirements of the colony exceed the resources of the media; *in vivo*, this may represent a mechanism by which cells release themselves from attachment sites and disseminate from the primary infection site once it has been established (Wesson *et al*., 1998).

The temporal regulation of the above factors is coordinated by a number of pleiotropically-acting regulators, most notably of the unlinked *sar* and *agr* loci, but also

including *sae* (Giraudo *et al*., 1997), *ssrAB* (Yarwood, *et al*., 2001), *mgr*, and several Sar homologs (*sarT*, *sarU*, *sarV* and *rot*) (reviewed in Novick, 2003). The agr locus (<u>a</u>ccessory <u>g</u>ene <u>r</u>egulator) consists of two adjacent, divergent transcripts, RNAII and RNAIII, expression of which are driven by the P2 and P3 promoters, respectively (Peng *et al*., 1988). RNAII encodes four genes (*agrABCD*), which comprise an autoregulatory quorum-sensing circuit with a classical two-component sensory transduction system having genomic sequence and organization similar to the *comAP* operon of *Bacillus subtilis* (Dubnau *et al*., 1994). AgrD, the signaling peptide, is post-translationally processed into an octapeptide pheromone, cyclized, and exported from the cell by AgrB (Ji *et al*., 1995). AgrC, a membrane-bound histidine kinase, senses the extracellular concentration of the signaling peptide, and, once it has reached the minimum threshold of concentration due to a cell density corresponding to late exponential phase in culture, phosphorylates the response regulator, AgrA. AgrA, thus activated, binds the P2 and P3 promoters within the *agr* intergenic region, and upregulates expression of both transcripts (Koenig *et al*., 2004). RNAIII, the effector molecule of the system, is an untranslated regulatory RNA molecule that incidentally includes the open reading frame (ORF) for δ-hemolysin (*hld*). RNAIII, by a largely unknown mechanism, operates at the level of transcription and translation to decrease the production of cell-surface proteins and initiate the secretion of exoproteins (Novick *et al*., 1993; Morfeldt *et al*., 1995; Korem *et al*., 2005). Mutants lacking any gene of the *agr* locus exhibit highly reduced virulence in animal models of disease (Abdelnour *et al*., 1993; Cheung *et al*., 1994; Gillaspy *et al*., 1995).

The *sar* (<u>s</u>taphylococcal <u>a</u>ccessory gene <u>r</u>egulator) locus was first described as a locus distinct from *agr*, which changed the overall pattern of virulence gene expression when inactivated (Cheung *et al*., 1992), and then as a gene required for optimal expression of *agr*

(Cheung and Projan, 1994). It contains three overlapping transcripts (*sarA*, *sarB*, and *sarC*) with a common termination signal (Bayer *et al*., 1996), and all encode the 14.5 kDa DNA-binding protein, SarA (Cheung and Projan, 1994). SarA forms a dimer in solution (Rechtin *et al*., 1999) and is shown to upregulate expression of both RNAII and RNAIII by binding *cis* to the P2 and P3 promoters (Morfeldt *et al*., 1996; Chien and Cheung, 1998; Chien *et al*., 1999). Yet *sar* also affects expression of virulence determinants independently of *agr* (Cheung *et al*., 1994; Blevins *et al*., 1999; Chien *et al*., 1999; Wolz *et al*., 2000), and mutant strains of *S. aureus* with *sar* or *agr* mutated can have quite different phenotypes (Cheung *et al*., 1992; Blevins *et al*., 1999; Blevins *et al*., 2002). Although the literature is inconsistent with regards to whether SarA represses or induces certain genes, particularly those encoding exoproteins, it does appear to repress *cna* and *spa* transcription (Cheung *et al*., 1997; Chan and Foster, 1998; Chien *et al*., 1999) and enhance fibronectin-binding capacity (FBC) (Cheung *et al*., 1994; Wolz *et al*., 2000). *sar* mutants show attenuated virulence compared to the wild-type, and *sar agr* double mutants have diminished virulence in comparison to *agr* mutants (Cheung *et al*., 1994).

For many years, SarA was believed to be a classical transcription factor; however, mounting evidence to the contrary indicates that it is not. SarA is thought to affect over 100 genes as either an activator or a repressor, and though much of its influence may be through other regulators, it has been shown to bind directly to promoter regions of a number of target genes. A number of laboratories have identified different binding sites for SarA within the *agr* intergenic region (Morfeldt *et al*., 1996; Chien and Cheung, 1998; Chien *et al*., 1999; Rechtin *et al*., 1999; Sterba *et al*., 2003). An AT heptad repeat identified by SELEX (selective evolution of ligands by exponential enrichment) as a consensus SarA binding site (Sterba *et al*., 2003) is present in over 2500 copies within the *S. aureus* genome (Roberts *et al*., 2006). SarA is present

in the cytoplasm in copy numbers far exceeding any known transcription factor (Fujimoto *et al*., unpublished data). SarA has also been shown to influence mRNA turnover, stabilizing *cna* and *spa* transcripts, evidence which suggests that genetic regulation by SarA may not be entirely at the level of transcription (Roberts *et al*., 2006).

Confounding an understanding of virulence gene regulation in *S. aureus* is the discovery by several laboratories of strain-dependent differences in *sar*- and *agr*-mediated gene expression (Li *et al*., 1997; Papakyriacou *et al*., 2000; Blevins *et al*., 2002; Peacock *et al*., 2002; Cassat *et al*., 2005). The bulk of genetic data on *S. aureus* has focused on the common laboratory strain RN6390, or its parent, NCTC8325-4 (Novick, 1967). However, this strain has long been known to carry an 11-bp deletion in *rsbU*, which encodes a positive regulator necessary for activation of the stress response sigma factor, σ^B, rendering it essentially a *sigB* mutant (Kullick *et al*., 1998). It would therefore be expected that RN6390 would be deficient in its defenses against environmental stresses, which may, in turn, attenuate virulence; indeed, this appears to be the case. It is also lacking in *cna*, which promotes collagen binding. Although wild-type RN6390 is virulent in animal models of disease, its pathogenicity is greatly attenuated, even when a chromosomal copy of *cna* is introduced, in comparison to clinical isolates of *S. aureus* (Blevins *et al*., 2002; Cassat *et al*., 2005).

Blevins *et al*. (2002) compared the effects of mutations of *agr* and/or *sar* within three laboratory strains, including RN6390, to those within four clinical isolates, and found a surprisingly different pattern of virulence gene regulation among the different strains. Although *sar* mutants all exhibited increased protease expression and decreased FBC and mutations of *agr* brought forth the opposite effect, the impact of these mutations was hardly uniform among the different strains. For instance, the decrease in protease expression was barely significant in

clinical isolates but quite obvious in RN6390; however, wild-type RN6390 was shown to be proteolytically hyperactive compared to wild-type clinical isolates (Blevins *et al.*, 2002). The significance of this finding is highlighted by the observation that epidemic strains of *S. aureus* generally limit exotoxin release to booster host protein-binding capacity, which may favor the colonization phase of infection (Papakyriacou *et al.*, 2000). Moreover, it was found that the *sar* mutant's diminished FBC was not due to decreased *fnb* transcription, but the rise in proteolysis, particularly by *sspA*. Mutations of *sar* also produced disparate phenotypes in regard to hemolysis: in RN6390, hemolysis was negatively impacted by both *sar* and *agr* mutations in accord with the findings of Cheung and Ying (1994); in all other strains tested, the *sar* mutants gained hemolysin activity. Again, the hemolysin production of wild-type clinical isolates was greatly reduced compared to that observed in wild-type RN6390 (Blevins *et al.*, 2002). Finally, in stark contrast to the findings of Cheung and Projan (1994), mutating *sar* seemed to have little effect on RNAIII expression in all strains except RN6390 (Blevins *et al.*, 2002).

Cassat *et al.* (2005) performed a genome-wide comparison of RN6390, seven sequenced strains, and two clinical isolates, and used hierarchical clustering to establish that RN6390 is actually an outlier strain in regards to genetic relatedness. All of the clinical isolates tested were closely linked, including EMRSA-16, the MRSA dominant in the United Kingdom (Moore and Lindsay, 2002). These strains were found to be more infective at lower doses than RN6390 and its close relatives. The researchers also determined that *cna*, which RN6390 lacks, is not only a marker for overall virulence, but for genetic relatedness as well (Cassat *et al.*, 2005). This agrees with an earlier report stating that *S. aureus* strains which encode *cna* account for the majority of human infections (Booth *et al.*, 2001).

It is apparent from these studies that the regulatory circuits controlled by *agr* and *sar* in RN6390 are not representative of those in clinical isolates, and further characterization of these global regulators within the background of the laboratory strain may be pointless. What is not readily apparent is the source or sources of this discrepancy. One possible explanation is that SarA is not maximally expressed in RN6390 due to the lack of *sigB* activity. Deora *et al*. (1997) purified σ^B from *S. aureus* and used it in *in vitro* transcription of SarA from all three promoters. They discovered that transcriptional activation from the P3 promoter of *sarC*, which is intermediate in size to the other two transcripts, is σ^B-dependent (Deora *et al*., 1997). Another laboratory verified this result *in vivo* using transcriptional fusion experiments (Manna *et al*., 1998). Blevins *et al*. (2002) also found reduced levels of *sarC* transcript in RN6390, but it did not result in decreased SarA production. Another laboratory determined that SarA activity was dependent not only on functional σ^B, but growth phase, as well, with maximal SarA expression occurring during late exponential growth in a *sigB*-positive strain. They also found *agr* expression was increased in *sigB* mutant strains. σ^B appeared to have an inhibitory effect on FBC in this study (Bischoff *et al*., 2001). It should also be mentioned that at least one laboratory concluded that σ^B plays no role in pathogenesis by *S. aureus* (Chan *et al*., 1998). So while it is tempting to suggest that RN6390 has a phenotype similar to a *sar* mutant because of the *rsbU* deletion, definitive evidence remains elusive. An otherwise isogenic strain of RN6390 encoding functional *sigB* does exist (Horsburgh *et al*., 2002). If experiments performed in RN6390 were replicated in this strain (SH1000), the role of σ^B in global gene expression could be better characterized.

The discovery by Bischoff *et al*. (2001) that optimal SarA expression was dependent not only on σ^B, but on growth stage also, suggests that environmental stimuli and stress response

may be involved in SarA expression and SarA-mediated expression. Chan and Foster (1998b) examined expression of *hla*, *tst*, and several putative extracellular proteases under aerobic and microaerobic conditions. Their results were rather inconsistent: a *sar* mutant had ten-fold accumulation of Hla compared to the wild-type as measured by NH_2-terminal sequencing of the exoprotein profile; the same mutant had 40 % activity relative to wild-type in a *hla:lacZ* transcriptional fusion experiment. They did find that SarA seems to be a general repressor of proteases, in accordance with the bulk of literature on *S. aureus*, and hypothesized that the discrepancy concerning Hla accumulation and activation could be a result of proteolysis of Hla by various secreted proteases, notably a prominent V8 serine protease. They also argued that differential protease expression could be a mechanism to control a wide range of responses, including to stresses and environmental stimuli. One rather intriguing piece of evidence pointing to a potential role for SarA in stress response was that *hla* activation, as measured by the *lacZ* fusion, was dampened in the *sar* mutant under microaerobic conditions even compared to normal growth conditions. They also noted that *hla* expression was induced earlier under microaerobic conditions (Chan and Foster, 1998b). It seems possible that exponential-phase cells could behave like post-exponential phase cells when undergoing environmental stress or as the media becomes depleted of nutrients and saturated with waste products, and this reaction could involve alternate sigma factors, SarA, and other regulatory networks.

Morfeldt *et al.* (1996) noted that the spacing between the -35 and -10 elements of P3 promoting RNAIII transcription was three base pairs too long for optimal recognition by RNAP, and hypothesized that SarA may function to change DNA topology so RNAP and possibly other regulators could interact with *S. aureus* promoters (Morfeldt *et al.*, 1996). Supporting this hypothesis is the intrinsic curvature of DNA within AT-rich tracts that SarA is known to bind to

(Sterba *et al*., 2003). SarA activates expression of a murein hydrolase encoded by *lrg*, and this activity was shown to be dependent on a region of intrinsic DNA curvature (Fujimoto *et al*., 2000). It must be stated, however, that while an early published report of the crystal structure of SarA binding DNA supported this theory showing SarA overwinding B-DNA into D-DNA upon contact (Schumacher *et al*., 2001), a later report found SarA and DNA made more traditional activator-promoter interactions (Liu *et al*., 2006). Yet evidence still accumulates that SarA may more resemble an accessory protein analogous to Xis from *Escherichia coli* or Integration host factor (IHF) from bacteriophage λ, which promote intramolecular excision recombination and Integrase-mediated recombination, respectively (Mumm *et al*., 2006), than a transcription factor in the usual sense. SarA could replace Xis in *in vitro* intramolecular recombination reactions, although with only modest stimulation of excisional activity, and could bind the bacteriophage λ attachment site, *attL*, forming complexes resembling intasomes (Fujimoto *et al*., unpublished data). The DNA substrates employed in these assays, *attL* and *attR*, contain a site, X, which is AT-rich (75%), mirroring the AT-richness of the *S. aureus* genome, and containing a SarA binding site previously identified by SELEX (Fujimoto *et al*., unpublished data; Sterba *et al*., 2003). This evidence suggests that SarA has a high affinity for AT-rich DNA, such as is found in UP elements (Ross *et al*., 1993), and that activation and repression of target genes by SarA may be an indirect consequence of its chromosomal accessory protein activity.

In the above study, Fujimoto *et al*. performed competitive enzyme-linked immunosorbent assays (ELISAs) with cell-free extracts of *S. aureus* competing against purified SarA for antibody, and discovered that there are approximately 50,000 SarA dimers per cell at all stages of growth (Fujimoto *et al*., unpublished data). This number far exceeds cellular quantities of known transcription factors, and is similar to amounts of nucleoid accessory proteins found in *E.*

coli (Johnson *et al*., 2005). Fujimoto *et al*. also determined that, contrary to previous reports (Bischoff *et al*., 2001; Manna and Cheung, 2001), SarA is equally active at all stages of growth *in vitro*, which caused the researchers to question why the literature continually shows SarA exerting its regulatory effects in late- and post-exponential stages of growth *in vivo*. Recalling the discovery by Chan and Foster (1998b) that SarA activity changed under microaerobic conditions, Fujimoto *et al*. performed electromobility shift assays (EMSAs) of SarA binding to three promoter regions (for *agr*, *cna*, and *sspA*) under oxidizing and reducing conditions. They observed a dramatic increase of SarA activity when thiol-specific reductants, dithiothreitol (DTT) and β-mercaptoethanol, were added to the reactions and a decline in activity when hydrogen peroxide was used. The redox response of SarA was shown to be mediated by its lone cysteine residue at position nine: the C9A mutant had maximal activity under all conditions tested. Similarly, SarA's DNA-binding affinity was also responsive to changes in pH, with affinity increasing as the pH decreased and optimal binding measured at a pH of 5.5. Again, this response appears to be dependent on C9, as the C9A mutant's activity was maximal at a pH of 7.5 (Fujimoto *et al*., unpublished data). *agr* expression was earlier found to be favored by low pH, which may be a consequence of SarA's binding *cis* to the *agr* promoter regions (Regassa and Betley, 1992; Regassa *et al*., 1992).

The enhancement of SarA activity caused by low pH and lack of oxygen may explain why researchers have generally concluded that SarA exerts its regulatory effects during post-exponential growth, as culture media tends to accumulate acidic waste products, and denser colonies may be less efficiently aerated. More importantly, however, is the logical inference that this may better represent the environment *S. aureus* encounters during an actual infection, where conditions will be far from the monospecific, highly aerated, nutrient-rich cultures typically used

in a laboratory. Within the host, an invading pathogen will encounter environmental stresses such as release of reactive oxidative species from macrophages, high temperatures induced by fever, acidic pH of phagolysosomes, and anaerobic conditions found within an abscess. *S. aureus*, being a facultative anaerobe, can grow without oxygen, and anecdotal evidence has pointed to a heightening of virulence when it does, as was seen with toxic shock syndrome. SarA may therefore be a chromosomal architecture protein which prepares the cell for environmental stress it will encounter during stationary phase growth while it coordinates virulence gene regulation with *agr* and other regulators.

Prokaryotes must contend with oxidative-reductive (redox) stress as a consequence of aerobic respiration, residence within a host, and competition from other microbes. In general, redox stress is caused by exposure to reactive oxygen intermediates (ROIs), such as the superoxide anion ($O_2 \cdot -$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO \cdot$) (reviewed in Bauer *et al*., 1999). $O_2 \cdot -$ and H_2O_2 are normal byproducts of oxidative phosphorylation, formed when molecular oxygen oxidizes electron carriers along the respiratory chain. They are also released from macrophages as part of the host defense to infection. Bacteria secrete redox-cycling antibiotics to inhibit growth of competitors. $O_2 \cdot -$ destroys iron-sulfur clusters found in many enzymes. Iron released can react with H_2O_2 to form $HO \cdot$, which damages DNA and oxidizes free thiol residues within proteins into disulfide bonds (reviewed in Storz and Imlay, 1999). Formation of nonnative disulfide bonds causes protein misfolding, and is a common mechanism for proteins to gain or lose activity (Åslund and Beckwith, 1999). Cytoplasmic proteins generally do not contain structural disulfide bonds due to the reducing environment within the cell, but are found in the periplasm (Stewart *et al*., 1998). $O_2 \cdot -$, which cannot cross the plasma membrane at neutral pH, can combine with nitric oxide ($NO \cdot$) to form HOONO.

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HOONO can diffuse across the membrane into the cytoplasm, where it is free to attack iron-sulfur clusters and free thiols, inactivating enzymes. Alone, NO· blocks respiration by binding hemes and metal cofactors of enzymes (Storz and Imlay, 1999).

Prokaryotes have evolved a number of different mechanisms to sense and respond to environmental or cellular increases in oxygen and ROIs (reviewed in Bauer *et al.*, 1999). The purpose of redox regulation is to maintain a steady state concentration of ROIs that is within the limit of tolerance (reviewed in Zheng and Storz, 2000). One method by which this aim is achieved is through induction of genes involved in transport, such as porins and pumps, which exclude redox-active enzymes to stabilize oxygen levels (Storz and Imlay, 1999). Two of the best characterized prokaryotic redox regulators are OxyR and SoxR from *E. coli*. OxyR functions as a tetramer, with each monomer possessing two cysteine residues. In the normal reducing environment of the cell, the cysteines exist as free thiols, but an increase in H_2O_2 concentration oxidizes them into disulfide bonds. OxyR, thus activated, binds upstream promoter elements of its regulon to upregulate transcription of genes encoding catalase (*katG*), alkyl hydroperoxide reductase (*ahpF*), glutaredoxin (*grx*), and glutaredoxin reductase (*gorA*) (Storz *et al.*, 1990; Zheng *et al.*, 1998). SoxR is a homodimer, which contains four iron-sulfur clusters. In its reduced form, it is inactive, but is activated by oxidation of the clusters by $O_2\cdot-$ or nitric oxide. Activated SoxR then upregulates the transcription of *soxS*, which relays the redox response by initiating transcription of genes such as superoxide dismutase *(sodA)*, aconitase (*acnA*), fumarase (*fum*), and DNA repair enzymes like *recA* (Ding and Demple, 1997). The oxidation events that lead to activation of both of these regulators are reversible, so the cascade of events ceases upon return of the cell to a reduced state (Zheng and Storz, 2000). Another redox sensor, ArcB, is an intramembrane histidine kinase, which phosphorylates its cognate

response regulator, ArcC, depending on the redox state of the plasma membrane (Bauer *et al.*, 1999).

S. aureus is a facultative anaerobe, meaning that it can grow aerobically or anaerobically, either by fermentation or by respiration, using an alternative terminal electron acceptor to oxygen, such as nitrate. Therefore, it must have sensory transduction pathways to gauge the oxygen content of its environment in order to optimize gene expression. One of these systems, characterized in *B. subtilis*, is ResDE (Nakano *et al.*, 1996), which has *S. aureus* homologs, SrrAB. In *B. subtilis*, ResDE is required for anaerobic respiration when nitrate is the terminal electron acceptor. The response regulator, ResD, is constitutively autophosphorylated, but ResE has phosphatase activity when oxygen is present. Upon oxygen limitation, ResE loses its activity, and ResD becomes active, inducing transcription of its regulon, which includes essential genes for anaerobiosis (Nakano *et al.*, 2000b). Another role of oxidoreductases in the cell is to provide reducing power for biosynthetic enzymes, such as the ribonucleotide reductases which are necessary for nucleic acid synthesis. The source of reducing power is generally NADPH (Reichard, 1993). In *S. aureus*, three classes of ribonucleotide reductases exist, and are differentially expressed depending on oxygen availability. Class Ib is oxygen-dependent, Class II is oxygen-independent, and Class III is only expressed during anaerobic growth. This ability of staphylococci to sense oxygen levels ensures that this essential activity will not be lost (Masalha *et al.*, 2001).

The first attempt to characterize the transcriptome of *S. aureus* cells experiencing oxidative stress was made by Chang *et al.* (2006). They subjected the cells to hydrogen peroxide for 10-20 minutes, and analyzed the pattern of gene expression during the onset of, and recovery from, stress, and noted an initial period of repression of iron storage and transport genes, during

which time genes involved in fermentation were being upregulated. They concluded that *S. aureus* has the ability to induce an anoxic state, and can thus limit its exposure to oxygen and resultant cytotoxicity (Chang *et al.*, 2006). Another group also reported that suppression of the tricarboxylic acid cycle through aconitase inactivation actually enhances stationary phase survival of *S. aureus*. Their hypothesis was that this is a naturally occurring event, where an irreversible loss of the iron-sulfur cluster in aconitase renders the pathogen less susceptible to ROIs produced by host macrophages (Somerville *et al.*, 2002). In light of this evidence, it seems highly likely that *S. aureus* can maximize its infectivity through modulation of redox-dependent gene expression, and that redox sensors and regulators might cooperate with virulence determinant factors, like SarA, to accomplish this aim.

The discovery that virulence gene regulation by SarA, and perhaps *agr,* is dependent on redox has led our laboratory to investigate a novel global redox regulator, *spx*. This gene was well characterized in *B. subtilis* by the group led by Peter Zuber. It has sequence and structural homology to the enzyme, arsenate reductase (*arsC*), first located on *S. aureus* plasmid pI258 (Martin *et al.*, 2001). Arsenate reductase functions in anaerobic respiration, allowing arsenate to be used as the terminal electron acceptor, and in staphylococci, its activity is dependent on hydrogen donation by thioredoxin (*trxA*) and thioredoxin reductase (*trxB*) (reviewed in Silver and Phung, 2005). However, Spx was found to be a transcriptional activator and repressor with a mode of activity unique among known prokaryotic transcription factors.

spx (originally named *yjbD*) was discovered in *B. subtilis* as the site of suppressor mutations that overcame growth and development deficiencies in *clpP* and *clpX* strains (Nakano *et al.*, 2001). The Clp (casinolytic proteolytic) complex is composed of the ClpP proteolytic subunit and one of three substrate-binding components: ClpX, ClpC, or ClpE. ClpX is an

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ATPase and unfoldase that denatures substrate proteins and translocates them into the proteolytic chamber of ClpP, imparting specificity in the energy-driven reactions (reviewed in Hecker *et al*., 1996). Alone, ClpX acts as a molecular chaperone. It also aids in the displacement of σ^A, the housekeeping σ, in favor of σ^H, the sporulation factor, and stimulates σ^H-dependent transcription of sporulation genes (Liu and Zuber, 2000). ClpP functions as a heptamer, and without an ATPase unit, can only degrade small peptides (Krüger *et al*., 2000). ClpXP is a crucial member of the cellular response team to heat and other stresses, and mutations of it are highly pleiotropic, restricting growth and preventing competence, sporulation, and biosynthesis. Six *clpXP* suppressor mutations (denoted *cxs*) were isolated, and mapped to two unlinked loci: in *rpoA*, encoding the α subunit of RNA polymerase (RNAP), specifically helix 1 of the α-carboxy terminal domain (α-CTD) (Nakano *et al*., 2000a), and a novel gene, *yjbD* (Nakano *et al*., 2001). Helix 1 is important for interaction with activator domains of transcription factors (Russo and Silhavy, 1992), and making contacts with UP elements of promoter DNA (Ross *et al*., 1993; Gourse *et al*., 2000). The mutations, Y263C (*rpoA* $^{cxs-1}$) and V260A (*rpoA* $^{cxs-2}$) changed two residues that are highly conserved in low GC-content Gram positive bacteria (Nakano *et al*., 2000a). Within *yjbD*, one nonsense (*cxs*$^{-10}$) and three missense (*cxs*$^{-5,-7,-12}$) mutations were isolated (Nakano *et al*., 2001), also in residues that are conserved in *yjbD* orthologs found in Gram positive bacteria (Zuber, 2004). *yjbD* was renamed *spx*, $\underline{s}$uppressor of *clp$\underline{X}$* and *clp$\underline{P}$* (Nakano *et al*., 2002a).

In addition to regulating proteolysis, ClpXP plays an important role in initiating transcription of genes required for competence. The development of competence in *B. subtilis* is regulated by the intricate *com* operon. The concentration of ComX, a quorum-sensing pheromone, is sensed by ComP, a membrane-bound sensory kinase. ComP phosphorylates its

cognate response regulator, ComA, which, in turn, activates transcription of *srf*. *srf* encodes a peptide, ComS, which liberates ComK, a transcription factor for late competence genes, from an inhibitory complex. ComK is sequestered by ClpCP and the adaptor protein, MecA, until ComS replaces it and presents itself as a target for proteolysis. ComK then activates transcription of itself and genes involved in DNA uptake, repair, and recombination (reviewed in Dubnau *et al*., 1994).

In *clpP* and *clpX* strains, which did not develop competence, but not in the wild type, Spx was discovered to bind to the proteolytic complex, and actually enhance the inhibition of ComK (Nakano *et al*., 2002a). Western analysis of wild type and mutant strains revealed that Spx is barely detectable in wild type and *clpC* cells, but accumulates in *clpX* and *clpP* mutants (Nakano *et al*., 2002b). Spx has a C-terminus resembling an SsrA peptide tag (Nakano *et al*., 2003a), which is co-translationally appended to a truncated peptide, targeting it for degradation by ClpXP or a similar protease (Weigert and Schumann, 2001). Based on this evidence, and the phenotypes of *clpX/P* strains with concurrent restoration of competence in a *clpX/P spx* double mutant (Nakano *et al*., 2001), it seemed likely that ClpXP was necessary for degradation of Spx in order for cells to overcome Spx-dependent blocks to competence, and, presumably, sporulation and growth, as well (Nakano *et al*., 2002b). However, Spx was not degraded by ClpXP in *in vitro* proteolytic reactions, but by ClpCP with an adaptor protein (Nakano *et al*., 2002b). This puzzling result turned out to be an artifact, caused by two amino acids appended to the C-terminus of the purified protein after the His-6-tag was cleaved. This was remedied by cloning the tag onto the N-terminus, where the extension did not affect substrate recognition by ClpXP, which then successfully proteolyzed Spx *in vitro* (Nakano *et al*., 2003b).

The activity of Spx was further characterized by examining its effect on expression on

srf-lacZ and *hmp-lacZ* fusion proteins (Nakano *et al*., 2003b). *hmp* encodes flavohemoglobin,

and is induced under oxygen-limiting conditions by the two-component ResDE activator

(Nakano *et al*., 2000b). A *clpX* strain showed repression of both genes, but this defect was

eliminated in *clp* cells bearing the *rpoA^cxs-1^*, *rpoA^cxs-2^*, *spx^cxs-16^* (R52G) or *spx* null mutations, and

also in the wild type, where Spx is presumably degraded. These data suggested that Spx might

inhibit gene expression by association with the α-CTD of RNAP, a hypothesis which was tested

using a yeast two-hybrid experiment to search for proteins which interact with wild type, but not

mutant alleles, of Spx. Two positive clones both encoded *rpoA*, and the result was verified with

a pull-down assay. Spx activity was further elucidated by IVT of *srf* and *hmp*, transcription of

which was repressed by the addition of Spx to reactions containing promoters with their cognate

activators and wild-type RNAP, but not with *cxs* alleles. The *rpoA* mutants, with or without Spx,

transcribed a control gene, *rpsD*, indicating it encodes an otherwise functional enzyme. The

mechanism by which Spx could interrupt activator-dependent transcription was analyzed using

EMSAs of *srf* and *hmp*, where Spx destabilized complexes formed by RNAP, promoters, and

activator proteins (Nakano *et al*., 2003b). Spx was also shown *in vivo* to repress genes involved

in transport and utilization of alternative sulfur sources when cells were grown in the presence of

the preferred source, sulfate (Erwin *et al*., 2005). These experiments convincingly demonstrated

that Spx affects gene expression by an apparently unique mechanism, blocking activator-

stimulated transcription by binding to the α-CTD of RNAP, rather than promoter DNA,

prompting Spx to be dubbed an "anti-α" factor (Nakano *et al*., 2003b).

In order to examine the effects of Spx *in vivo* in a *clpXP+* background, a mutant allele of

Spx (*spx^DD^*) was constructed, which removed the SsrA peptide tag-like amino acids from the

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carboxyl terminus (A130D, N131D), rendering it unrecognizable as a substrate for ClpXP. Spx^{DD} accumulation in otherwise isogenic cells mirrored that of *clp* strains, and its repression of *srf* and nonproductive interaction with *rpoA^{cxs}* was unchanged. Total RNA was isolated from *B. subtilis* strains bearing Spx^{DD} and either wild type RNAP or *rpoA^{cxs-1}*, and used for microarray analysis of genome-wide expression. 176 genes were found to be repressed at least three-fold in cells carrying the wild type allele of *rpoA*: as expected, these included the *srf* operon, and also many genes involved in growth and metabolic processes, such as purine, pyrimidine, and amino acid biosynthesis (Nakano *et al*., 2003a). More surprising was the number of genes (106) induced at least three-fold by the Spx-RNAP interaction (Nakano *et al*., 2003a), several of whose products function to regulate thiol homeostasis in the cell, chief among them *trxA* (thioredoxin) and *trxB* (thioredoxin reductase). Nearly half of the affected genes in the transcriptome of *spx^{DD}*–expressing cells (Nakano *et* al., 2003a) followed a similar pattern of induction and repression to that of *B. subtilis* cells treated with a thiol-specific oxidant, diamide (Leichert *et al*., 2003). Diamide reacts with free thiols to form a disulfide bond with hydrazine as its end product (reviewed in Kosower and Kosower, 1995). This strong correlation led the researchers to examine Spx-dependent regulation of *trxA*, *trxB*, and *srf* in cells undergoing diamide-induced stress using primer extension analysis. As predicted, Spx inhibited transcription of *srf,* and activated *trxAB* when native RNAP was the catalyst. Cells bearing *cxs* or *spx* null mutations were also hypersensitive to growth on 50 μM diamide, indicating Spx to be an important component of the cellular response to oxidative stress (Nakano *et al*., 2003a).

Thus, Spx was characterized as a unique global transcriptional regulator that functions to halt normal growth and metabolism in order to devote the cell's transcriptional machinery to cope with thiol-specific oxidative stress, such as that incurred by treatment with diamide. Under

normal conditions, Spx is proteolyzed by ClpXP, but accumulates under disulfide stress to block basal transcription, which explains the growth defects observed in *clp* mutants (Nakano *et al*., 2003b). These effects are transient: at the onset of stress, cell growth is arrested, but resumes after diamide is consumed and detoxified (Leichert *et al*., 2003). Although suppression of transcription by Spx is easily envisioned, the mechanism by which it activates genes involved in the stress response is more mysterious, as it has no DNA-binding domain (Zuber, 2004). Besides *trxAB*, Spx induces *tpx* (thiol peroxidase), *msrA* (peptide methionine sulfoxide reductase), and various thioredoxin-like proteins (Nakano *et al*., 2003a). It could inhibit expression of a repressor of a target gene, or recruit RNAP to an activator through protein-protein interactions (Zuber, 2004). Also unclear is how Spx is stabilized under disulfide stress. ClpXP could be diverted to focus on proteolysis of proteins damaged by oxidation or it could be inactivated itself. If so, it is likely the ClpX subunit that is affected, because ClpP and ClpC, but not ClpX, are induced over ten-fold upon diamide treatment (Leichert *et al*., 2003). The N-terminal of ClpX has a zinc-finger domain, in which a zinc atom is chelated by five cysteine residues, the oxidation of which could result in irreversible loss of the bound zinc, and therefore, ClpX activity (Banecki *et al*., 2001).

Nakano *et al*. (2005) explored the mechanism by which Spx activates transcription of *trxB* by mutating the highly conserved cysteine residues at positions 10 and 13. These form a CXXC motif, which is a common feature of redox sensors (OxyR), and a likely candidate for oxidative sensing and response (Åslund *et al*., 1999). Cells bearing Spx with either C to A mutation were hypersensitive to diamide, and could not induce transcription of *trxAB*. These residues were not involved in stability of Spx, as either mutant accumulated at the onset of diamide treatment, indicating that they are involved in transcriptional activation of and/or

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binding to the α-CTD of RNAP. IVT of *trxB* could only be accomplished when wild-type Spx was present in the reaction and was abolished with inclusion of the thiol-specific reductant, DTT, leading the researchers to conclude that formation of a disulfide bond activates Spx in regards to positive transcriptional regulation of *trxB*. They convincingly established that the cysteine residues at positions 10 and 13 form an intramolecular disulfide bond by performing electrospray ionization mass spectrometry (ESI-MS) on Spx in the presence of an alkylating reagent, iodoacetamide. Iodoacetamide alkylates free thiol residues. No alkylation was detected unless DTT was present (Nakano *et al.*, 2005), even when a strong denaturant was added to remove the possibility that free thiols could be inaccessibly buried within the protein's hydrophobic core (Messens *et al.*, 2004). When DTT was added, acetylation occurred, and the resultant species had a mass similar to an Spx monomer, indicating that an *intra*molecular, not *inter*molecular, disulfide bond had been created. Thus, it was demonstrated that Spx, like OxyR (Storz *et al.*, 1990), is activated by a thiol-disulfide switch at the onset of thiol-specific oxidative stress, and this activation is necessary for positive transcriptional regulation (Nakano *et al.*, 2005). By this mechanism Spx acts as both the sensor and transducer of oxidative stress, as was shown with OxyR (Storz *et al.*, 1990). The mechanism by which Spx activates transcription is still not clear, however, as DNase I footprinting revealed no DNA-binding ability of even oxidized Spx at the *trxB* promoter (Nakano *et al.*, 2005).

Newberry *et al.* (2005) solved the crystal structure of Spx in complex with the α-CTD of RNAP. Spx is composed of two domains: a central domain which interacts with RNAP, and a redox domain consisting of the N- and C- termini, including the CXXC motif. Oddly, the site of interaction between Spx and the α-CTD was found to be on the distal face from the redox-sensing motif, opening up the possibility that Spx could induce a conformational change in

RNAP allosterically that would allow initiation of transcription. Another surprising discovery

was that the affinity of Spx for α was unchanged regardless of oxidation state (Newberry *et al*.,

2005), in agreement with other reports that reduced Spx could still bind α in footprinting

experiments, though the complex formed was unproductive (Nakano *et al*., 2005) and that the

redox domain of Spx is nonessential for repression of target genes (Zhang *et al*., 2006).

Residues within the region of interaction of α with Spx correspond to residues of the *E. coli* α-

CTD that are necessary for transcriptional initiation by activator proteins (Chen *et al*., 2003),

supporting the Spx-dependent repression model (Nakano *et al*., 2003b; Zhang *et al*., 2006). Spx

also interacts with residues of *B. subtilis* α corresponding to the "261" determinant of *E. coli* α, a

necessary element for recruitment of σ^{70}, the *E. coli* housekeeping σ (Savery *et al*., 2002),

prompting the investigators to hypothesize that Spx could activate transcription by facilitating

recruitment of σ to the RNAP core enzyme to form the holoenzyme (Newberry *et al*., 2005).

As *clpXP* mutations have such pleiotropic effects in a nonvirulent organism such as *B.*

subtilis, Frees *et al*., (2003) investigated whether mutations of either gene could affect virulence

in the pathogen, *S. aureus*. The researchers constructed deletions of both genes in the common

laboratory strain, 8325-4, which is the parent strain of RN6390 (Novick, 1967), and examined

the resultant change in the overall pattern of expression of genes involved in both virulence and

stress response. Predictably, the stress response was dampened in the *clpP* mutant, particularly

to heat shock (Frees *et al*., 2003), as ClpP has long been known to play a crucial role in the

turnover of denatured and otherwise nonnative proteins (Frees and Ingmer, 1999; Krüger *et al*.,

2000). The strain harboring a *clpX* deletion was actually more susceptible to many types of

environmental stress, including oxidative stress by hydrogen peroxide. If Spx accumulation is

controlled by ClpXP in *S. aureus* as it is in *B. subtilis*, one would assume that these mutants were

Spx-overproducers and that they would gain oxidoreductase activity, at least in response to thiol-specific oxidative stress. However, the response to diamide was not examined in this study. The researchers did note an attenuation of virulence in both mutants, probably by both *agr*-dependent (represented by decreased hemolysis) and *agr*-independent (represented by decreased *spa* transcription) pathways. Whether these effects involve Spx is not clear (Frees *et al*., 2003). Examining the interconnectedness of virulence gene regulatory circuits involving ClpXP, *agr*, and SarA and its homologs, Frees *et al*., (2005) established an absolute requirement of ClpX for *spa* and *sspA* transcription. They also observed repression of *clfB* and *fnbA* by both ClpX and ClpP, although they could not conclude that virulence gene expression was systematically controlled by ClpXP. Instead they proposed that ClpXP affects virulence through molecular chaperone activity of other regulators, such as Rot (Frees *et al*., 2005). Based on the work of the Zuber laboratory, Spx would surely be another candidate.

The same laboratory constructed a *spx* deletion strain of 8325-4, and examined changes in growth and development, stress response, and biofilm formation compared to the *clp* mutants and wild-type. Like *B. subtilis*, *S. aureus* lacking *spx* is hypersensitive to diamide; unlike *B. subtilis*, *S. aureus* lacking *spx* is deficient in biosynthesis and more sensitive to other stresses like hydrogen peroxide, sub- and superoptimal temperatures, and increased osmotic pressure. Although *S. aureus* Spx accumulates in a *clp* background, indicating it is likely a substrate for ClpXP, transcription seems to be autoregulated in *S. aureus*, and induction of stress response by Spx is not accompanied by increased *spx* transcription (Pamp *et al*., 2006) as Peter Zuber's group found in *B. subtilis* (Nakano *et al*., 2003a). Spx in *S. aureus* appears to be an important housekeeping gene, unlike Spx in *B. subtilis*; this may be because *trxB* is essential in *S. aureus* even under non-oxidizing conditions (Uziel *et al*., 2004), and its two promoters are both

dependent on Spx and σ^A (Pamp *et al*. 2006). In *B. subtilis*, *trxB* transcription is activated by either σ^B or σ^A and Spx (Nakano *et al*., 2005). Most interestingly, the *spx* mutant had greatly enhanced ability to form a biofilm compared to wild-type, even without biofilm-promoting additives to the media. The researchers concluded that Spx negatively impacts biofilm production by modulating the expression of *icaR*, a repressor of the *ica* operon, which encodes gene products necessary for the synthesis of polysaccharide intercellular adhesin (PIA) (Cramton *et al*., 1999). It is fairly obvious, even from this rather preliminary study, that regulatory events by Spx from *S. aureus* are not identical to those by Spx from *B. subtilis*. This may be due, at least in part, to its role within the complex layer of regulatory circuits promoting virulence in *S. aureus*.

In this study, we have cloned *spx* from *S. aureus* laboratory strain RN6390, overexpressed it in *E. coli*, and purified the recombinant protein. We also isolated Spx in the form of inclusion bodies, and used these for production of polyclonal antibodies in rabbits for western analysis of purified Spx and expression of Spx in whole-cell lysates of RN6390. We have partially purified the RNAP holoenzyme from RN6390, and created an *S. aureus in vitro* transcription (IVT) system for analysis of Spx-dependent activation of *trxB*. These experiments were also performed in a *B. subtilis* IVT system previously described (Liu and Zuber, 2000; Nakano *et al*., 2003b; Nakano *et al*., 2005). *S. aureus* Spx was highly active in the *B. subtilis* IVT system, stimulating transcription of *trxB* in the absence of a reducing agent. Although active RNAP was extracted from *S. aureus*, the preparation had a high level of contaminating proteins, which may have hindered Spx activity, as Spx-dependent initiation of *trxB* transcription could not be demonstrated in the *S. aureus* IVT system.

Materials and Methods

Bacterial strains and growth conditions:

Escherichia coli strain One Shot TOPO 10F' (Invitrogen) was used for TA-cloning of the *spx* gene amplified from *S. aureus* RN6390 genomic DNA. *E. coli* strain XL1-Blue (Stratagene) was used for mutagenesis reactions. *E. coli* strain BL21 (DE3) pLysS (Novagen) was used for expression of recombinant Spx. *E. coli* strain DH5α was used for all other sub-cloning reactions and storage of all plasmids. All *E. coli* strains except BL21 (DE3) pLysS were grown in Luria-Bertani (LB) media at 37 °C with shaking at 225 rpm. For soluble expression of Spx in BL21 (DE3) pLys, cells were grown in Terrific Broth supplemented with 1 M D-sorbitol and 10 mM betaine HCl at 25 °C while shaking at 150 rpm. For expression of Spx in inclusion bodies for antibody production, BL21 (DE3) pLys cells were grown as other *E. coli* strains. For selection of transformants with TOPO 10F' and pET9a vectors, kanamycin (Km) was added to the media at a concentration of 25 μg mL^{-1}. For selection of pLysS in BL21 (DE3) pLysS, chloramphenicol (Cm) was added to a concentration of 30 μg mL^{-}1. For selection of transformants with pLL28 in *E. coli*, spectinomycin (Sm) was added to the media at a concentration of 50 μg mL^{-1}. *S. aureus* strain RN6390 was used for partial purification of RNA polymerase and extraction of genomic DNA. *S. aureus* cells were grown in brain-heart infusion (BHI) (Bacto) or tryptic soy broth (TSB) (Bacto) at 37 °C with shaking at 225 rpm and without antibiotic selection. All strains were stored at -80 °C in growth media with dimethylsufoxide (DMSO) added to a final concentration of 7 % and antibiotics where necessary.

Purification of genomic DNA:

2 mL of an overnight culture of *S. aureus* strain RN6390 was pelleted at 6000 g for 5 minutes and resuspended in 500 μL cold P1 buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA) with 100 μg

RNase A, 200 μg Proteinase K, and 20 μg lysostaphin. The lysate was incubated at 37 °C until

the solution became clear. Phenol extractions were performed with 500 μL phenol equilibrated

to a pH of 8.0 until the interface between organic and aqueous layers was clear. Phenol was

removed from solution by chloroform extractions performed likewise. For all extractions, the

solution was centrifuged for 5 minutes at 15,000 rpm, and the organic layer was discarded. DNA

was subsequently precipitated with 1/10 volume 3 M sodium acetate, pH 5.2, and ¾ volume neat

isopropanol, and the solution was centrifuged for 20 minutes at 15,000 rpm, 4 °C. The pellet

was washed once with 1 mL ice-cold 70 % ethanol, centrifuged for 5 minutes at 15,000 rpm,

4 °C, and dried in a 37 °C incubator. DNA was dissolved in 100 μL EB buffer (10 mM Tris-Cl,

pH 8.5) overnight at room temperature.

Preparation of plasmid DNA:

Plasmid DNA was purified with the Qiagen miniprep and midiprep kits (Qiagen, Inc.) according

to manufacturer's instructions.

Construction of Spx expression vector:

The Spx coding region was amplified from *S. aureus* genomic DNA by PCR using primers

oJKM1 and oJKM2, which incorporated Nde I and Bam H1 restriction sites, respectively. The

PCR product was visualized by agarose gel electrophoresis to verify that a product of the correct

size (approximately 400 bp) had been amplified (results not shown).   The PCR product was TA-

cloned into pCR 2.1-TOPO vector (Invitrogen) according to manufacturer's instructions to make

pJKM10. The Spx coding region was excised from the vector by sequential restriction

digestions with Nde I and Bam H1 enzymes (New England BioLabs) followed by agarose gel

electrophoresis. The band corresponding to Spx was excised from the gel and purified with the

QIAEX II kit (Qiagen) according to manufacturer's instructions. Purified insert was ligated into

pET9a expression vector (Novagen) cut with the same enzymes with 1 μL T4 DNA ligase (400 U μL^{-1}) (New England BioLabs) to make pJKM11. The correct nucleotide sequence of the recombinant gene was confirmed by DNA sequencing (University of Arkansas for Medical Sciences, Little Rock, AR).

Overexpression and purification of Spx:

Expression host BL21 (DE3) pLysS was transformed with pJKM11 and grown up in 5 mL LB-Km-Cm media overnight at 37 °C. The entire overnight culture was then used to inoculate 500 mL Terrific Broth containing 1 M D-sorbitol and 10 mM betaine and appropriate antibiotics. Cells were grown in 2 L baffled polycarbonate flasks at 25 °C with shaking until the cell density reached an OD_{600} of 0.4. Spx overexpression was induced by adding 1 mM isopropy-β-D-thiogalactopyranoside (IPTG), and the cells were grown an additional 24 hours. Cells were harvested by centrifugation for 10 minutes at 5000 rpm, 4 °C, and pellets were frozen at -20 ºC. Cells were thawed in 5 mL ice-cold MDJ-50 buffer (50 mM Tris-Cl pH 6.9, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) per gram of wet cell paste on ice. Cell lysis was achieved by the release of endogenous lysozyme encoded within the pLysS plasmid carried by the expression host. Chromosomal DNA was sheared by sonication at 30 % power in 15 second intervals until the lysate lost viscosity. The lysate was cleared by centrifugation for 20 minutes at 14,000 rpm, 4 °C. The supernatant was brought to 55 % of saturation with solid ammonium sulfate at a rate of 5 g hr^{-1} while stirring in 4 °C with an additional hour of stirring, and centrifuged at 10,000 rpm for 10 minutes, 4 °C. The insoluble fraction was discarded, and the supernatant was brought to 70 % of saturation the same way. This precipitate was resuspended in 8.5 mL cold MDJ-50 buffer, centrifuged again to remove insoluble particulates, and desalted into 12 mL of the same buffer with an Econo-Pac 10 DG

chromatography column (BioRad) in three-3 mL batches according to manufacturer's instructions. The desalted solution (12 mL) was applied to a Macro-Prep High S exchange support column (BioRad). The column was washed first with MDJ-50 buffer until the OD_{280} of the wash fell below 0.02, and then washed with MDJ-100 buffer (same buffer with 100 mM NaCl) the same way. Spx was eluted from the column with MDJ-200 buffer and the column was cleared with 15 mL MDJ-1000 buffer. 10 µL of each fraction was mixed with an equal volume of sample buffer, boiled at 95 °C for 5 minutes, and analyzed by 15 % Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with Coomassie Brilliant Blue R-250 (**Figure 1**). The Spx-containing fraction was desalted on an Econo-Pac 10 DG chromatography column into MDJ-50 buffer, and stored in small aliquots at -20 °C with glycerol added to a final concentration of 10 %. The purity of Spx was estimated to be over 99 %, with an average concentration of 0.5 – 1 mg mL^{-1}, as determined via the Coomassie assay (Pierce) against bovine serum albumin (BSA) standards and spectrophotometric measurement at 280 nm using an extinction co-efficient of 10,870. The identity of the protein was verified by western blot analysis and automated Edman degradation of the NH_2-terminal ten amino acids (University of Louisiana Health and Sciences Core Labs, New Orleans, La).

Polyclonal anti-Spx antibody production and western blots:

Recombinant Spx was expressed as described above except that cells were grown in 500 mL LB media with antibiotics at 37 ºC, and induced with 1 mM IPTG for 3 hours.   When cells were cultured this way, Spx was found in the insoluble fraction of the lysate, likely in inclusion bodies. An inclusion body isolation was performed for antibody production. Cells were harvested by centrifugation at 5000 rpm for 10 minutes, 4 °C, and frozen at -20 °C. The pellet was resuspended in lysis buffer (50 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT) at

room temperature for 20 min, and centrifuged at 5000 rpm for 10 minutes, 4 °C. The

spheroplasts were resuspended in 10 mL of the same lysis buffer with 0.1 % sodium

deoxycholate and incubated on ice. 8 mM $MgCl_2$ and 100 μg DNase I were added at 4 ℃. The

inclusion bodies were removed by centrifugation, and washed twice with 10 mL lysis buffer plus

1 % Triton-X. The resultant pellet was dissolved in 2.5 mL of Tricine sample buffer, and boiled

at 95 ℃ for 10 min. The entire sample was loaded onto a 15 % Tricine SDS-polyacrylamide gel,

and resolved by electrophoresis. A vertical strip of the gel was stained with Coomassie Brilliant

Blue R-250 and aligned with the gel to reveal the location of Spx, which was excised from the

gel, and sent to Sigma Aldrich for injection into each of two rabbits. The rabbits were injected

with the homogenate three times at two-week intervals, and given an additional booster five days

prior to the final bleed. The serum from the rabbits was used as the primary antibody in

immunoblotting with no further modifications. Purified Spx was resolved by SDS-PAGE, and

transferred onto a polyvinylidene difluoride membrane (PVDF) overnight at 20 V in 4 °C in

Towbin solution (25 mM Tris, 192 mM glycine, 20 % methanol). After blocking non-specific

binding with a 10 % powdered milk phosphate-buffered saline solution with 0.05 % Tween-20

(PBST), the membrane was incubated with anti-Spx primary antibody diluted 1:5000 in PBST

containing 1 % powdered milk. Secondary antibody was goat anti-rabbit linked with horseradish

peroxidase (Amersham) diluted 1:100,000. Antibodies were detected with substrates from the

ECL kit (Amersham) used according to manufacturer's instructions, and scanned on the

Amersham Biosciences Storm 840 scanner (**Figure 2**).

Site-directed mutagenesis of Spx:

pJKM11 was used as the template for PCR with the Quick Change site-directed mutagenesis kit

(Stratagene). oJKM3 and oJKM4 introduced a C10A mutation in pJKM11 to make pJKM12.

oJKM5 and oJKM6 created a C13A mutation to make pJKM13. oJKM7 and oJKM8 mutated

both residues, resulting in a C10/13A change in pJKM14. All reactions were performed

according to manufacturer's instructions for one amino acid change reactions (16 PCR cycles),

followed by Dpn I digestion of methylated parental template. Reactions were subsequently

purified with the Qiagen PCR Clean-Up kit (Qiagen, Inc.), and used to transform *E. coli* XL1-

Blue supercompetent cells (Stratagene). Nucleotide changes were verified by DNA sequencing.

BL21 (DE3) pLysS expression host was transformed by mutant vectors, and expression of

mutant alleles of Spx was attempted using the same protocol as for wild-type Spx (**Figure 3**).

Partial purification of RNA polymerase holoenzyme from *S. aureus* and western blots:

4 mL of an overnight culture of RN6390 grown in BHI were used to inoculate 4 x 1 L BHI broth

pre-warmed to 37 °C, which was grown at 37 °C on 225 rpm until cells reached late log phase

(OD_{560} = 1.2). Cells were harvested by centrifugation at 3500 rpm for 10 minutes, 4 °C, and

frozen at -80 °C overnight. Cells were thawed on ice in 30 mL cold lysis buffer (50 mM Tris pH

7.8, 100 mM NaCl, 5 mM $MgCl_2$, 20 % glycerol, 5 mM β-mercaptoethanol) for 30 minutes, and

resuspended by vortexing. PMSF was added to a final concentration of 1 mM, and cells were

lysed by passing through a French pressure cell press three times at 10,000 psi. The lysate was

cleared by centrifugation at 14,000 rpm for 20 minutes, 4 °C. The cleared lysate was brought to

30 % of saturation with ammonium sulfate, and the resultant pellet was brought to 60 % of

saturation, as stated previously. This pellet was resuspended in 9 mL lysis buffer and dialyzed

overnight in 1 L lysis buffer in 4 °C using a Slide-A-Lyzer dialysis cassette with 7,000 MWCO

(Pierce.) The dialyzed sample was centrifuged at 14,000 rpm for 10 minutes, 4 °C to clear it of

insoluble particulates, and loaded onto a heparin-Sepharose column at the rate of 1 mL min^{-1}.

The column was washed with at least 50 mL of lysis buffer, and proteins were eluted with a 90

mL linear gradient of lysis buffer containing 100 mM – 1 M NaCl. Column fractions were analyzed for the presence of β and β' subunits of RNA polymerase by 12 % SDS-PAGE and peak fractions were combined. The molarity of NaCl in pooled fractions was calculated, and the solution was diluted to 100 mM NaCl with lysis buffer containing no salt. The diluted solution was applied to an Econo-Pac High Q Strong Anion exchange column (BioRad) at a rate of 2 mL min^{-1}. Proteins were eluted with a linear gradient performed and analyzed as before. Peak fractions were combined and concentrated in a Centricon Y-10 (Ambion) at 5000 rpm, 4 °C until volume ≤ 50 µL. Buffer exchange was performed in the concentrator with 1 mL storage buffer (10 mM Tris pH 8.0, 10 mM MgCl$_2$, 100 mM KCl, 0.1 mM EDTA, 20 % glycerol) and concentrated to about 50 µL. Neat glycerol was added to a final concentration of 50 %, and the enzyme was stored at -20 °C. Protein concentration was determined via the Coomassie assay (BioRad) according to manufacturer's instructions. Western analysis was performed on concentrated heparin fractions and dialyzed ammonium sulfate pellet with polyclonal antibody raised against purified α subunit from *B. subtilis* (results not shown) and on concentrated High-Q fractions with anti-Spx from *S. aureus* generated as described previously (**Figure 5**). Anti-α was diluted 1:1000, and secondary antibody, goat anti-rabbit alkaline phosphatase (BioRad), was used at a dilution of 1:30,000. Anti-Spx was diluted 1:2000. Reactions were developed in alkaline phosphatase (AP) color development solution, and AP conjugate substrate was added according to manufacturer's instructions (BioRad).

In vitro transcription of *rpsD* and *trxB*:

Linear DNA templates for *in vitro* transcription (IVT) were generated by PCR from RN6390 genomic DNA. oJKM9 and oJKM10 amplified a 305 bp fragment including promoters for *rpsD*, which encodes a 129 nt major run-off transcript and a 93 nt minor transcript. A 228 bp fragment

with *trxB* promoter elements amplified by oJKM10 and oJKM11 encodes a 91 nt run-off

transcript (Uziel *et al*., 2004). IVT was performed as previously described (Nakano *et al*., 2005).

Briefly, IVT reactions (20 µL) using *B. subtilis* RNAP contained 10 mM Tris pH 8.0, 50 mM

NaCl, 5 mM MgCl$_2$, 50 µg mL^{-1} BSA, 50 nM Spx-free RNAP, 100 nM purified *B. subtilis* σ^A,

and 20 nM *rpsD* or 10 nM *trxB* in diethylpyrocarbonate (DEPC) water. Reactions catalyzed by

RNAP from *S. aureus* (approximately 50 nM) were the same but excluded σ^A. Reactions were

incubated in the presence or absence of 300 nM Spx from *S. aureus* or *B. subtilis* and/or 5 mM

DTT for five minutes at room temperature to allow open-complex formation. A ribonucleotide

cocktail (200 µM each GTP, ATP, and CTP, 250 µM UTP, and 5 µCi [α-^{32}P]UTP) was added to

the reaction, which was incubated at 37 °C to initiate transcription. After 20 minutes, the

reactions were precipitated with 2.5 volumes of ice-cold absolute ethanol and 1/10 volume of 5

M ammonium acetate at **-**20 °C for 30 minutes, and then centrifuged at 14,000 rpm for 20

minutes. Precipitated pellets were resuspended in 5 µL formamide loading dye, boiled at 90 °C

for three minutes, and resolved by denaturing electrophoresis through a 7.6 M urea 8 %

polyacrylamide gel at 550 V. Transcripts were visualized by storage phosphorimaging and

scanned on a phosphorimager (BioRad).

Construction of *E. coli*-*S. aureus* shuttle vector to make *spx*::*cat* mutant:

spx locus (*spx* gene with two flanking genes, *mecA* and *trpS*) was amplified by PCR from

RN6390 genomic DNA using oJKM13 and oJKM14. The PCR product of approximately 2.9 kb

was precipitated with ethanol, resolved by agarose gel electrophoresis, and excised from the gel.

DNA was purified from the agarose using the QIAEX II kit, and TA-cloned into pCR 2.1-TOPO

to make pJKM20, which was then used to transform TOPO One Shot competent cells**.** Plasmid

DNA was purified, and the presence of an insert was verified by restriction digest and

sequencing. pJKM20 was used as the template for PCR with oJKM15 and oJKM16, which

amplified *trpS*, incorporating Sac I and Not I restriction sites, and with oJKM17 and oJKM18,

which amplified *mecA*, adding in Bam H1 and Hind III sites. PCR products (the former of 1.6

kb and the latter being 1.3 kb in size) were cloned into pCR 2.1-TOPO as stated above, to make

pJKM21 and pJKM22, respectively. *trpS* was separated from the vector by sequential digestion

with Not I followed by Sac I, and the product was resolved on 0.7 % low melting-point agarose

gel. *E. coli-S. aureus* shuttle vector, pLL28, was digested likewise, and electophoresed

alongside *trpS*. The two bands (*trpS* and linearized pLL28) were excised from the gel, placed in

separate microcentrifuge tubes, heated to 65 °C for ten minutes, and then removed to 37 °C

heating block. 10 µL of each dissolved gel plug was used in a 50 µL ligation reaction, which

included 5 µL 10 X T4 DNA ligase buffer and 25 µL 10 mM Tris, pH 7.6 at 37 °C. 1 µ T4 DNA

ligase was added to the mixture while vortexing. The reaction was incubated overnight at room

temperature, stopped at 65 °C for ten minutes, and removed to a 37 °C heating block. 10µL of

the ligation product, pJKM23, was used to transform 100 µL *E. coli* strain DH5α. Plasmid DNA

was purified from an overnight culture of a transformant by miniprep. *mecA* was sequentially

digested from pCR 2.1-TOPO with Bam H1 followed by Hind III, and ligated into pJKM23 cut

with the same enzymes using the protocol stated above to make pJKM24. Presence of all inserts

was verified by visualization by gel electrophoresis followed by staining with ethidium bromide

and restriction digest. pJKM30 was made like pJKM21 except that oJKM15 and oJKM16 were

used for PCR, which amplified nucleotides 1-163 from the 5' end of *spx* along with *trpS.*

pJKM31 was made likewise, with PCR of pJKM21 amplifying the 3' end of *spx* encompassing

nucleotides 250-396 along with *mecA*. The insert was digested from pJKM31 and ligated into

pJKM30 to make pJKM32. All restriction enzymes and buffers were purchased from New

England BioLabs.

<div align="center">**Results**</div>

Purification of RN6390 genomic DNA:

Genomic DNA was extracted from *S. aureus* strain RN6390 in order to amplify the gene

encoding Spx to clone into an expression vector and to amplify its flanking genes, *trpS* and *mecA*,

for the purpose of creating a vector to delete chromosomal *spx*. A typical extraction from a 2 mL

overnight culture yielded approximately 20 µg of purified DNA as determined via

spectrophotometric measurement of OD_{260} using an extinction co-efficient of 50 µg mL^{-1}.

Quality was judged by the ratio of OD_{260}:OD_{280}, which was typically around 1.9, and also by an

OD_{220} to OD_{280} spectrophotometric wavelength scan, which showed very low levels of RNA and

protein contamination (results not shown). DNA was also visually inspected by agarose gel

electrophoresis followed by staining with ethidium bromide, and fidelity of DNA was checked

by using it as the template for PCR with oJKM1 and oJKM2, which generated a product of the

correct size (results not shown). Although this yield is rather low, the high quality of DNA

extracted indicates the protocol used was fairly efficient.

Soluble expression and purification of wild-type Spx:

Spx was expressed in recombinant form without any heterologous tags or fusions and purified to

use in IVT of redox genes in order to characterize its transcriptional activity in *S. aureus*. For

overexpression of recombinant *S. aureus* Spx, the highly efficient bacteriophage T7 RNAP

encoded within the genome of *E. coli* expression host was utilized.   Induction of protein

expression was accomplished by the addition of 1 mM IPTG after cells reached mid-exponential

growth ($OD_{600} = 0.4$), to activate expression of T7 RNAP under control of *lacI***.** Cells were

autolysed by endogenously-expressed lysozyme encoded in the pLysS plasmid carried by the

expression host, which was released by the slow freeze-thaw method. When the expression host

carrying pJKM11 was cultured in LB broth at 37 °C, overexpressed Spx was found in the insoluble fraction of the lysate, likely in inclusion bodies (**Figure 1, Lanes 1-2**). Solubility problems were surmounted by growing the cells under osmotic shock conditions in 1 M D-sorbitol with 10 mM betaine at 25 °C (Blackwell and Horgan, 1991). When cells were grown under these conditions, Spx was almost completely soluble in the cleared lysate (**Figure 1, Lanes 3-4**). Spx was then purified by a two-step ammonium sulfate precipitation followed by ion exchange chromatography. The cleared lysate was brought to 55 % of saturation with ammonium sulfate and pelleted, and this pellet was then brought to 70 % of saturation. This first separation step rid the preparation of approximately 70 % of contaminating proteins. The desalted 70 % pellet was bound to the High S-strong cation exchange chromatography column. At a pH of 6.9, most of the remaining proteins in the *E. coli* lysate were apparently negatively-charged: they did not bind to or bound very weakly to the column, nearly all the contaminants being discarded in the flow-through or initial wash (**Figure 1, lanes 8-9**). The wash with buffer containing 200 mM NaCl eluted Spx of apparent homogeneity, resulting in a single band which migrated to 15 kDa as seen by SDS-PAGE, in agreement with the published molecular weight of Spx (15.4 kDa) (Nakano *et al.*, 2001) (**Figure 1, lane 11**). Some Spx remained bound to the High S column, as evidenced by stripping the column of all remaining bound proteins with buffer containing 1 M NaCl (**Figure 1, lane 12**), but the protocol was still deemed quite efficient. Raising the salt concentration of the elution buffer would increase the likelihood of contamination by proteins that came off in the final wash, as seen by SDS-PAGE. A typical yield of active Spx was 3 – 5 mg liter of culture $^{-1}$. The identity of the purified protein was confirmed by sequencing of the ten NH$_2$-terminal residues via automated Edman degradation
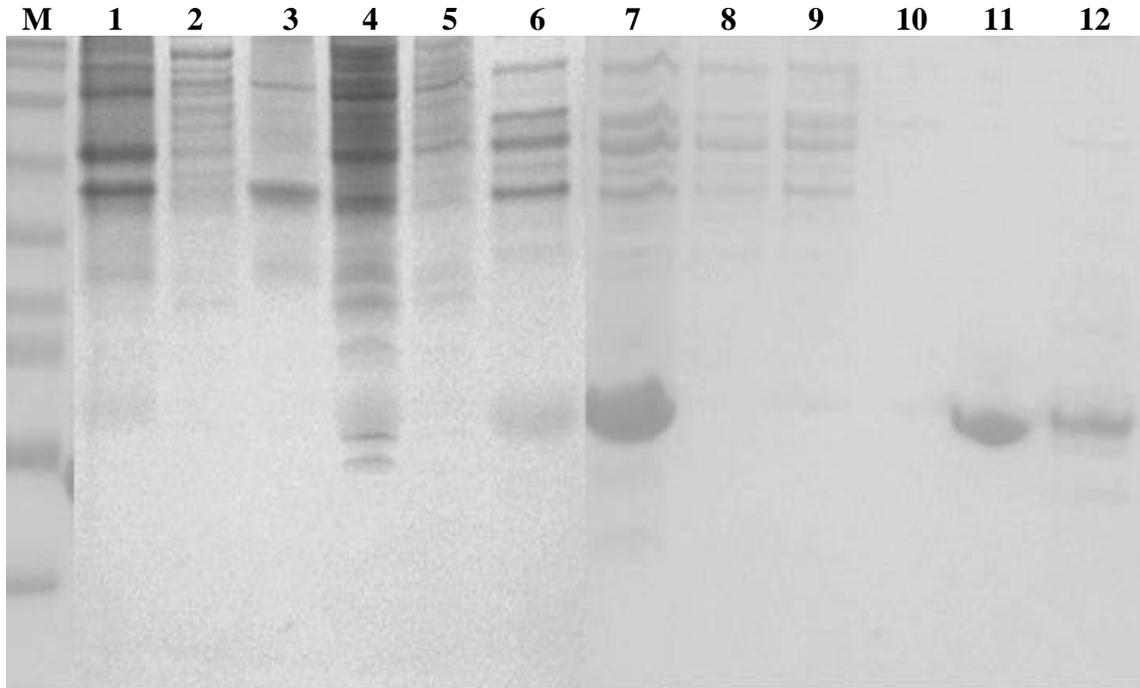
Figure 1: Soluble expression and purification of recombinant Spx

SDS-PAGE: (10 µL of each sample)
Lane M: Full-Range Rainbow molecular weight marker (Amersham); Lane 1: Insoluble fraction of lysate from culture grown in LB at 37 °C; Lane 2: Soluble supernatant of same culture; Lane 3: Insoluble fraction of lysate from culture grown in Terrific Broth with 1 M D-sorbitol and 10 mM betaine at 25 °C; Lane 4: Soluble supernatant of same culture; Lane 5: 55 % ammonium sulfate precipitate; Lane 6: 70 % ammonium sulfate precipitate; Lane 7: Desalted 70 % ammonium sulfate precipitate; Lane 8: High-S column flow-through; Lane 9: High-S 50 mM NaCl wash; Lane 10: High-S 100 mM NaCl wash; Lane 11: High-S 200 mM NaCl wash; Lane 12: High-S 1 M NaCl elution buffer

(data not shown) and immunoblotting with polyclonal antibodies raised against purified Spx (**Figure 2**). Spx is 131 amino acids in length, and has a predicted isoelectric point (pI) of 6.13.

Expression of mutant alleles of Spx:

To better elucidate the mechanism by which Spx activates transcription of redox genes, we mutated the CXXC region within Spx which comprises the redox domain essential for its activation activity, mutating each cysteine residue both singly and concertedly. The Quick Change site-directed mutagenesis kit (Stratagene) was used to mutate the *spx* coding region in pJKM11 to construct pJKM12, pJKM13, and pJKM14. Each or both of the two cysteine residues in Spx was changed to an alanine (C10A in pJKM12, C13A in pJKM13, and C10/13A in pJKM14). Each of the pET9a constructs expressing mutant alleles of Spx was used to transform expression host, BL21 (DE3) pLysS. Expression of the mutant Spx alleles was attempted using the same protocol as for wild-type Spx: slow growth at room temperature in osmotic shock conditions (1 M D-sorbitol and 10 mM betaine) (Blackwell and Horgan, 1999) followed by induction of protein expression with 1 mM IPTG. The frozen cell pellets were autolysed and sonicated as stated for wild-type Spx. The lysate was cleared by centrifugation, and aliquots of the soluble supernatant and insoluble pellet were analyzed by 15 % Tricine SDS-PAGE (**Figure 3**). All three mutant alleles of Spx were expressed entirely in insoluble form, likely in inclusion bodies. Repeated attempts to express the mutant proteins did not result in soluble expression of any of the C to A mutants.

Partial purification of RNA polymerase holoenzyme from *S. aureus*:

To catalyze Spx-dependent IVT of redox genes, crude preparation of DNA-dependent RNA polymerase (RNAP) was made from wild-type *S. aureus* RN6390 cell cultures. Due to its magnitude in size (437 kDa) (Deora and Misra, 1995) and number of subunits (five), RNA

37
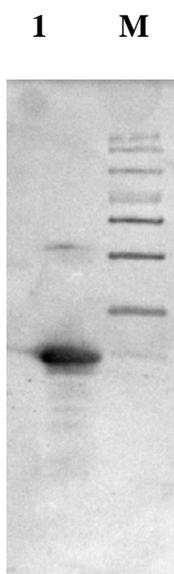
Figure 2: Western blot of purified Spx with anti-Spx antibody

Lane 1: 1 µg purified Spx   Lane M: Magic-Mark molecular weight marker (Invitrogen)
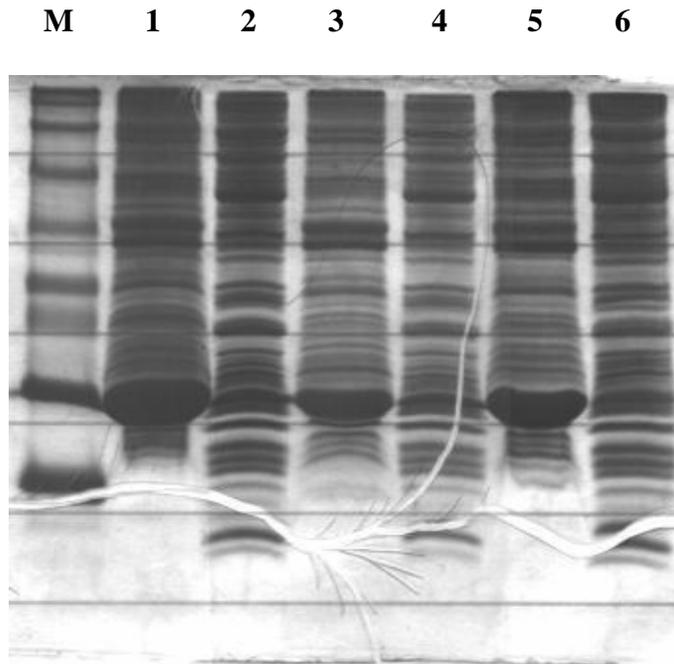
Figure 3: Expression of mutant alleles of Spx was insoluble

SDS-PAGE: (15 μL of each sample)
Lane M: Full Range Rainbow molecular weight marker (Amersham); Lane 1: Insoluble fraction of lysate from cells expressing C10A allele of Spx; Lane 2: Soluble supernatant of same culture; Lane 3: Insoluble fraction of lysate from cells expressing C13A allele of Spx; Lane 4: Soluble supernatant of same culture; Lane 5: Insoluble fraction of lysate from cells expressing C10/13A double mutant allele of Spx; Lane 6: Soluble supernatant of same culture

polymerase cannot be easily overexpressed in recombinant form using the T7 polymerase system in *E. coli*, as was employed for Spx overexpression. There was also no readily available assay for RNAP activity with which to identify purified fractions of polymerase, although one has been published (Deora and Misra, 1995). No antibodies to any of the four different subunits (α, β, β', or σ) were obtained, except one to *B. subtilis* α which proved unreactive in western blots (results not shown). Therefore, visual inspection of SDS polyacrylamide gels for the presence of β and β' subunits from all of the various fractions collected was the only indicator of the efficacy of this highly empirical protocol. *S. aureus* β and β' have been reported to migrate to 151 and 147 kDa, respectively, by SDS-PAGE (Deora and Misra, 1995). As they are among the largest proteins found in the prokaryotic proteasome, they are readily identifiable. *S. aureus* α (42 kDa) and σ (55 kDa) (Deora and Misra, 1995) are not as easily apparent on gels, as more contaminating proteins will be found in this moderately-sized region. α and σ subunits also often show aberrant mobility in SDS-PAGE (Losick *et al.*, 1970; Lowe *et al.*, 1979). Cells were grown until their approximate entry into late-exponential growth (OD_{560} = 1.2) to ensure that protein quantity would be sufficient, but to minimize the likelihood of isolating RNAP with σ^B or other alternate sigma factors associated with stress response (Peter Zuber, personal communication). After harvesting, cell pellets were frozen overnight at -80 °C, and then lysed by passage through a French pressure cell press as stated in Materials and Methods. The first purification step employed was precipitation with ammonium sulfate. The initial cut was to 30 % of saturation (results not shown)**,** and the supernatant was subsequently brought to 60 % of saturation, and centrifuged. The insoluble fraction of the 60 % saturated lysate was used for further purification (**Figure 4, Lane 2**) after dialysis to remove salt. Previously, the soluble supernatant of the 60 % fraction had been brought to 80 % of saturation with ammonium sulfate (results not shown)**.**
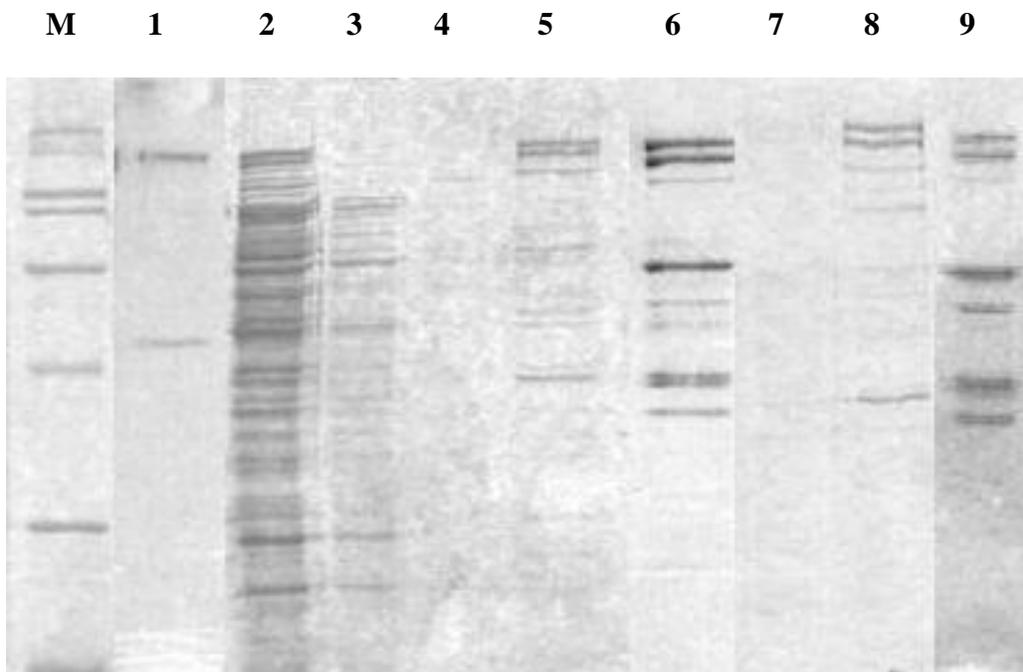
Figure 4: Partial purification of RNA polymerase from *Staphylococcus aureus*

Lane M: Broad-range molecular weight marker (BioRad) Lane 1: Purified 6-His-tagged *B. subtilis* RNAP; Lane 2: 60 % ammonium sulfate precipitate of cleared lysate; Lane 3: Heparin-Sepharose column flow-through; Lane 4: Heparin 100 mM NaCl wash; Lane 5: Heparin combined peak fractions Lane 6: Heparin concentrated peak fractions Lane 7: High-Q column flow-through Lane 8: High-Q combined peak fractions; Lane 9: High-Q concentrated peak fractions

However, visual inspection of electrophoresed ammonium sulfate fractions did not unambiguously reveal purified or enriched β and β' subunits due to the amount of proteins present. As the 60 % precipitate contained most of the protein in the lysate (**Figure 4, Lane 2**), this was the fraction chosen for subsequent purification. The next purification step employed was binding of the dialyzed 60 % precipitate to a heparin-sepharose column, and elution with a 0.1 – 1 M NaCl gradient. The heparin-sepharose resin consists of a weak cation exchanger with an affinity ligand, and is routinely used for RNA polymerase preparations (Deora and Misra, 1995; Babcock *et al.*, 1997), though RNA polymerase from heterologous organisms does not uniformly bind this column (Mani *et al.*, 2006). β and β' subunits were easily seen on SDS polyacrylamide gels, as the heparin column chromatography was a much more effective isolation step than ammonium sulfate precipitation (**Figure 4, Lane 5**). However, the salt concentration of the fractions where RNA polymerase eluted was highly variable over the three attempts undertaken to extract it. In two of the trials, the fractions which eluted at a NaCl concentration of around 0.85 – 0.95 M were chosen (results not shown); in the other trial, the polymerase eluted around 0.35 M NaCl (**Figure 4, Lane 5**). As the latter produced the most homogeneous preparation, this is the one represented in figures. The only published protocol of *S. aureus* RNA polymerase purification reported elution from the heparin-sepharose column at a KCl concentration of 0.4 – 0.55 M, but this protocol did not involve initial precipitation with ammonium sulfate (Deora and Misra, 1995). Likely, dialysis did not result in a uniform starting salt concentration among the three trials, and this led to the wide discrepancy in salt concentrations of elution buffers. However, this was still the most effective purification step, as it rid the preparation of approximately 90 % of contaminating proteins. The final purification step involved binding to the High Q Strong Anion exchanger after dilution of the heparin elutate

to a NaCl concentration of 0.1 M with salt-free lysis buffer. Bound proteins were eluted with a linear gradient of 0.1 – 1 M NaCl as before. Fractions containing the β and β' subunits eluted at NaCl concentrations between 0.45 – 0.6 M, and were combined (**Figure 4, Lane 8).** This step initially seemed to increase the purity of the preparation (**Figure 4,** compare **Lane 5** to **Lane 8**), but comparison of concentrated and combined heparin fractions (**Figure 4, Lane 6**) to the concentrated and combined Q fractions (**Figure 4, Lane 9**) shows that the Q column chromatography only rid the preparation of an estimated 10 - 20 % of contaminating proteins. Moreover, the Q column, which is highly-positively charged, binds DNA that co-purifies with RNA polymerase through the heparin column. This benefit, however, is frequently counterbalanced by the unwanted dissociation of σ factors (Peter Zuber, personal communication). The combined fractions from the Q column were concentrated and stored at -20 °C in a buffer containing 50 % glycerol, which prevents dissociation of σ from the core enzyme (Burgess and Jendrisak, 1975). The storage buffer also contained 10 mM $MgCl_2$. This is within the optimal Mg^{2+} concentration for activity of *B. subtilis* RNAP (8 – 12 mM) (Avila *et al.*, 1971), although *S. aureus* RNAP has been reported to have maximal activity at Mg^{2+} concentration between 10 – 30 mM (Deora and Misra, 1995). Overall, the RNA polymerase preparation was very successful in light of the short time available to optimize the protocol. Active enzyme was isolated, as evidenced by successful IVT of the *rspD* gene, but the protocol was very long, reproducibility was poor, and the yield was quite low. Concentration of RNA polymerase was determined via the Bradford assay, although non-RNAP proteins may constitute up to 50 % of the least pure preparation. The preparation of highest fidelity was shown to be quite dilute, with a concentration of approximately 0.3 μM (**Figure 4, Lane 9**). A four liter culture only yielded enough enzyme for 22 IVT reactions from this preparation. A second

preparation from a six liter culture was much more concentrated (6.2 μM), and produced enough

RNAP to perform over 250 IVT reactions, but with an almost proportionate loss of purity (results

not shown).

Western analysis of partially purified *S. aureus* RNA polymerase:

To ascertain if RNAP had in fact been isolated from RN6390, western detection of the α subunit

was performed on the polymerase preparation. A western blot was performed on an aliquot of

the combined and concentrated heparin fractions and an aliquot of the dialyzed 60 % ammonium

sulfate pellet from the purest *S. aureus* RNAP preparation (results not shown). The primary

antibody used was a polyclonal antibody raised against the α subunit of *B. subtilis* RNAP (a gift

from Peter Zuber). The serum was from the first bleed of the inoculated rabbit, and had never

been tested for activity. Concentrated 6-His-tagged *B. subtilis* RNAP (a gift from Peter Zuber)

was used as a positive control. Although the primary antibody did react with the purified RNAP

from *B. subtilis* at the expected molecular weight as judged by comparison with the molecular

weight standards, the resultant band was very weak, and did not appear for nearly one hour after

the addition of AP substrate and conjugate. Three bands appeared in the lane corresponding to

the heparin fractions from the *S. aureus* RNAP preparation but none migrated to the expected

weight for the α subunit of *S. aureus* RNAP (42 kDa) (Deora and Misra, 1995). Multiple bands

appeared in the lane corresponding to the dialyzed 60 % ammonium sulfate pellet, and it was

futile to predict if any of them might represent a true reaction with the primary antibody. More

likely, these are the result of a cross-reaction with the goat anti-rabbit secondary antibody with

various antigens from *S. aureus*, as the ammonium sulfate precipitate contained most of the

proteins found in the *S. aureus* lysate. Therefore, it proved impossible to predict from this

experiment if RNA polymerase had in fact been extracted. Another western blot was performed

on the concentrated High-Q fractions from the purest *S. aureus* RNAP preparation (**Figure 5**)

using a polyclonal antibody raised against purified *S. aureus* Spx to determine if Spx co-purifies

with *S. aureus* RNAP as it seems to in *B. subtilis* (Peter Zuber, personal communication).

Purified Spx from *B. subtilis* (a gift of Peter Zuber) (**Figure 5, Lane 1**) and *S. aureus* (**Figure 5,**

Lane 4) were loaded as positive controls, and both reacted with the primary antibody, producing

a single band at the expected molecular weight (15.4 kDa). Spx-free 6-His-tagged *B. subtilis*

RNAP was loaded as a negative control, and no reaction with anti-Spx antibody was observed

(**Figure 5, Lane 2**). The concentrated High-Q fractions from the preparation of RNAP from *S.*

aureus (**Figure 5, Lane 3**) did not react with the antibody against Spx, indicating that Spx did

not co-purify with RNAP, at least according to the limits of detection in this experiment.

***In vitro* transcription of *S. aureus rpsD*:**

The activity of partially purified RNAP from *S. aureus* was tested in a defined *in vitro*

transcription system. The *rpsD* gene encodes ribosomal protein S4, and its transcription has

been shown to be unaffected by either Spx activation or repression (Nakano *et al*., 2003b). It

was therefore used as an indicator of polymerase activity and a negative control for Spx-

dependent transcriptional regulation. The reactions were performed using a protocol optimized

for IVT with *B. subtilis* RNAP (Nakano *et al*., 2005), which differs from published protocols

optimized for IVT with *S. aureus* RNAP (Deora and Misra, 1995; Deora and Misra, 1996). All

reactions performed with partially purified RNAP from *S. aureus* were repeated with Spx-free 6-

His-tagged *B. subtilis* RNAP holoenzyme which was reconstituted from the core enzyme by the

addition of purified *B. subtilis* σ^A (a gift of Peter Zuber). To determine if the *S. aureus* σ^A

subunit was present in the RNAP preparation, one reaction was performed where the *B. subtilis*

σ^A subunit was added. Based on the nucleotide sequence of the region upstream of the *rpsD*
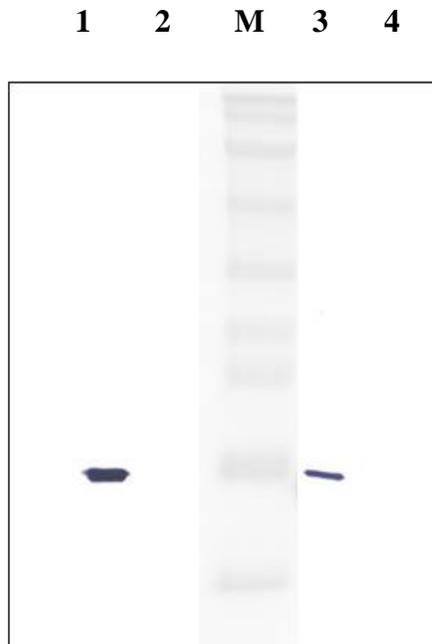
**Figure 5:** *S. aureus* **Spx did not co-purify with RNA polymerase**

gene, a major transcript of 129 nt and a minor transcript of 93 nt were predicted from the 305 bp template. The major transcript was predicted from a perfect match to the strong *B. subtilis* -35 promoter consensus sequence (TTGACA) and a near perfect match (AATAAT) to the -10 promoter consensus sequence (TATAAT). The elements were separated by a 17 bp linker for optimal RNAP recognition (Moran *et al.*, 1982). The minor transcript was predicted from a lesser match to the promoter consensus sequences: the -35 promoter (TGTGAA) and -10 promoter (TATATT) were separated by an 18 bp linker. IVT of the *S. aureus rpsD* gene did result in two distinct products using both *B. subtilis* and *S. aureus* RNAP (**Figure 6**). Sequencing reactions were electrophoresed alongside the IVT reactions to verify the sizes of the transcripts (**Fig. 6, Lanes 1-4?**). As predicted, a very strong band was seen with an approximate size of 130 nt based on comparison to the sequencing reactions, and a weaker band migrated to approximately 90 nt (**Figure 6, Lane 1-4**) when *S. aureus* RNAP was used. The addition of 100 nM *B. subtilis* σ^A to the *S. aureus* RNAP preparation produced bands of greater intensity, but of the same size (results not shown). A reaction catalyzed by the *B. subtilis* RNAP holoenzyme produced the major product, but the minor product was not seen, indicating that the less perfect consensus promoter might not be well recognized by the polymerase from a heterologous organism (Helmann, 1995). The addition of *S. aureus* Spx and/or DTT did not affect transcription of *S. aureus rpsD* (**Figure 6, Lanes 2-4**), as was observed for the *B. subtilis* gene (Nakano *et al.*, 2005).

In vitro transcription of *S. aureus* and *B. subtilis trxB*:

The activation activity of Spx was tested by transcription of *trxB*, which is highly induced both *in vivo* and *in vitro* by Spx, both in *B. subtilis* and *S. aureus*, in a redox-dependent manner (Nakano *et al.*, 2003a; Nakano *et al.*, 2005; Pamp *et al.*, 2006). IVT was performed from the *B.*

	1	2	3	4
Template (20 nM)		*S. aureus rpsD*		
RNAP (50 nM)		*S. aureus*		
S. aureus Spx (300 nM)	---	+	---	+
DTT (5 mM)	---	---	+	+
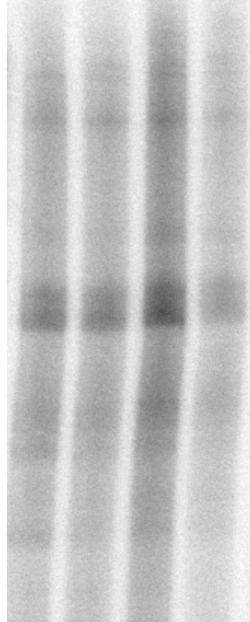
Figure 6: *S. aureus* **RNA polymerase was active in** *in vitro* **transcription of** *rpsD*

subtilis trxB template, which produces a transcript of 88 nt (a gift of Peter Zuber). When the 6-His-tagged *B. subtilis* RNAP holoenzyme catalyzed the IVT reactions, no transcription occurred unless *S. aureus* Spx was included (**Figure 7, Lane 3**). Purified Spx from *B. subtilis* did not induce transcription from the *B. subtilis trxB* template (**Figure 7, Lane 2**), in contrast to previous findings (Nakano *et al*., 2005). This failure may be due to the age of the protein preparation, or repeated freezing and thawing cycles it may have been exposed to. When 5 mM DTT was added to the reactions, no transcription occurred whether Spx was present or not (**Figure 7, Lanes 4-6**). As the *S. aureus trxB* promoter had been mapped previously (Uziel *et al*., 2004), a 91 nt transcript was predicted from the 221 bp template. *B. subtilis* RNAP was able to recognize the *S. aureus* promoters, and produced transcripts of the appropriate size when either *B. subtilis* Spx (**Figure 7, Lane 8**) or *S. aureus* Spx (**Figure 7, Lane 9**) were employed in the reactions, although *S. aureus* Spx proved to be a much stronger inducer. Again, no transcription resulted without the addition of Spx (**Figure 7, Lane 7**) or when DTT was included (**Figure 7, Lanes 10-12**). IVT of *S. aureus trxB* was attempted using partially purified *S. aureus* RNAP. A comparison of the reactions catalyzed by the two different polymerases is presented in Figure 8. As shown in the previous figure, a transcript was only produced by *B. subtilis* RNAP in the presence of *S. aureus* Spx and absence of DTT (**Figure 8, Lane 2**). No obvious bands are seen in the reactions which employed *S. aureus* RNAP even in the reaction which included *S. aureus* Spx and excluded DTT (**Fig. 8, Lane 6**), in which a transcript was expected. Therefore, although active polymerase was extracted from *S. aureus* as shown by the successful transcription of *rpsD*, the preparation was not faithful enough to catalyze Spx- and redox-dependent transcription of *trxB*. This may be due to other transcriptional factors that contaminated the RNAP preparation, or perhaps the dissociation of the σ subunit.

	1	2	3	4	5	6	7	8
Template (10 nM)				*S. aureus trxB*				
RNAP (50 nM)		*B. subtilis*				*S. aureus*		
Spx (300 nM)	---	*S.a.*	---	*S.a.*	---	*S.a.*	---	*S.a.*
DTT (5 mM)	---	---	+	+	---	---	+	+
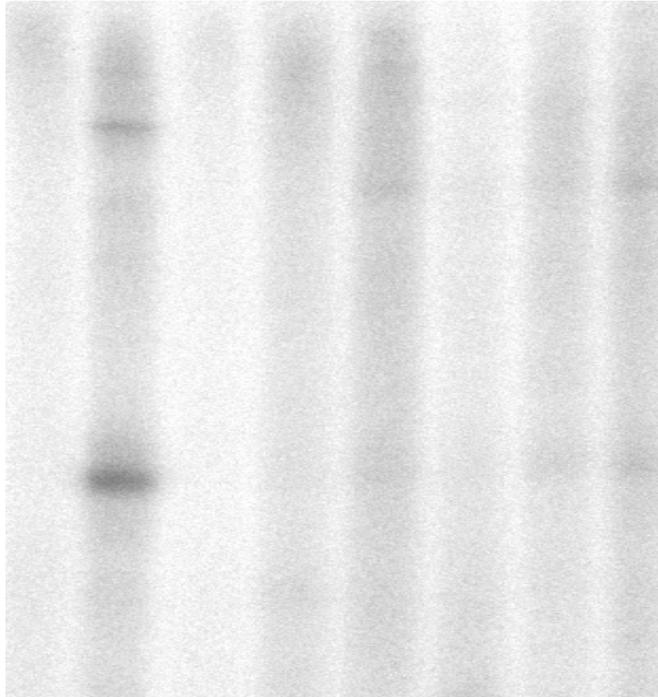
Figure 7: *S. aureus* **Spx activates** *trxB* **in a** *Bacillus subtilis* **transcription system**

	1	2	3	4	5	6	7	8	9	10	11	12
RNAP (50 nM)							*B. subtilis*					
Template (10 nM)				*B. subtilis trxB*			*S. aureus trxB*					
Spx (300 nM)	---	*B.s.*	*S.a.*	---	*B.s.*	*S.a.*	---	*B.s.*	*S.a.*	---	*B.s.*	*S.a.*
5 mM (DTT)	---	---	---	+	+	+	---	---	---	+	+	+
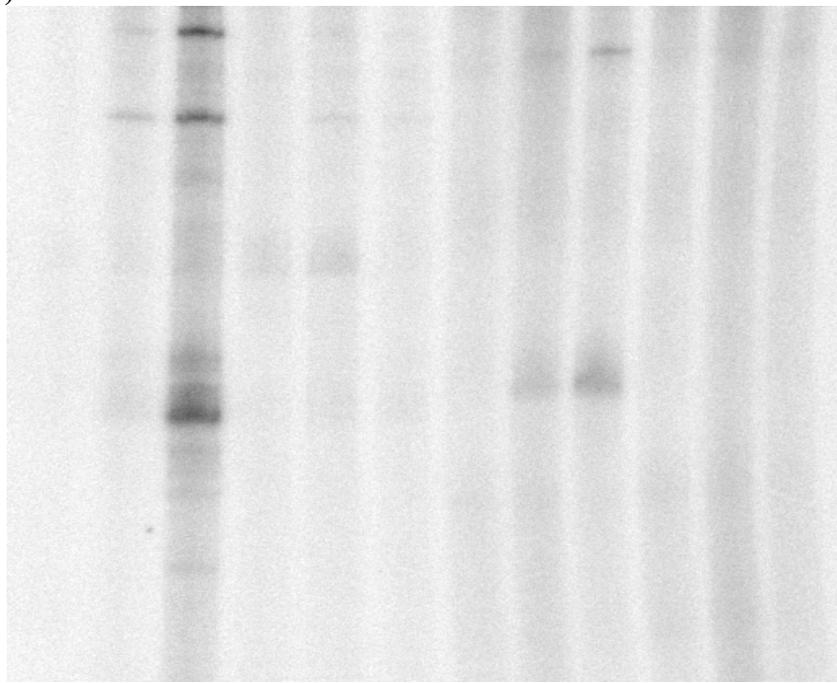
Figure 8: Spx from *S. aureus*, not *B. subtilis*, activated *trxB* transcription

Discussion

We have expressed recombinant, native Spx and purified it to apparent homogeneity using a two-step ammonium sulfate precipitation followed by strong cation exchange chromatography. To our knowledge, this is the first report of purification of Spx from *S. aureus*, and the first purification of native Spx from any organism. Characterization of Spx purified without any tags or heterologous fusions should allow a more thorough characterization of its activity, as some of the preliminary data on Spx may have been compromised by artifacts of the 6-His-tag employed for purification of various proteins. For example, *in vitro* proteolysis of Spx could only be accomplished by ClpCP with an adaptor protein, MecA, due to the loss of the SrrA peptide tag-like recognition sequence on the C-terminus of 6-His-tagged Spx, while a *clpC* mutant showed no concurrent restoration of Spx accumulation (Nakano *et al*., 2002b). Cloning the 6-His-tag onto the N-terminal of Spx permitted *in vitro* proteolysis by ClpXP, in accord with the *in vivo* data (Nakano *et al*., 2003b). The same laboratory also used the inducible, self-cleaving intein tag to purify Spx through a chitin column (Nakano *et al*., 2001). Spx purified by this method was reported to bind to a complex consisting of 6-His-tagged MecA, ClpCP, and ComK in a protein interaction experiment performed using a pull-down assay. Spx was thus initially thought to repress competence by enhancing the sequestration of ComK by ClpCP and MecA (Nakano *et al*., 2002a). However, it was later shown that Spx represses competence by blocking transcriptional activation of *srf* by ComK (Nakano *et al*., 2003b). Moreover, a yeast two-hybrid experiment which searched for proteins that interact with Spx only yielded positive clones bearing *rpoA* (Nakano *et al*., 2003b). It is possible that Spx could have bound the Ni-NTA column used in the pull-down assay; indeed, the researchers reported that Spx purified with the intein tag could bind the Ni-NTA column unless the Spx-expression host was grown in

copper-free media (Nakano *et al*., 2002a). Although the intein tag is completely cleaved after purification (Chong *et al*., 1997), we still believe that the potential for erroneous data posed by these heterologous fusions outweighs the convenience of affinity purification, and that purification of the native protein should always be attempted unless expression of the native gene would prevent growth of the expression host. Thus, the purification of native Spx reported here will be a valuable tool in the further study of this important protein.

We have reported purification of DNA-dependent RNA polymerase from *S. aureus* to approximately 50 % apparent homogeneity. To our knowledge, this is only the second report of purification of native RNAP from *S. aureus*, and no commercial source is available. This preparation was used for IVT from promoters for the *rpsD* and *trxB* genes from *S. aureus*. Although RNAP can be easily purified from *B. subtilis* by overexpressing a 6-His-tagged *rpo* subunit (Babcock *et al*., 1997; Qi and Hulett, 1998), stable transformants in *S. aureus* are not readily obtained due to the general inability of staphylococci to undergo double cross-over recombination (Novick, 1991). Use of native RNAP from *S. aureus* should lead to a better understanding and more thorough characterization of its gene regulation, as RNAP from heterologous organisms might not recognize *S. aureus*-specific promoters. Also, regulation of a certain gene in one organism might not involve the same factors as that of another organism, even if the two are closely related. For instance, transcription of *trxB* in *B. subtilis* is activated by either σ^B alone or σ^A in conjunction with Spx (Nakano *et al*., 2005); in contrast, *trxB* in *S. aureus* is wholly dependent on Spx and σ^A (Pamp *et al*., 2006).

The RNAP preparation described in this report contains contaminating proteins when compared to the 6-His-tagged RNAP from *B. subtilis* upon polyacrylamide electrophoresis. The contaminating species were of moderate size, and may be transcription factors or DNA-binding

accessory proteins. The protocol used to isolate RNAP is expected to purify proteins that bind

DNA, as the heparin column mimics the polarity and negative charges of the phosphate-ribose

backbone of nucleic acids. The protocol employed for RNAP purification is very similar to the

one used to purify recombinant *S. aureus* SarA, a DNA-binding protein (Rechtin *et al.*, 1999).

Both protocols begin with ammonium sulfate precipitation. The precipitate obtained by 60 %

saturation was used for further purification of RNAP, whereas SarA remains soluble in a solution

saturated to 70 % with ammonium sulfate (Rechtin *et al.*, 1999; this report). However, it was not

directly tested if RNAP was completely insoluble in the solution saturated to 60 % with

ammonium sulfate, and some of the holoenzyme might have been discarded in the supernatant.

Likewise, SarA may be present in the 60 % ammonium sulfate precipitate of the *S. aureus* lysate.

Both protocols follow the ammonium sulfate treatment with heparin column chromatography.

Since both proteins bind DNA, they bind to the heparin column, and are eluted with a salt

gradient (Rechtin *et al.*, 1999; this report). The final step in both protocols is anion exchange

column chromatography with the High Q column. The High Q column is strongly positively-

charged, and its purpose is essentially to remove contaminating DNA that may have co-purified

with the DNA-binding species isolated by binding to the heparin column, as the negatively-

charged phosphate-ribose backbone in nucleic acids will bind the positively-charged resin. Due

to the similarity between the two protocols, it is expected that SarA may, to some extent, co-

purify with *S. aureus* RNAP prepared by this method. No band migrated to the molecular weight

of SarA (14.5 kDa) (Cheung and Projan, 1994) as seen by SDS-PAGE analysis (Fig. 4), but the

acrylamide percentage of the gel (12 %) was likely too low to detect a protein of such small size.

Even if a small fraction of cellular SarA co-purifies with *S. aureus* RNAP, its effect on IVT

could be substantial, due to the extremely high copy number of SarA (Fujimoto *et al.*,

unpublished data) and the large proportion of the transcriptome it is thought to regulate (Dunman *et al.*, 2001).

One contaminating protein that was predicted to co-purify with *S. aureus* RNAP is Spx; Spx from *B. subtilis* is found in preparations of RNAP purified via the 6-His-tagged *rpoC* subunit (Peter Zuber, personal communication). The presence of contaminating Spx in the holoenzyme is believed to be due to Spx's binding to α, essentially rendering it an additional subunit of RNAP. Therefore, this group purifies RNAP from a mutant strain of *B. subtilis* that lacks Spx (Peter Zuber, personal communication). However, it seems likely that the binding of Spx to α would be transient, and that the stability of the complex would wane once transcription had been initiated or blocked. The σ subunit, which dissociates from the core enzyme once the elongation stage of transcription begins, is frequently lost during purification of RNAP, and often has to be overexpressed and purified alone, and then added to the core enzyme to reconstitute the holoenzyme (Hager and Burgess, 1980; Deora and Misra, 1996). Another plausible explanation for the presence of Spx in the RNAP preparation is that native Spx can, to some degree, bind the Ni-NTA column used to bind the 6-His-tagged β subunit, as intein-tagged Spx was shown to do (Nakano *et al.*, 2002a). A western blot was performed on the RNAP preparation from *S. aureus* described in this report using antibodies raised against Spx, and no Spx was detected. However, a second preparation of RNAP was not tested for the presence of Spx. When IVT was catalyzed by enzyme from this second preparation, weak transcripts were made from the *trxB* promoter whether or not Spx was included in the reaction (results not shown), prompting speculation that Spx had co-purified with RNAP. Due to time constraints, the experiments could not be repeated to obtain reproducible results. A probable cause of this discrepancy may be the transient nature of Spx's binding to α; depending on the stringency of

this interaction, which itself will rely upon variable conditions such as salt, temperature, and protein concentration, Spx may dissociate from or co-purify with the holoenzyme. Thus, different results could be obtained, which may, in part, explain the inconsistency of the data reported here.

Although the two preparations of RNAP obtained from *S. aureus* lysates had sufficient activity to catalyze IVT of the control gene, *rpsD*, redox- and Spx-dependent transcription of *trxB* was not successful. The possible reasons for this failure are numerous, but the impurity of the holoenzyme probably plays a role in the inefficacy of these experiments. The protocol employed for purification could be modified to include a final step of size-exclusion chromatography, as Deora and Misra (1995) utilized in their published report of *S. aureus* RNAP purification. The authors used a size-exclusion column with a molecular weight cutoff of 200 kDa, which should remove virtually all contaminating species, particularly since DNA-binding proteins tend to be rather small. The authors followed heparin column chromatography with elution through a single-stranded DNA cellulose column (Deora and Misra, 1995). This is expected to further enrich the solution of DNA-binding proteins, and may also aid in discarding double-stranded DNA that co-purified with proteins eluted from the heparin column, as the authors did not make use of the High Q column in this report (Deora and Misra, 1995). A later report from the same laboratory purified *S. aureus* RNAP using a modified protocol that omitted the single-stranded DNA cellulose column, and added ammonium sulfate precipitation and strong anion exchange column chromatography through the High Q column (Deora and Misra, 1996). These changes render the RNAP purification protocol of Deora and Misra (1996) essentially identical to the one in this report, except that they included size-exclusion chromatography as a final step. Since RNAP is one of the largest known prokaryotic proteins,

this step is expected to be quite efficient in polymerase isolation. It is likely that if our protocol were modified to include size-exclusion chromatography, the resultant RNAP preparation would be pure enough to obtain consistent and reproducible data from IVT. We would then expect IVT from the *S. aureus trxB* promoter to be successful if *S. aureus* Spx was included in the reaction without DTT, as we demonstrated using *B. subtilis* RNAP.

Another possible explanation of our inability to show Spx-dependent transcription from *trxB* is lack of the σ^A subunit in the RNAP enzyme preparation, as transcription of *trxB* in *S. aureus* relies on the presence of both σ^A and Spx (Pamp *et al*., 2006). However, we do not believe this to be true, as the *rpsD* gene was successfully transcribed using the same RNAP preparation. When the *S. aureus* holoenzyme was supplemented with *B. subtilis* σ^A, the transcripts obtained from the *rpsD* promoter were of greater intensity, but the banding pattern was identical to that from the reaction which did not include exogenous σ factor, indicating that the holoenzyme had been extracted from *S. aureus*. Adding *B. subtilis* σ^A to the IVT reactions from the *trxB* promoter likely would have complicated the data by adding further variables, and did not seem necessary.

Although the *S. aureus* RNAP preparation was not sufficiently pure to demonstrate Spx-dependent transcription of *trxB*, recombinant *S. aureus* Spx proved to be highly active in IVT using *B. subtilis* RNAP purified via 6-His-tagged *rpoC*. When 5 mM DTT was included in the reactions, *trxB* transcription was inhibited. This finding suggests that, as was shown for *B. subtilis* Spx (Nakano *et al*., 2005), the activation activity of *S. aureus* Spx is dependent on the presence of a disulfide bond between cysteine residues 10 and 13. Addition of DTT can quench transcriptional activation by reducing the disulfide bond to free thiol residues, as occurs with another redox-sensing prokaryotic transcription factor, OxyR (Storz *et al*., 1990). Since the vast

majority of genes that Spx has been shown to positively regulate are involved in thiol homeostasis (Nakano *et al.*, 2003a), it is logical that once a cell previously suffering from oxidative stress had returned to its normal, reduced state, transcriptional activation of target genes by Spx would no longer be necessary or even desirable. The fact that cytoplasmic proteins rarely contain disulfide bonds due to the reducing environment of the cell (Stewart *et al.*, 1998) reinforces this notion that gene regulation by Spx should be a transient reaction to cellular stress, since recovery from oxidative stress will be followed by Spx inactivation.

Spx purified from *B. subtilis* by a post-translationally cleaved intein tag (Nakano *et al.*, 2001) had little or no activity in the experiments reported here using this same *B. subtilis* IVT system to induce transcription from the *trxB* promoters of either *B. subtilis* or *S. aureus*. We do not believe, however, that this failure to demonstrate transcriptional activation of *trxB* with *B. subtilis* Spx is scientifically relevant, as *B. subtilis* Spx purified by this method has been shown to be active in this IVT system, both in regards to repression and activation of target genes, including *trxB*, and other assays as well (Nakano *et al.*, 2003b; Nakano *et al.*, 2005; Zhang *et al.*, 2006). Moreover, *B. subtilis* Spx from the same preparation as was used in the experiments reported here was found to be active at the time these experiments were performed in IVT of other target genes (Dindo Reyes, personal communication). However, the preparation was several years old, and the specific aliquots of protein employed in the experiments reported here may have been subjected to repeated cycles of freezing and thawing, rendering them inactive or less active. Therefore, it is our belief that the failure to show transcriptional activation of *trxB* by *B. subtilis* Spx in this report is either due to experimental error or reduced activity of the Spx preparation.

Since the redox-sensing activity of *B. subtilis* Spx is dependent on two cysteine residues which constitute a thiol-disulfide "switch" (Nakano *et al*., 2005), we attempted to express and purify recombinant *S. aureus* Spx mutants that lack one or both of these residues. However, soluble expression of the three C to A Spx mutants was not achieved. The mutant forms of Spx were expressed insolubly, probably as inclusion bodies, even when using the modified osmotic shock protocol (Blackwell and Horgan, 1991) that permitted soluble expression of wild-type Spx. This raises the possibility that the CXXC motif in Spx is necessary for the translated protein to fold into the proper conformation, since the C to A alleles of *B. subtilis* Spx were soluble when expressed as fusions (Nakano *et al*., 2005), and we attempted to express the mutant proteins without tags or fusions. Having these mutant alleles of Spx for IVT from the *trxB* promoter could elucidate the mechanism by which the addition of DTT represses transcription, as was shown in *B. subtilis* (Nakano *et al*., 2005). A possible solution to the problem presented by the inability to express the Spx mutants in soluble form would be to denature the insoluble protein in urea, and then allow it to refold into the proper conformation. However, this avenue was not explored as there appeared to be no positive marker for activity of these mutant proteins to ensure that *trxB* was not transcribed *in vitro* due to the loss of the redox sensing motif, and not merely because the denatured protein was innately inactive. But it was later shown that the redox sensing motif in *B. subtilis* Spx is inessential for its repression activity (Zhang *et al*., 2006). So it might be possible to denature the insoluble alleles of Spx and test the activity of the refolded protein in IVT of a gene Spx is known to repress, such as *srf* in *B. subtilis* (Nakano *et al*., 2003b).

If repression of such a gene could be demonstrated by these refolded mutant alleles of Spx, then IVT from promoters of genes Spx is thought to activate based on *in vivo* data could be

attempted. One could then form a clearer model of the redox dependence of Spx for transcriptional activation. Many genes in the *S. aureus* transcriptome that Spx appears to regulate do not seem to involve redox processes; therefore, it seems likely that formation of an intramolecular disulfide bond may be only one mechanism by which Spx activates gene expression. For example, an *spx* null mutant of *S. aureus* had enhanced biofilm formation ability, and this was suggested to be the result of Spx upregulating the biofilm repressor gene, *icaR*, apparently independent of oxidation state (Pamp *et al*., 2006). An advantage of IVT is that the oxidation state of the reaction can be controlled by the addition of agents such as DTT and diamide. This control is not easily achieved *in vivo*, where processes are more dynamic and conditions harder to measure. The existence of Spx mutants that lack redox sensing ability but are nevertheless active could facilitate the elucidation of other mechanisms of transcriptional activation by Spx, if they exist.

Another aim of this project that was not brought to fruition was the creation of an *spx* null mutant of *S. aureus*. A plasmid (pJKM24) was constructed that included the genes which flank *spx* on the *S. aureus* chromosome, *trpS* and *mecA*, surrounding *cat*, which encodes chloramphenicol acetyltransferase, making it a marker for chloramphenicol resistance on the staphylococcal integration plasmid, pLL28. It was originally intended that introduction of this plasmid into *S. aureus* laboratory strain RN6390 would result in incorporation of *cat* into the chromosome at the site of *spx* following double cross-over recombination events between the genes flanking *spx* on plasmid and chromosome. However, another laboratory reported this event to be extremely rare (Mark Smeltzer, personal communication), which agrees with the overall consensus that double cross-over recombination is inefficient or nonexistent in staphylococci (Novick, 1991). Another plasmid (pJKM33) was then constructed in the same

way except that the 5' and 3' ends of *spx* flanked the *cat* gene along with *trpS* and *mecA*. This new vector can knock out *spx* on the chromosome by single cross-over recombination events followed by excision of the plasmid (Mark Smeltzer, personal communication). Due to time constraints and the difficulty of transforming staphylococci, the mutant strain of *S. aureus* was not made. However, another laboratory recently published a report of a creation of an *spx* null mutant of 8325-4 (Pamp *et al*., 2006), and has offered to share the clone with our group. This mutant strain could be used in the future to inoculate mice to determine whether Spx contributes, either positively or negatively, to virulence in *S. aureus*. We would also like to create a *sar spx* double mutant and investigate whether these two important global regulators have a synergistic or antagonistic effect upon each other.

Some evidence exists that, at least in regards to biofilm production, Spx might attenuate virulence, and this activity is in opposition to biofilm promotion by SarA. Spx in *S. aureus* was found to repress biofilm formation by upregulating transcription of *icaR* (Pamp *et al*., 2006), a repressor of the *ica* operon, which encodes the enzymes necessary for the synthesis of the polysaccharide intracellular adhesin (PIA) (Cramton *et al*., 1999). An earlier report postulated that SarA promotes biofilm formation by activation of *ica*, and that σ^B might antagonize SarA-dependent biofilm development. The authors speculated that SarA and σ^B were indirectly affecting *ica* expression, and that another regulator was involved, which would repress *ica* in a σ^B-dependent manner or activate it through SarA (Valle *et al*., 2003). Biofilm factor expression is difficult to characterize because so many factors contribute to its regulation: global regulators, cell density, media additives such as glucose and antibiotics, and environmental cues like osmolarity, temperature, and redox. In a global analysis of changes in the *S. aureus* transcriptome in response to hydrogen peroxide, Chang *et al*. (2006) noted a halt in *ica*

transcription. Based on the later report by Pamp *et al*. (2006), Spx may be responsible for this oxidative stress-induced repression of *ica*, by activation of *icaR*. In light of this evidence, Spx appears to be yet another regulator in what is clearly a complex regulatory circuit.

Pamp *et al*. (2006) also noted important differences in the phenotype of an *spx* null mutant of *S. aureus* compared to the phenotype of an *spx* mutant of *B. subtilis* as reported by Peter Zuber's group. Whereas inactivation of the *spx* locus in *B. subtilis* alleviated growth and development deficiencies caused by *clp* mutations (Nakano *et al*., 2001), *spx* mutants of *S. aureus* were obtained with very low frequency, and the colonies exhibited slow and reduced growth (Pamp *et al*., 2006). This is due, at least in part, by the inability of the *spx* mutant to express *trxB*. *trxB* is an essential gene in *S. aureus* even in the absence of oxidative stress (Uziel *et al*., 2004), and both of its promoters rely on Spx in conjunction with σ^A (Pamp *et al*., 2006). In contrast, *trxB* in *B. subtilis* has a σ^B-dependent promoter in addition to the Spx-dependent one. It is only essential for survival under oxidizing conditions, as *B. subtilis*, like *S. aureus*, lacks the glutathione/glutaredoxin system found in *E. coli* (Scharf *et al*., 1998; Uziel *et al*., 2004). The growth defects observed in the *spx* mutant strain of *S. aureus* may also arise from the inactivity of various biosynthetic reductases, as thioredoxin is a hydrogen donor system for these enzymes (Reichard, 1993). Since *S. aureus* has no alternative thiol redox system like glutathione and *trxB* expression depends on Spx, it is expected that many housekeeping functions would be hindered or halted in a strain lacking *spx*. Indeed, it was found that purine, pyrimidine, and amino acid biosynthesis genes and DNA metabolism genes were both positively and negatively affected by deleting *spx* in *S. aureus* (Pamp *et al*., 2006), while these housekeeping genes were only upregulated in *spx* mutants of *B. subtilis* (Nakano *et al*., 2001). Lastly, inactivation of *spx* in *S. aureus* resulted in hypersensitivity to numerous environmental stresses like sub- and

superoptimal temperatures, salt, and hydrogen peroxide, in addition to diamide (Pamp *et al*., 2006), which is the only agent to which *spx* mutants of *B. subtilis* are known to exhibit hypersensitivity (Nakano *et al*., 2005). Based on this evidence it appears that Spx plays a greater role in survival and general stress protection in *S. aureus* than in *B. subtilis*.

In its role as a global regulator of the general stress response in *S. aureus*, Spx may carry out some of the functions that σ^B performs in other organisms where it has been identified. The σ^B regulon of *B. subtilis* contains many of the components necessary for protection of the cell from damage caused by a number of environmental stresses (Hecker *et al*., 1996), and the discovery of a related alternative sigma factor in *S. aureus* (Kullick and Giachino, 1997) led to the assumption that it controlled the general stress response in this close relative of *Bacillus*. But although the responses to heat shock, hydrogen peroxide, and acidic pH are controlled by σ^B in both organisms, responses to ethanol, salt, and osmotic shock are mediated via σ^B in *B. subtilis* (Boylan *et al*., 1993) and independently of it in *S. aureus* (Chan *et al*., 1998). For instance, despite the presence of a σ^B recognition site in the promoter for *katA*, the sole catalase of *S. aureus*, catalase expression and activity was unchanged in the *rsbU*-positive strain, SH1000, compared to its otherwise isogenic parent, RN6390, even in cells experiencing oxidative stress (Horsburgh *et al*., 2002). A global analysis of gene expression in response to diamide in *B. subtilis* found that, although over 20 % of the transcriptome was affected, expression of only a few σ^B–dependent genes was altered (Leichert *et al*., 2003). The transcriptome followed a strikingly similar pattern of induction and repression to that of *clp* mutants in which Spx accumulates (Nakano *et al*., 2003a). Spx could assume the role of σ^B in directing gene expression in response to certain types of stresses, such as disulfide stress, in both organisms. It is conceivable that this regulation may involve an as-yet undiscovered alternative sigma factor.

A novel sigma factor, σ^R, was discovered in *Streptomyces coelicolor* that induces the thioredoxin system to combat oxidizing conditions (Paget *et al*., 1998). Its activity is controlled by a redox-sensing anti-sigma factor, RsrA, by a mechanism analogous to the anti-alpha activity of Spx (Kang *et al*., 1999). Under normal conditions, RsrA binds σ^R, rendering it inactive. When exposed to diamide or hydrogen peroxide, reversible intramolecular disulfide bond formation within RsrA causes dissociation of the complex and transcription of the σ^R regulon. Re-establishment of thiol homeostasis within the cell leads to reduction of RsrA and resumption of σ^R repression, completing the negative feedback regulatory loop (Kang *et al*., 1999).

Repression of target gene activation by Spx has been thoroughly characterized in *B. subtilis*, and there is no evidence that it operates differently in *S. aureus*. The unique mechanism of Spx-dependent repression is to block activator-stimulated activation of RNAP by physically hindering protein-protein interaction (Nakano *et al*., 2003b). The two residues within the α-CTD of RNAP found to be essential for Spx-dependent repression are V260 and Y263, which lie within helix 1 of the α-CTD. Helix 1 is important for both activator-dependent transcriptional activation and the binding of RNAP to UP elements of promoter DNA (Russo and Silhavy, 1992, Ross *et al*., 1993). More evidence supporting this "interference model" exists: Spx binds α at the same residues which are necessary for ComA-dependent activation of *srf* (Zhang *et al*., 2006), and this operon is repressed by Spx both *in vivo* and *in vitro* (Nakano *et al*., 2003b; Zhang *et al*., 2006). What remains unclear is whether oxidation of Spx is required for its repression activity. It is logical to assume that only oxidized Spx should accumulate in the cell, perhaps due to the diversion of the ClpXP proteolytic machinery. The general view of Spx activity is that Spx accumulates at the onset of disulfide stress to repress the expression of general housekeeping functions in order that the cell might devote its resources to combating damage caused by the

stress, while also activating expression of thioredoxin and other genes that restore thiol balance (Zuber, 2004). If this is true, then repression and activation by Spx would occur simultaneously; however, this is hard to test, as the redox motif of Spx has been shown to be unnecessary for transcriptional repression (Zhang *et al.*, 2006), and the affinity of Spx for α is unchanged by oxidation state (Nakano *et al.*, 2005). Another gene Spx represses is *hmp* (Nakano *et al.*, 2003b), which encodes flavohemoglobin, the expression of which is induced under oxygen-limiting conditions to allow anaerobic growth (Nakano *et al.*, 2000b). It would seem appropriate that expression of this gene should be inhibited when oxygen is in excess; however, evidence from *S. aureus* suggests that transient anoxic growth in response to oxidative stress can be beneficial to the cell by limiting additional cytotoxicity caused by ROIs produced during the normal course of oxidative phosphorylation (Chang *et al.*, 2006).

The activation activity of Spx is more mysterious, and a model to explain it does not yet exist. It is not apparent how Spx could activate transcription of specific genes when it has no DNA-binding ability and has not been found to interact with any activator proteins productively (Zuber, 2004). The crystal structure of oxidized *B. subtilis* Spx in complex with α-CTD was solved recently, and the report did suggest a possible mechanism (Newberry *et al.*, 2005). Structural and genetic data indicate that Spx might activate gene expression by facilitating recruitment of the σ subunit to the core enzyme (Newberry *et al.*, 2005; Zhang *et al.*, 2006). Spx was found to interact with residues on α homologous to the "261" determinant of *E. coli* α (Newberry *et al.*, 2005), which is important for its interaction with region 4 of σ^{70}, the housekeeping σ of *E. coli* (Savery *et al.*, 2002). The site of Spx's contact with α was shown to be distal to the CXXC motif, which introduces the possibility that binding of oxidized Spx could

result in a conformational change within the α-CTD, which would then allow for α-σ interaction, thus permitting transcriptional activation to proceed (Newberry *et al.*, 2005).

It is also unknown whether disulfide bond formation between cysteines 10 and 13 is the only mechanism by which transcription is activated by Spx. Newberry *et al.* (2006) compared the crystal structure of Spx-α-CTD to the published structure of *E. coli* ArsC, an Spx homolog, bound to an arsenate ion (Martin *et al.*, 2001). C10 of Spx aligns with the conserved arsenate-ligating cysteine in ArsC, and these cysteines are proximal to a conserved arginine residue in both proteins. The purported role of this residue in both proteins is to lower the pK_a of the reactive cysteine, which would allow for nucleophilic attack of the thiol at physiological pH, favoring either ligation of the arsenate ion (Martin *et al.*, 2001) or oxidative stress sensing (Newberry *et al.*, 2005). In both structures it was reported that a sulfate molecule which originated from the crystallization solution interacted with this arginine residue. The significance of this finding is highlighted by the discovery that when *B. subtilis* is grown with sulfate as its sole sulfur source, Spx regulates expression of genes involved in sulfur assimilation and metabolism (Erwin *et al.*, 2005). This suggests that activation of Spx could be affected by other mechanisms in addition to thiol oxidation, which may be especially relevant when considering environmental sensing by *S. aureus* Spx, which controls the response to more types of stress than does its homolog in *B. subtilis* (Pamp *et al.*, 2006).

Another factor complicating a model of transcriptional control by Spx in *S. aureus* is the global regulator, SarA. This study did not attempt to discover a link between the regulatory pathways of Spx and SarA, but that is a future research directive of our group. SarA does share some of the defining characteristics of Spx. It affects the expression of hundreds of genes as both an activator and a repressor, and is responsive to redox. Unlike Spx, SarA is a DNA-

binding protein. But, although consensus sequences of preferred SarA recognition sites have

been found, SarA also can bind DNA promiscuously (Sterba *et al*., 2003), and may do so *in vivo*

in a non-sequence specific manner. SarA binds AT-rich DNA with high affinity (Sterba *et al*.,

2003). Since UP elements which are bound by α-CTD are comprised of AT-rich sequences,

SarA's repression activity may be to outcompete RNAP for these activation elements, a mode of

repression with obvious analogies to that of Spx. SarA experiences a great increase in activity

upon reduction of its sole cysteine residue, while Spx is activated by oxidation of its cysteine

residues. Since Spx induces the expression of genes involved in thiol reduction, it is possible

that Spx enhances regulation by SarA. Another scenario posits that only one of the two global

regulators would be active at any specific timepoint, depending on growth phase and external

conditions, such as pH and redox. In this manner, SarA and Spx could coordinate virulence gene

regulation and stress response. This might explain why preliminary data shows antagonistic

gene regulation by SarA and Spx in regards to biofilm production (Valle *et al*., 2003; Pamp *et al*.,

2006).

      In order to develop an accurate model of global gene regulation by SarA and Spx, it will

be necessary to construct *S. aureus* strains lacking either and both of these regulators. Although

in vitro experiments can be helpful in elucidating a particular mechanism of activity, the

potential for mistaking artifacts for real data is always present, and hypotheses formulated based

on *in vitro* results should be verified *in vivo*. Most importantly, for the same reasons that

pathogenesis should be studied using clinical, rather than laboratory, strains, examination of

virulence gene expression should not be separated from the stress response. Pathogens produce

virulence factors within the confines of a host, which is concomitantly mounting an immune

response and defense. This defense can involve a number of factors, such as changes in

temperature, pH, redox, and cellular responses like phagocytosis and release of complement and cytokines, any or all of which can antagonize or aid in the offense presented by the pathogen. In essence, it can be postulated that both host and pathogen present an offense and a defense, and each must synchronize its own gene expression to maximize the potential of both in order to survive. If studies of virulence expression by prokaryotes were moved away from the ideal conditions of a laboratory into an environment which resembles one that these organisms actually encounter, perhaps more meaningful discoveries would follow.

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Primers used:

oJKM1: amplify *spx* from RN6390 incorporating Nde I site

5' AGAGTGAGACATATGGTAACATTATTTACT 3'

oJKM2: reverse primer for oJKM1 incorporating Bam H1 site

5' TAGTAAAGTAGGATCCATAGTTAAATGGTT 3'

oJKM3: introduce C10A mutation into Spx

5' TTTACTTCACCAAGTGCTACATCTTGCCGTAAAG 3'

oJKM4: reverse primer for oJKM3

5' CTTTACGGCAAGATGTAGCACTTGGTGAAGTAAA 3'

oJKM5: introduce C13A mutation into Spx

5' CCAAGTTGCACATCTGCTCGTAAAGCGAAAGC 3'

oJKM6: reverse primer for oJKM5

5' GCTTTCGCTTTACGAGCAGATGTGCAACTTGG 3'

oJKM7: introduce C10/13A mutation into Spx

5' CCAAGTGCTACATCTGCTCGTAAAGCGAAAGC 3'

oJKM8: reverse primer for oJKM7

5' CGTTTCGCTTTACGAGCAGATGTAGCACTTGG 3'

oJKM9: amplify *rpsD* fragment with promoter from RN6390 incorporating EcoR1 site

5' GCGAATTCCGGCGTCTTAGATATCGATGCC 3'

oJKM10: reverse primer for oJKM9 incorporating Bam H1 site

5' CGGGATCCGCATGCTTACTAAGATATATTG 3'

oJKM11: amplify *trxB* fragment with promoter from RN6390 incorporating Eco R1 site

5' CCGAATTCCCTCGAAACGTTACAAAGAAGC 3'

oJKM12: reverse primer for oJKM11 with Bam H1 site

5' GCGGATCCCATACCAGCTGGACCTGCACCG 3'

oJKM13: amplify *spx* with flanking genes, *trpsS* and *mecA*, from RN6390

5' GAGGCACTAGTTAATTCT 3'

oJKM14: reverse primer for oJKM13

5' AGACTCCAAAGAGTCTCC 3'

oJKM15: amplify *trpS* from pJKM20 incorporating Sac I site

5' CTTTATTTGAGCTCTTACGTCCTAAACCCATCGC 3'

oJKM16: reverse primer for oJKM13 incorporating Not I site

5' GATTTCATCGCGGCCCGCTTCAGTCATTTT 3'

oJKM17: amplify *mecA* from pJKM20 incorporating Bam H1 site

5' CAAGAGGATCCTGGCTTATTACGTC 3'

oJKM18: reverse primer for oJKM15 incorporating Hind III site

5' AGAGACTCCAAAGCTTCTCCTTTCATAG 3'

Plasmids:

Plasmid	Description	Source
pCR 2.1-TOPO	*E. coli* subcloning vector	Invitrogen
pET9a	Recombinant protein overexpression vector	Novagen
PJKM10	Wild-type Spx coding region in pCR 2.1-TOPO	This study
pJKM11	Wild-type Spx coding region in pET9a	This study
pJKM12	Spx coding region bearing C10A mutation in pET9a	This study
pJKM13	Spx coding region bearing C13A mutation in pET9a	This study
pJKM14	Spx coding region bearing C10/13A mutation in pET9a	This study
pLL28	*E. coli-S. aureus* shuttle vector to replace gene with *cat* marker	Chia Lee
pJKM20	*spx* locus with flanking genes, *trpS* and *mecA,* in pCR 2.1-TOPO	This study
pJKM21	*trpS* in pCR 2.1-TOPO	This study
pJKM22	*mecA* in pCR 2.1-TOPO	This study
pJKM23	*trpS* in pLL28	This study
pJKM24	*trpS*/*mecA* in pLL28	This study
pJKM30	*trpS*/*spx*5' in pCR 2.1-TOPO	This study
pJKM31	*mecA*/*spx*3' in pCR 2.1-TOPO	This study
pJKM32	*trpS*/*spx*5' in pLL28	This study
pJKM33	*trpS*/*spx*5'/*mecA*/*spx*3' in pLL28	This study

Vita

Jane Kathleen McBride was born in Redwood City, California in 1976. She briefly attended the University of California at Berkeley before attending the University of New Orleans, where she received her B.S. in Biological Sciences in 2003. She is currently a Medical Research Specialist at Tulane University Health Sciences Center. She lives in New Orleans, Louisiana with her dog and cat.