Rifampin Resistance *rpoB* Alleles or Multicopy Thioredoxin/Thioredoxin Reductase Suppresses the Lethality of Disruption of the Global Stress Regulator *spx* in *Staphylococcus aureus*

Maite Villanueva,^a Ambre Joussetin,^a Kristoffer T. Baek,^c Julien Prados,^a Diego O. Andrey,^b Adriana Renzoni,^b Hanne Ingmer,^c Dorte Frees,^c William L. Kelley^a

Department of Microbiology and Molecular Medicine, University Hospital and Medical School of Geneva, Geneva, Switzerland^a; Service of Infectious Diseases, University Hospital and Medical School of Geneva, Geneva, Switzerland^b; Department of Veterinary Disease Biology, University of Copenhagen, Frederiksberg, Denmark^c

ABSTRACT

Staphylococcus aureus is capable of causing a remarkable spectrum of disease, ranging from mild skin eruptions to life-threatening infections. The survival and pathogenic potential of *S. aureus* depend partly on its ability to sense and respond to changes in its environment. Spx is a thiol/oxidative stress sensor that interacts with the C-terminal domain of the RNA polymerase RpoA subunit, leading to changes in gene expression that help sustain viability under various conditions. Using genetic and deep-sequencing methods, we show that *spx* is essential in *S. aureus* and that a previously reported Δ *spx* strain harbored suppressor mutations that allowed it to grow without *spx*. One of these mutations is a single missense mutation in *rpoB* (a P-to-L change at position 519 encoded by *rpoB* [*rpoB*-P519L]) that conferred high-level resistance to rifampin. This mutation alone was found to be sufficient to bypass the requirement for *spx*. The generation of rifampin resistance libraries led to the discovery of an additional *rpoB* mutation, R484H, which supported strains with the *spx* disruption. Other rifampin resistance mutations either failed to support the Δ *spx* mutant or were recovered at unexpectedly low frequencies in genetic transduction experiments. The amino acid residues encoded by *rpoB*-P519L and -R484H map in close spatial proximity and comprise a highly conserved region of RpoB. We also discovered that multicopy expression of either *trxA* (encoding thioredoxin) or *trxB* (encoding thioredoxin reductase) supports strains with the deletion of *spx*. Our results reveal intriguing properties, especially of RNA polymerase, that compensate for the loss of an essential gene that is a key mediator of diverse processes in *S. aureus*, including redox and thiol homeostasis, antibiotic resistance, growth, and metabolism.

IMPORTANCE

The survival and pathogenicity of *S. aureus* depend on complex genetic programs. An objective for combating this insidious organism entails dissecting genetic regulatory circuits and discovering promising new targets for therapeutic intervention. In this study, we discovered that Spx, an RNA polymerase-interacting stress regulator implicated in many stress responses in *S. aureus*, including responses to oxidative and cell wall antibiotics, is essential. We describe two mechanisms that suppress the lethality of *spx* disruption. One mechanism highlights how only certain rifampin resistance-encoding alleles of RpoB confer new properties on RNA polymerase, with important mechanistic implications. We describe additional stress conditions where the loss of *spx* is deleterious, thereby highlighting Spx as a multifaceted regulator and attractive drug discovery target.

Staphylococcus aureus is a major human pathogen that causes a diversity of disease ranging from relatively minor to invasive and systemic disease with significant morbidity and mortality (1–7). *S. aureus* is common both in the community and in hospital environments and has the ability to transiently colonize the skin and mucous membranes of most humans, unnoticeably or with mild clinical features. An estimated 20% of the world's population are persistent carriers. The worldwide distribution of *S. aureus* coupled with its asymptomatic carriage have precluded any effective eradication strategy (8, 9).

The survival of a versatile pathogen like *S. aureus* requires that it effectively combat environmental dangers, as well as effectors of the innate and adaptive immune response. Among the important environmental sensors responsive to oxidative and thiol stress is Spx (10, 11). Extensive studies revealed that, in *Bacillus subtilis*, Spx functions as a redox-sensitive thiol disulfide switch by virtue of an N-terminal C₁₀XXC₁₃ motif (11, 13, 15, 17, 19, 21). The interaction of Spx with RNA polymerase (RNAP) is thought to activate transcription through an incompletely understood mech-

anism that results in enhanced RNA polymerase promoter recognition and open promoter complex formation (17, 19, 21–23). Until recently, no evidence suggested that Spx was itself a DNA binding protein; however, Spx-DNA contact by cross-linking at

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Address correspondence to William L. Kelley, William.Kelley@unige.ch.

M.V. and A.J. contributed equally to this work.

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an AGCT element situated near position −44 of an *spx*-regulated promoter was reported (17, 22). Thus, Spx likely acts as a facilitator/appropriator promoting the engagement of the C-terminal domain of the α subunit (α -CTD) of RNAP with so-called UP elements positioned upstream from its −35 promoter region, thereby affecting sigma factor positioning at canonical promoter elements (23, 25–28).

The adaptor protein YjbH interacts with Spx and helps direct proteolytic turnover of Spx, together with ClpXP (30–34). The deletion of *yjbH*, *clpP*, or *clpX* or the addition of stress agents like diamide or cell wall-active antibiotics has been shown to stabilize Spx, which is otherwise maintained at very low levels (35, 36). As in *B. subtilis*, Spx in *S. aureus* is regulated posttranslationally by YjbH (17, 35, 38, 39). A recent comprehensive study using chromatin immunoprecipitation (ChIP) analysis in *B. subtilis* also suggests a role for Spx in the basal expression of certain genes even under nonstressed conditions (17, 27).

The Spx regulon has not been as extensively studied in *S. aureus*. *S. aureus* Spx is 80% identical at the protein level and 96% similar to its *B. subtilis* counterpart, suggesting that important biochemical properties are conserved. Disruption of *spx* in *S. aureus* led to pronounced pleiotropic effects, suggesting its role as a more global regulator and pointing to multiple functions besides thiol/oxidative homeostasis (36). Recent work demonstrated roles for Spx in modulating resistance to various cell wall-active antibiotics and in the regulation of protein turnover, including that of certain toxins, through its transcriptional regulation of the adaptor protein TrfA, an ortholog of *B. subtilis* MecA (42, 76).

S. aureus contains multiple redox buffering systems, such as the thioredoxin and thioredoxin reductase system encoded by *trxA* and *trxB* and the redoxin and thioredoxin reductase system encoded by *nrdH* and *trxB*, which collectively serve to mitigate the extent of damage to oxidized thiols (43). The genes encoding thioredoxin (*trxA*) and thioredoxin reductase (*trxB*) are essential in *S. aureus* (6, 45–47). Thioredoxin also serves as an electron donor for aerobic ribonucleotide reductase and, thus, serves an important role in the production of deoxynucleotides (48). *S. aureus* may also accomplish additional thiol protection by the use of L-cysteine pools or coenzyme A (CoA) and CoA reductase (45, 49, 50) and bacillithiol (15, 51, 53–57). Phylogenomics suggests that additional thiol oxidoreductases are encoded in genomes possessing the biosynthetic machinery for bacillithiol (54, 56). For example, YtxJ may function as a bacillithiol, together with bacillithiol, to combat hypochlorite stress (10). Notably, all *S. aureus* strains derived from strain NCTC8325, a vast majority of standard laboratory strains, lack bacillithiol because of disruption of the *bshC* (*yllA*)-encoded cysteine ligase (61, 63).

Spx is not essential in *B. subtilis* (13, 15), and yet, despite the strong similarity in sequences, posttranslational regulation by YjbH, and role as mediator of thiol/oxidative stress defenses, we discovered that Spx was essential in *S. aureus*. In the work presented here, we investigated the mechanism underlying the survival of *S. aureus* strains lacking *spx*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this study are listed in Table 1 or in Table S3 in the supplemental material. *Escherichia coli* strains were grown in Luria-Bertani broth, and *Staphylococcus aureus* strains were grown in Mueller-Hinton broth or agar (MHB or MHA; Difco) or tryptic soy broth or agar (TSB or TSA; Difco) as indicated

TABLE 1 Bacterial strains used in the study

<i>S. aureus</i> strain	Relevant genotype and/or characteristics	Reference or source
8325-4	NCTC8325 lacking ϕ 11, ϕ 12, and ϕ 13; sequenced strain is named AR1089	7
ISP794	NCTC8325 derived, <i>pig131 rsbU</i> mutant	102
Newman	ATCC 25904, MSSA, ^a <i>rsbU</i> ⁺	103
AJ850	AR739 with <i>ermC</i> near Δ <i>spx</i> , Tet ^r Erm ^r	This work
AJ862	CB1188 <i>geh::pCL25spx</i> ⁺ Tet ^r	This work
AJ866	CB1349 <i>geh::pCL25 spx</i> ⁺ Tet ^r	This work
AJ1008	AR1089 with <i>kanA</i> near <i>rpoBC</i>	This work
AJ1009	AR1089 with <i>kanA</i> near <i>rpoB</i> -P519L	This work
AJ1010	Independent isolate identical to AJ1009	This work
AJ1011	AJ1008 <i>geh::pCL25spx</i> ⁺ Tet ^r	This work
AJ1022	AR739 with <i>ermB</i> near <i>rpoBC</i>	This work
AR738	8325-4 Δ <i>spx</i> (Δ 27–131)	36
AR739	AR738 <i>geh::pCL25spx</i> ⁺ Tet ^r (previously named <i>spx</i> -c)	36
AR1089	8325-4 sequenced strain; isogenic precursor of AR738	7
CB1188	AR1089 SAOUHSC_01464 with P37S mutation	This work
CB1348	AR1089 <i>geh::pCL25spx</i> ⁺ Tet ^r	This work
CB1349	AR1089 <i>ytxJ</i> promoter mutant, markerless	This work
CB1400	AR739 Δ <i>spx::kanA</i>	This work
CB1401	AR738, wild-type SAOUHSC_01464 with <i>kanA</i> nearby	This work
DA500	ISP794 <i>geh::pCL25spx</i> ⁺ Tet ^r	This work
DA600	Newman <i>geh::pCL25spx</i> ⁺ Tet ^r	This work
MV30	AJ1009 Δ <i>spx</i> , <i>ermC</i> near <i>rpoB</i> -P519L	This work
MV36	AR738 <i>ytxJ::ermB</i>	This work
MV40	AR1089, <i>ermB</i> near <i>rpoB</i> -P519L	This work
MV42	AR1089, <i>ermB</i> near <i>rpoB</i> ⁺	This work
MV45	MV40 Δ <i>spx::kanA</i>	This work
MV54	AJ1009 revertant, <i>rpoB</i> ⁺ with <i>ermB</i> nearby	This work
MV80	WLK8-1R <i>rpoB</i> -A477V <i>geh::pCL25spx</i> ⁺ Tet ^r	This work
MV82	WLK8-4R <i>rpoB</i> -S486L <i>geh::pCL25spx</i> ⁺ Tet ^r	This work
MV85	MV42 derivative, <i>rpoB</i> -R484H Δ <i>spx::kanA</i>	This work
MV94	AR1089 derivative, <i>rpoB</i> -R484H with <i>ermB</i> nearby	This work
MV98	MV94 Δ <i>spx::kanA</i>	This work
MV120	AR1089/pMK4-pGlyS- <i>trxA</i>	This work
MV121	AR1089/pMK4-pGlyS- <i>trxB</i>	This work
MV122	AR1089/pMK4-pGlyS- <i>gfpuv4</i>	This work
MV126	MV120 Δ <i>spx::kanA</i>	This work
MV128	MV121 Δ <i>spx::kanA</i>	This work
MV141	MV30 <i>geh::pCL25 spx</i> ⁺ Tet ^r	This work
MV142	MV45 <i>geh::pCL25 spx</i> ⁺ Tet ^r	This work
MV144	MV98 <i>geh::pCL25 spx</i> ⁺ Tet ^r	This work
NE1281	USA300-derived strain JE2 with <i>ytxJ::ermB</i>	Narsa
WLK8-4R	AJ1008 <i>rpoB</i> -S486L	This work
WLK8-3R	AJ1008 <i>rpoB</i> -D471Y	This work
WLK8-1R	AJ1008 <i>rpoB</i> -A477V	This work

^a MSSA, methicillin-susceptible *S. aureus*.

below. The growth media were supplemented with ampicillin (100 μ g/ml), spectinomycin (100 μ g/ml), kanamycin (40 μ g/ml), tetracycline (1 to 3 μ g/ml), erythromycin (3 to 5 μ g/ml), or chloramphenicol (15 μ g/ml) when appropriate. Recombinant lysostaphin was obtained from AMBI Products LLC (Lawrence, NY). *S. aureus* strain NE1281 (strain USA300 JE2 with an *ermB* insertion in *ytxJ*) was obtained through the Network of Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program, supported under NIAID/NIH contract number HHSN272200700055C. Rifampin (product number R3501; Sigma) was prepared as a 20-mg/ml stock solution in dimethyl sulfoxide (DMSO) and diluted in DMSO as necessary prior to agar plate supplementation.

Plasmid constructions for site-specific genome modifications. Details on the construction of all plasmids and genetically engineered strains are supplied in the supplemental material.

Genome sequencing and SNP analysis. Genomic DNA of strain AR738 (*Δspx*) was prepared from an overnight culture of a strain obtained directly from the D. Frees/H. Ingmer laboratory strain archives, University of Copenhagen, which was grown in MHB at 37°C essentially as described previously (35, 76). The strain was sequenced on an Illumina Hi-Seq 2000 instrument (Fasteris SA, Geneva, Switzerland). Paired-end reads were mapped on the NCTC8325 genome sequence (NCBI accession number NC_007795) using the Burrows-Wheeler Alignment tool, giving a raw coverage depth of approximately 2,400-fold. The mapping covered >98.7% of NCTC8325, and 99.95% when corrected for prophage loss. Variant-base calling relative to the sequence of NCTC8325 was initially performed with SAMTOOLS and the CLC Genomics Workbench and identified 125 single nucleotide polymorphisms (SNPs). SNPs supported by less than 99% of the reads were reexamined by capillary sequencing. Subsequent analysis used filtering of potential SNP/indel differences using our previously sequenced strain 8325-4 genome as the reference (7), which reduced the number of SNPs identified to four. Importantly, the 8325-4 genome used for filtering was the immediate precursor of AR738 and was also derived from strain archives in the Frees laboratory. The complete details of the sequence of this strain and its refinement, correction of NCTC8325 reference genome database errors, and revised annotations of single nucleotide changes and insertions/deletions are thoroughly reported in our previously published work (7). Additional confirmatory sequence verification was performed by sequencing the corresponding regions in the *Δspx* mutant-derived, *spx*⁺-complemented strain *spx-c* (AR739) (36). Insertions/deletions were examined using the Integrated Genomics Viewer (Broad Institute) using tiled data file (tdf) conversion of AR738 Illumina sequence reads. Seven expected indels were detected, which arose from the loss of prophages ϕ 11, ϕ 12, and ϕ 13, the disruption of *spx*, the previously noted 63-bp deletion in the *sarS-spa* intergenic region, SA0282 homolog cluster repeat variation, and the excinuclease ABC β subunit region genome assembly artifact (7). Thus, collectively, apart from the *Δspx* disruption, we detected no additional indels in the *Δspx* strain (AR738) compared with the sequence of its isogenic 8325-4 precursor (AR1079). The sequence reads obtained for AR738 were also *de novo* assembled using the string graph assembler (86) running on the Vital-IT high-performance computing platform (www.vital-it.ch). Gap filling of contigs was accomplished by BLASTN alignment and comparison to NCTC8325 (accession number NC_007795) and our 8325-4 reference sequence (7). The assembled AR738 sequence is 2,687,774 bp.

SNP verification. Genomic DNA was prepared and used as a template for PCR amplification of the region surrounding each suspected nucleotide change. Upstream and downstream primers for SNP verification (see Table S1 in the supplemental material) were designed to create convenient fragment sizes (100 to 600 bp) for subsequent agarose gel purification and sequence analysis using either PCR driver primer. Strain construction used similar methods for all SNP verifications.

Cloning of *trxA* and *trxB* with heterologous promoter control. The *trxA* (346-bp) and *trxB* (967-bp) products were amplified by PCR using forward and reverse primers and strain 8325-4 chromosomal DNA as the template. Forward primers carried a KpnI tail, and reverse primers carried an NsiI-BamHI tail. The PCR products were amplified with *Pfu* DNA polymerase (Promega) and then purified and cloned using KpnI and BamHI in pBluescript II (KS+). Fragments were then ligated using KpnI and NsiI/PstI enzymes in a custom-designed pMK4-based vector containing the *PglyS* promoter. Following sequence verification, the entire polylinker cassette was excised with MfeI and BglII and ligated with the *E. coli*/*S. aureus* shuttle vector pMK4 cleaved with EcoRI-BamHI.

Random rifampin resistance mutation library. Libraries were generated using the same selection strategy as described above but instead starting with an *ermB*-marked (MV42) *rpoB*⁺ strain. Plates grown for 48 h were flooded with MHB, and colonies scraped and combined into four

independent pools, each representing >1,100 independent colonies from 10 85-mm petri plates each. The pools were prepared as recipient cells for bacteriophage-mediated transduction of *Δspx::kanA* from strain CB1400. Transductions were performed on plates with 40 μ g/ml kanamycin. Colonies were restreaked and sequenced. We obtained in this manner three independent isolates carrying *rpoB*-R484H (an allele with an R-to-H change at position 484 encoded by *rpoB* [R484H]) from three independent library pools. To confirm that mutants with this allele could support the *Δspx* mutation, cells bearing the allele were backcrossed to fresh 8325-4 cells using the *rpoB*-linked *ermB* marker and then retested.

Rifampin MIC assays. MIC assays were performed with rifampin-supplemented MHA plates. Rif MICs were defined essentially as described previously (24), corresponding to the concentration of rifampin on MHA plates at which a spot inoculation (10 μ l) of 1×10^{-6} CFU showed no detectable colony formation at 48 h. Each overnight strain culture was first normalized to a McFarland standard of 2 (corresponding to 6×10^8 CFU/ml) using a bioMérieux Densimat instrument and sterile 0.9% saline. Normalized cultures were then further diluted sixfold to 1×10^8 CFU/ml for reference stock solutions.

5' RACE transcript mapping. The 5' transcriptional start sites of SAOUHSC_01464 and SAOUHSC_00755 were determined essentially as described previously (90), using the SMARTer rapid amplification of cDNA ends (RACE) kit (Clontech). The start sites for these genes were also confirmed by comparison with transcription start sites determined by the exact mapping of transcript ends (EMOTE) method (92), with minor modifications, and unpublished data of P. Redder (University of Geneva).

Colony formation counting. CFU counts were performed by spotting aliquots of 10-fold serial dilutions onto agar plates and counting after 24 and 48 h of growth at 37°C unless otherwise indicated (see Fig. 5). Serial 10-fold dilutions of overnight cultures adjusted to a McFarland standard of 1 were made using a Densimat apparatus (bioMérieux) and sterile saline. Aliquots (10 μ l) of, typically, 10^{-2} , 10^{-4} , 10^{-6} , and 10^{-7} dilutions were spotted onto plates. After incubation, colonies were counted and CFU titers computed by dividing the test titer by the reference titer. For efficiency of plating (EOP) computation, the control condition was defined as the number of CFU obtained at 37°C on MHA. For *Δspx* strains compared to their isogenic precursors, EOP was measured within less than 1 log₁₀ difference, typically less than a fivefold fluctuation in titer. For phenotype analysis, the anaerobic culture conditions used mini-Anaerocult A sealed sacs together with the gas generator system as described by the manufacturer (Merck). All sacs used internally fixed BBL dry anaerobic resazurin indicator strips (Becton Dickinson). Nitrate supplementation for anaerobic respiration used 20 mM (final concentration) sodium nitrate in MHA plates. Diamide [bis(*N,N*-dimethylamide); Sigma Aldrich] was freshly prepared as an aqueous 50 mM stock solution and filter sterilized through Millipore 0.22- μ m cartridges prior to use. The stock solutions were diluted as necessary for MHA plates.

RESULTS

***Spx* is essential in *S. aureus*.** Multiple experiments consistently revealed an inability to cross the *Δspx* allele into other *S. aureus* strain backgrounds using standard bacteriophage-mediated general transduction procedures. These results and consideration of the difficulty encountered when obtaining the original *Δspx* disruption in strain 8325-4 (36) led us to test the hypothesis that *spx* is essential in *S. aureus* and that the *Δspx* strain previously described (36) contains a suppressor mutation(s) that permits its viability in the absence of *spx*.

We designed a test for essentiality based upon genetic linkage and bacteriophage-mediated transduction (95). For this, we used three different *S. aureus* genetic backgrounds: 8325-4, ISP794, and Newman (recipient strains). For each strain, we engineered control merodiploid derivatives in which a second copy of *spx*⁺ under the control of its native promoter sequence had been inserted into

FIG 1 Physical map of the *spx* genomic region and features used for genetic analysis. Ordered sequence tags for strain NCTC8325 (NCBI database accession no. NC_007795) are shown, together with arrows that depict the direction of transcription. The limits of the original *spx* disruption in AR738 are shown, together with the *Δspx::kanA* allele engineered in this study. The position of the erythromycin resistance gene, *ermC*, placed in the intergenic region near *spx* (5 kb upstream) and used for bacteriophage-mediated transduction experiments, is also shown.

a chromosomal site—specifically, in single copy at the *geh* (lipase) locus (36, 96). The original *spx*-c (AR739) construction containing this complementing copy of *spx*⁺ had been shown to compensate fully or partially for *spx* disruption phenotypes (36). The *Δspx* mutation (deletion of amino acids 27 to 131 [Δ27–131]) was originally constructed by markerless recombination, and so, to facilitate genetic analysis, we constructed a donor strain for transducing bacteriophage propagation where we site specifically placed an erythromycin resistance marker 5 kb downstream from the *Δspx* allele in the *spx*⁺-complemented strain termed *spx*-c (hereinafter called strain AR739) (35, 36). This strain was named AJ850, and the *spx* locus is depicted schematically in Fig. 1.

The transducing bacteriophage, propagated on AJ850 (the donor strain), carry genomic DNA with the erythromycin resistance marker, *ermC*, in proximity to *Δspx*. Infection of the recipient strains described above was performed with selection for erythromycin resistance. We expected (Fig. 1) to measure the cotransduction frequency of the selected erythromycin marker in close proximity to *Δspx* by screening for the presence of the cotransduced *Δspx* deletion by PCR assay.

The results are shown in Table 2. For the merodiploid recipient strains, we obtained a measured cotransduction frequency of 50 to 70% (global average of 62% for *n* = 62) with the *Δspx* mutation, whereas in no case did we obtain the *Δspx* mutation in the haploid strains. Because the *Δspx* mutation could not be crossed by generalized bacteriophage-mediated transduction into any of the three *S. aureus* strains tested, whereas it could readily be crossed into the identical strains provided they carried a second unlinked

TABLE 2 Bacteriophage-mediated cotransduction frequency assay shows that *spx* is essential in three *S. aureus* strain backgrounds

Strain	Background	Cotransduction frequency (no. of <i>Δspx ermC</i> colonies/total no. of <i>ermC</i> colonies tested)	<i>P</i> value ^a
AR1089	8325-4	0/26	<0.001
CB1348	AR1089 <i>geh::pCL25spx</i> ⁺	21/30	
ISP794	NCTC8325 derivative	0/20	<0.001
DA500	ISP794 <i>geh::pCL25spx</i> ⁺	10/20	
Newman	Clinical strain	0/12	<0.001
DA600	Newman <i>geh::pCL25spx</i> ⁺	6/12	

^a Exact binomial test. Data are compiled from at least 3 independent experiments.

TABLE 3 Sequence analysis and data refinement summary

Parameter	Value
Sample reads (no.)	69,030,392
Mapped bases (no.)	6,903,039,200
Paired reads (no.)	68,099,169
Properly paired reads (orientation and distance) (no.)	66,604,338
Raw coverage depth (avg)	2,568
Read mapping to NCTC8325 sequence (NC_007795) (%)	98.00
Read mapping corrected for phage loss (%)	99.95
SNPs uncorrected vs NCTC8325 sequence (NC_007795) (no.)	125
Indels uncorrected vs NCTC8325 sequence (NC_007795) (no.)	7
SNPs vs AR1089/8325-4 (no.)	4
Indels vs AR1089/8325-4 (no.)	1
bp in AR738 assembled genome (no.)	2,687,774

copy of *spx*, we concluded that *spx* was essential. This finding is further supported by the failure of global transposon insertion analysis to uncover *spx* disruption (1, 3, 5, 6).

Deep sequence analysis reveals only four SNP differences between 8325-4 and its *Δspx* derivative. To identify genetic changes that occurred in the *Δspx* strain (AR738) compared with its parent strain, 8325-4, we prepared genomic DNA and deep sequenced AR738 using Illumina/Solexa technology. Importantly, the *Δspx* parent strain 8325-4 (AR1089) was archived in one of our laboratories (D. Frees) and represented the immediate isogenic precursor used for the original production of *Δspx*. The complete genomic 8325-4 sequence was determined in our laboratories in a previous comprehensive study (7) and greatly facilitated the detection of genetic changes occurring specifically in the *Δspx* strain.

A summary of the sequence analysis results and refinement is shown in Table 3. The four confirmed point mutations found in AR738 compared with the sequence of its isogenic 8325-4 parent are shown in Table 4. The single nucleotide changes represent three transitions and one transversion. Three mutations occur in coding sequences, resulting in two missense mutations and one silent mutation. The fourth mutation occurs in an intergenic region corresponding to the region between the –10 and –35 motifs of the *ytjX* promoter upstream from a putative monothiol bacilliredoxin (10). The positions of the missense and intergenic mutations that were detected are depicted on physical maps of the corresponding gene regions (Fig. 2). We did not further consider the FtsY-N98N silent mutation in our analysis.

The *rpoB*-P519L mutation within the RNA polymerase β subunit was found by sequence alignment to correspond to P444 in *Thermus aquaticus* RNA polymerase or to P564 in *E. coli* RNA

TABLE 4 Single nucleotide changes detected by deep-sequence comparison of strains AR1089 (8325-4) and AR738 (*Δspx*)^a

Mutation	Genome position	Ordered locus tag in NCTC8325	Site or result of change
C/T	523715	SAOUHSC_00524	RpoB-P519L
C/A	738135	SAOUHSC_00755	<i>ytjX</i> promoter
T/C	1156631	SAOUHSC_01205	FtsY-N98N
G/A	1420281	SAOUHSC_01464	Hypothetical protein with P37S

^a Reference sequence coordinates are from *S. aureus* NCTC8325, NCBI accession number NC_007795.

FIG 2 Physical map depicting the positions of single nucleotide changes detected by deep-sequencing analysis of strain AR738 Δ *spx*. (A) *rpoB*-P519L. (B) *ytxJ* promoter transition mutation. (C) P37S in SAOUHSC_01464 and nearby open reading frames in three different reading frames. Conceptual translation reveals that loci SAOUHSC_01464 and SAOUHSC_01463 overlap by four nucleotides. Within the presumptive SAOUHSC_01463 reading frame, a stem-loop (predicted ΔG of -11.7) was detected in close proximity to the *gpsB* promoter. The orientations of transcription are shown by arrows, together with the positions of kanamycin or erythromycin resistance markers site specifically engineered in the chromosome and used for genetic analysis. Ordered sequence tag numbers for the indicated reading frames are shown as in Fig. 1.

polymerase. High-resolution X-ray crystal structures are available for the *T. aquaticus* and *E. coli* RNA polymerases (12, 14, 16, 18, 20), and this proline occurs in the highly conserved D region that forms the RNA exit channel and in close proximity to amino acids found to complex with rifampin (16). The equivalent mutations of P564 in *E. coli* and P519 in *S. aureus* strain 8325-4 have been shown to confer high-level resistance to rifampin (16, 24). The measured MIC for rifampin of strain AR738 was 512 μ g/ml, confirming that this strain possesses a high level of resistance to this drug. Importantly, the construction of the Δ *spx* strain (AR738) in *S. aureus* at no time involved exposure to rifampin and the precursor strain 8325-4 is rifampin sensitive (MIC of 0.008 μ g/ml) and does not have a mutation in *rpoB* (7).

The missense mutation resulting in P37S in SAOUHSC_01464 occurs in a hypothetical protein whose gene position is the first in a three-gene cluster (29). The intergenic-region point mutation occurs in the SAOUHSC_00755 *ytxJ* promoter, 97 nucleotides upstream from the putative *ytxJ* start codon. A single transcription start site for *ytxJ* was determined, and the SNP position places it within the boundaries of the -10 and -35 canonical housekeeping promoter elements (Fig. 2). The open reading frame for *ytxJ* does not appear to be an operon in *S. aureus*: 154 nucleotides (nt) separate it from its upstream neighbor and 424 nt from its downstream neighbor.

The *rpoB*-P519L mutation alone can suppress *spx* deletion lethality. In order to determine which of the detected mutations, or possibly their combinations, was suppressing the lethality of the Δ *spx* mutation in AR738, we first *de novo* reengineered each point mutation separately in the chromosome of *S. aureus* parental

strain 8325-4 (see the materials and methods in the supplemental material). As described above, we also engineered merodiploid derivatives of each single mutant strain harboring a second unlinked copy of *spx*⁺ at the *geh* locus.

Using the same strategy outlined above, we conducted bacteriophage-mediated transduction linkage experiments by first selecting for erythromycin resistance in both the haploid and merodiploid strains and then screening colonies for *spx*⁺ or Δ *spx* alleles by the position of the crossover point of homologous recombination. The results are shown in Table 5.

Our results showed that strain AJ1009, harboring *rpoB*-P519L by itself, could support *spx* disruption just as well as AJ1010, an independent *rpoB*-P519L strain prepared at the same time as AJ1009. In contrast, strains CB1188 and CB1349, containing the other two point mutations in either SAOUHSC_01464 or the promoter region of SAOUHSC_00755, could not support *spx* disruption. In control experiments, the Δ *spx* allele was readily transferred to each of the respective merodiploid strains, consistent with the expected cotransduction frequency. Notably, the Δ *spx* allele could not be transferred to AJ1008, the otherwise isogenic parent of AJ1009 and AJ1010; however, the *spx* disruption could be introduced into the *spx* merodiploid AJ1008-derived strain AJ1011, as expected.

Although the strategy of cotransduction in principle selects only for the applied drug resistance and subsequently screens for the acquisition of the Δ *spx* allele, we were nevertheless concerned that an unknown mutation(s) besides *rpoB*-P519L may have arisen during the construction of AJ1009 which could conceivably account for the observed suppression of Δ *spx* allele lethality. To

TABLE 5 Bacteriophage-mediated cotransduction assay using reconstruction of strains with single SNPs for detection of suppression of Δ *spx* lethality

Allele with SNP	Strain; relevant genotype	Cotransduction frequency (no. of Δ <i>spx</i> <i>ermC</i> colonies/total no. of <i>ermC</i> colonies tested)	<i>P</i> value ^a
SAOUHSC_01464	CB1188; P37S mutation AJ862; CB1188 <i>geh::pCL25spx</i> ⁺	0/20 13/27	<0.001
<i>ytxJ</i>	CB1349; <i>PytxJ</i> [*] AJ866; CB1349 <i>geh::pCL25spx</i> ⁺	0/20 11/20	<0.001
<i>rpoB</i>	AJ1008; <i>rpoB</i> ⁺ with <i>kanA</i> nearby AJ1011; AJ1008 <i>geh::pCL25spx</i> ⁺ AJ1009; AJ1008 <i>rpoB</i> -P519L with <i>kanA</i> nearby AJ1010; like AJ1009 but obtained independently	0/22 7/13 14/39 9/16	<0.001
	WLK8-4R; <i>rpoB</i> -S486L MV82; WLK8-4R <i>geh::pCL25spx</i> ⁺ WLK8-3R; <i>rpoB</i> -D471Y WLK8-1R; <i>rpoB</i> -A477V	0/43 15/20 4/24 5/43	<0.001

^a Exact binomial test. Data are compiled from at least three independent experiments.

address this possibility, we allele specifically reverted strain AJ1009 (*rpoB*-P519L) to *rpoB*⁺ using bacteriophage-mediated transduction and an exchange of a kanamycin resistance marker for the erythromycin resistance marker placed downstream from the *rpoBC* locus. The resulting revertant strain was called MV54. We screened for loss of rifampin resistance in strain MV54 and verified reversion to *rpoB*⁺ by sequence analysis. Multiple independent experiments using phage transduction of the Δ *spx::kanA* allele from donor strain CB1400 failed to generate viable colonies in MV54. We conclude that strain AJ1009 did not harbor an additional mutation(s) besides *rpoB*-P519L that was sufficient by itself to support the deletion of *spx*.

Viability of the sequenced AR738 strain following reversion of the P37S mutation and disruption of *ytxJ*. Our results described above predicted that mutations detected by our deep-sequencing analysis of strain AR738 could be eliminated, apart from the P519L mutation, and still yield a viable strain. We used a combination of allelic exchange for SAOUHSC_01464 and transposon disruption, in the case of *ytxJ*, to create Δ *spx* strains derived from AR738 that harbored reversions of SAOUHSC_01464 with the P37S mutation to the wild type, insertional disruption of *ytxJ* (*ytxJ::ermB*), or both. We were able to produce strains with each of the single mutant variants as viable derivatives of AR738 (CB1401 and MV36), as well as the double mutant derivative strain MV38 that contains only the original *rpoB*-P519L (see Fig. S1 in the supplemental material). Collectively, our results from two complementary genetic methods, mutant reconstruction and sequential SNP elimination/gene disruption, demonstrated that the *rpoB*-P519L mutation detected in AR738 was, by itself, sufficient to support the otherwise lethal deletion of *spx*.

Analysis of additional rifampin resistance alleles of *rpoB*. We next asked whether the suppression of Δ *spx* was allele specific for *rpoB*-P519L. As a first step, we selected several random spontaneous rifampin-resistant mutants derived from AJ1008 and sequenced the *rpoB* region. Three mutants, bearing S486L, D471Y, and A477V mutations, that were found to have a varied rifampin MIC range (4 to 1,024 μ g/ml) and whose mutations were spatially located in various regions of the rifampin binding pocket (Fig. 3)

were retained for further study. The results (Table 5) revealed that WLK8-4R *rpoB*-S486L consistently failed to support the acquisition of Δ *spx*, whereas its merodiploid derivative strain MV82 could support the acquisition of Δ *spx*. Multiple experiments with WLK8-1R *rpoB*-A477V revealed only 5 instances where we obtained stable viable strains harboring Δ *spx* out of 43 (11%) independent erythromycin-resistant transductants tested, considerably below the expected (50 to 60%) experimental cotransduction frequency. In control experiments, the merodiploid WLK8-1R *rpoB*-A477V-derived strain MV80 readily accepted Δ *spx*. A similar observation was made for WLK8-3R *rpoB*-D471Y, in that a low number of colonies (4/24, 17%), well below the expected cotrans-

FIG 3 RpoB rifampin resistance mutations that support deletion of *spx* are located in close spatial proximity. Shown is a spatial projection of amino acids of *S. aureus* RpoB surrounding the rifampin binding pocket, using *S. aureus* coordinates as in the alignment shown in Fig. 4 but based upon the X-ray structure determination of the *T. aquaticus* RNA polymerase core in complex with rifampin (16). The rifampin binding pocket is oriented with the rifampin functional groups as follows: naphthol ring (planar and perpendicular to the page, upper left), methyl-piperazine (below the naphthol ring), and O-acetyl (center right). Amino acids mutated in this study are boxed: the amino acids whose mutations support the viability of Δ *spx* strains (P519L and R484H) are highlighted in dark blue; those whose mutations support Δ *spx* strains less effectively (A477V and D471Y) are shown in light blue; and the amino acid whose mutation is not a Δ *spx* suppressor mutation (S486L) is not highlighted.

FIG 4 The amino acid residues encoded by the *rpoB* mutations P519L and R484H are located in a highly conserved region. Shown is a sequence alignment of RNAP β subunit regions comprising the discontinuous rifampin binding pocket regions I and II (16) in various bacteria (marked *rpoB*) or eukaryotic organisms, together with their corresponding amino acid coordinates. The positions of mutations described herein for *S. aureus* are depicted with colored circles consistent with the highlighting in Fig. 3. Regions that are boxed and highlighted in gray are highly conserved. Sa, *S. aureus*; Ec, *E. coli*; Bs, *B. subtilis*; Mt, *Mycobacterium tuberculosis*; Ta, *T. aquaticus*; Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*; Sc, *Saccharomyces cerevisiae*.

duction frequency, were viable and contained *Δspx*. We conclude that rifampin resistance *per se* is not sufficient to support the loss of *spx* and that certain alleles are required. The *rpoB*-P519L allele consistently supported *spx* disruption with the expected cotransduction frequency, whereas *rpoB*-A477V and *rpoB*-D471Y apparently support disruption of *spx*, but at a much lower frequency than expected.

To extend these findings and discover whether additional rifampin resistance alleles could be uncovered which support the deletion of *spx*, we next performed a genetic selection experiment using CB1400 *Δspx::kanA* as a donor strain (depicted in Fig. 1) for bacteriophage-mediated transduction.

Multiple independent experiments using recipient cells without prior selection for rifampin resistance consistently failed to give viable colonies following transduction with phage lysates from a *Δspx::kanA* donor strain, whereas *Δspx::kanA* could be crossed, as expected, into an *spx*⁺ merodiploid strain, CB1348. The failure to obtain viable colonies by direct *Δspx::kanA* transduction in the absence of prior rifampin exposure suggested that the frequency of spontaneous rifampin-resistant alleles in the unselected population was too low to detect by direct selection methods under our experimental conditions. This finding may also be explained by the fitness cost of rifampin mutation in the absence of selection within a growing population of cells used as the recipient strain in transduction experiments. This finding is also in complete agreement with the original difficulty encountered when constructing the original *Δspx* strain (36).

In contrast, when we created a library of >4,500 independent rifampin-resistant mutants selected in strain MV42 (*rpoB*⁺ with *ermB* nearby), we readily obtained kanamycin-resistant colonies that remained viable upon subcultivation. Sequence analysis of three independent *Δspx::kanA* isolates chosen at random from three independent MV42-derived rifampin-resistant-mutant pools contained the same rifampin resistance mutation, *rpoB*-R484H. We did not sequence all viable colonies obtained, leaving open the possibility that additional suppressor alleles await discovery. Rifampin resistance studied in other organisms has shown

that rifampin mutations are likely biased for only a handful of alleles (16), and thus, defining the complete landscape of rifampin resistance mutations that support the viability of *Δspx* strains was not further pursued in this analysis.

RpoB rifampin resistance mutations that support strains with a deletion of the *spx* cluster are in a highly conserved region. Figure 4 shows an expanded alignment of the D region of RpoB, including the locations of mutations conferring rifampin resistance in various organisms and of rifampin resistance mutations tested in this study. Strikingly, the two mutated amino acids P519L and R484H are located in close spatial proximity (Fig. 3) within a region that has been noted as highly conserved from bacteria to humans (16). R484 makes direct contact with O1 of the naphthol ring of rifampin, and P519 is positioned underneath R484. Of note, an equivalent of the P519L mutation has been described in *E. coli* (P564L, also historically called *ack-1* [41]), and this amino acid is in close proximity to the secondary channel of the polymerase where nucleoside triphosphates (NTPs) enter. Amino acid S486, located close to R484 (Fig. 3), also makes contact with rifampin naphthol ring O2. Notably, despite the spatial proximity of R484 and S486 and their presumably similar modes of disruption of rifampin binding, mutation of these amino acids results in opposite phenotypes with respect to their support of strains with an *spx* disruption.

Multicopy expression of thioredoxin or thioredoxin reductase can also support strains with a disruption of *spx* in the absence of a mutation conferring resistance to rifampin. *S. aureus* strains containing the *Δspx* disruption are consistently associated with a slow-growth phenotype. We considered the possibility that *Spx*-dependent gene transcription, such as is known for the essential thioredoxin reductase gene, *trxB* (36), explains the lethality of *spx* deletion. However, little is known concerning the regulation of the thioredoxin gene, *trxA*, in *S. aureus*. Using reverse transcription-quantitative PCR (qRT-PCR) analysis, we discovered that *trxA* is also transcriptionally regulated by *spx* (see Fig. S2 in the supplemental material). Thus, both *trxA* and *trxB* are in the *spx* regulon. In the absence of stress, *S. aureus* has evolved to survive in

FIG 5 Colony-forming assay showing certain concordant phenotypes attributable to the Δ *spx* allele. (A) Phenotypic analysis of rifampin-resistant suppressor strains and their Δ *spx* derivatives. Those marked P519L* still possess the original mapped SNPs. Strains marked +*spx* are the complemented strains. Spots made with the 10⁻⁴ dilution from serial dilutions of overnight cultures are depicted for all phenotypes except growth with diamide (0.2 mM) and NaCl (2.2 M), for which we show spots made with the 10⁻² dilution. (B) Phenotypic analysis of *trx*-dependent suppressor strains and their Δ *spx* derivatives.

the presence of basal levels of Spx, which are kept at extremely low levels by proteolytic degradation orchestrated by YjbH and ClpXP (35, 38). Nevertheless, these basal Spx levels are most likely responsible, in part, for the set points of certain critical promoters, especially those involved in controlling the cell redox state and contributing to the biosynthesis of deoxynucleoside triphosphates (dNTPS) via ribonucleotide reductase. We hypothesized that disruption of *spx* limited thioredoxin/thioredoxin reductase to such an extent that it compromised cell viability.

To address this hypothesis, we cloned the unlinked genes encoding thioredoxin (*trxA*) or thioredoxin reductase (*trxB*) on multicopy plasmids and placed the genes under the control of the T box riboswitch of the glycyl-tRNA synthetase gene promoter *PglyS* (SAOUHSC_01666) (44, 46). This promoter is not known to be regulated by Spx and would be expected to exert housekeeping levels of gene expression, since *S. aureus* encodes only one glycyl-tRNA synthetase. As a control, we also cloned emission-enhanced green fluorescent protein (GFPuv4) under the control of the same *PglyS* promoter. The plasmids were introduced into 8325-4, and the resulting strains (MV120, MV121, and MV122, respectively) used as recipients for transduction of the Δ *spx::kanA* allele.

We repeatedly recovered viable Δ *spx::kanA* strains from strains containing *trxA* or *trxB* plasmids but not from the control strain, MV122, expressing GFPuv4 from the same promoter. The Δ *spx::kanA* derivative strains MV126 and MV128 were tested and found to be phenotypically rifampin sensitive (MICs of <0.008 μ g/ml) and *rpoB*⁺ by direct sequence analysis. We conclude from this analysis that at least one more solution exists for the suppression of *spx* disruption lethality that is independent of rifampin resistance and a particular rifampin resistance allele. This second mechanism requires heterologous promoter-dependent expression of either thioredoxin or thioredoxin reductase.

Using serial dilutions of *spx*⁺ and Δ *spx* strains, we determined CFU counts on agar plates and determined the Δ *spx*-to-*spx*⁺ strain efficiency-of-plating (EOP) ratios (see Table S2 in the supplemental material). The results revealed that the suppressor strains (*rpoB* or *trx*) and their Δ *spx* derivatives had comparable plating efficiencies (the CFU titer ratios [Δ *spx*-to-*spx*⁺ strain ratios] ranged from 0.3 to 0.68). We have also noted that despite slow growth of Δ *spx* strains, the endpoint values for stationary-phase optical density at 600 nm (OD₆₀₀) of *spx*⁺ and Δ *spx* strains were also comparable. Thus, we conclude that the acquisition of the Δ *spx* mutation by the suppressor strains does not engender significant alterations in overall strain viability.

Phenotypic comparison of suppressor strains with the Δ *spx* mutation. Previous work (36) attributed several diverse phenotypes to the Δ *spx* strain AR738. In light of our findings that this particular strain contained additional mutations in addition to the *rpoB*-P519L suppressor allele, we wished to examine phenotypes associated with the Δ *spx* mutation in the context of either its *rpoB* or *trxA*/*trxB* suppressor to determine whether they were consistent irrespective of the suppressor mechanism. The results are shown in Fig. 5.

We observed that Δ *spx* strains grew aerobically or anaerobically (both respiration and fermentation) but displayed hypersensitivity to the thiol-specific oxidant diamide and to hypersaline conditions (2.2 M NaCl) compared with their *spx*⁺ control suppressor strains. Furthermore, we found that Δ *spx* strains have a strong cold sensitivity phenotype. Colonies with the Δ *spx* mutation grew to pinpoint size and displayed an initial efficiency of plating compared to that of the 37°C control of approximately 0.5 to 1; however, these colonies did not continue to grow, even after 10 days of incubation, nor did cells resume growth following replacement at 37°C. This result suggests that the growth of Δ *spx*

strains at a cold temperature results in a time-dependent depletion of an essential component(s), culminating in cell death.

The strong phenotypes we have documented for the Δ spx strains (Fig. 5) must be considered with the caveat that the successful disruption of *spx* occurs in the context of a suppressor mechanism that may itself contribute to the phenotypes tested. Notably, as shown by the data presented in Fig. 5, control strains harboring only a suppressor mutation and otherwise wild type for *spx* did not show these strong phenotypes. Thus, since we observed concordant strong phenotypes arising from *spx* disruption with both of the suppressor mechanisms we describe herein (*rpoB* based and *trx* based), it is likely that the observed effects arise primarily from the loss of *spx*, with negligible contribution from the suppressor mutation itself. A comprehensive examination of *spx*-dependent phenotypes and the *spx*-dependent gene expression that underlies precisely why *spx* is essential will be the subject of future study.

DISCUSSION

We have shown that the RNA polymerase-interacting regulator Spx is essential in *S. aureus* and that the viability of the strain (AR738) in which Δ spx was first described (36) could be traced to a single mutation in *rpoB*, the gene encoding the β subunit of RNA polymerase. Additional analysis showed that this mechanism of *spx* suppression required certain specific changes in RpoB, most notably P519L or R484H, conferring high-level resistance to rifampin. Another high-level rifampin resistance mutation, RpoB-S486L, failed to suppress the Δ spx disruption, and so rifampin resistance mutations in *rpoB* *per se* do not underlie the suppression mechanism, which points instead to genetic suppression that is dependent upon specific alleles.

We repeatedly failed to uncover *rpoB* mutations that could support the viability of a strain with the Δ spx mutation without prior selection for rifampin resistance. This suggests that the frequency of rifampin resistance mutations or alternative mutations that could conceivably suppress the deleterious effects of the Δ spx mutations and which map outside *rpoB* and are or are not related to mutations conferring rifampin resistance occur infrequently. With regard to rifampin resistance, this finding is consistent with the fitness cost of rifampin resistance mutations in *S. aureus* strain 8325-4, where almost all strains with such mutation show impaired growth compared to that of wild-type controls (24, 52).

A second mechanism of suppression was uncovered in our study, which showed that multicopy plasmids harboring a heterologous housekeeping promoter, a PglyS T box riboswitch driving *trxA* or *trxB*, could support the viability of strains with a disruption of *spx*. This result suggests that adequate minimal expression of thioredoxin and thioredoxin reductase is required to create an environment wherein *spx* can be dispensed with. Both *trxA* and *trxB* are *spx* regulated in *S. aureus*, as described herein and elsewhere (36, 45), and *trxA* and *trxB* are also *spx* regulated in *B. subtilis* (15). While we report these two mechanisms in this study, we cannot exclude the possibility that there are additional means to suppress Δ spx lethality that await discovery.

A survey of rifampin resistance mutations in other organisms that are synonymous to those we describe herein resulted in the remarkable finding that mutations of both P564 and R529 (equivalent to *S. aureus* P519 and R484) in *Salmonella enterica* are very strongly associated with an enhanced ability to survive in aging cultures compared with the ability of wild-type *rpoB*⁺ rifampin-

sensitive cells to do so (58). Intriguingly, cells with another mutation, S531F (equivalent to *S. aureus* S486, for which we show no ability to suppress Δ spx disruption), showed no enhanced survival advantage. The authors made these findings in the course of an effort to explain an apparently enhanced mutation frequency in aging cultures of *E. coli* or *Salmonella enterica* that was ultimately traced to the selective growth advantage of these mutations on acetate rather than altered mutation rates, using rifampin resistance mutation as a proxy (58, 60). P564L in *E. coli* has also been described as an *rpoB* allele that helps bypass starvation-induced replication arrest in *E. coli* cells lacking *dksA* (62).

Additional consideration of rifampin resistance alleles, especially those equivalent to *S. aureus* RpoB-P519L, uncovered yet further evidence of linkage to a wide variety of changes in RNAP transcription properties and their physiological consequences. These alterations include transcription slippage, sign epistasis, Rho-dependent terminator read-through, attenuation bypass, global transcriptome changes, and alterations in metabolic profiles (64–75). It appears that common solutions to confront diverse stress conditions have occurred in various organisms, hinting at a potentially fundamental process.

Of particular note, the rifampin resistance allele *rpoB*-H481Y in *S. aureus* is associated with extensive changes in the transcriptome profile compared with that of its isogenic parent strain, and this partially helps to explain the changes in virulence, persistence, and capsule formation in a clinical methicillin-resistant *S. aureus* (MRSA) strain (59). Further study showed that the *rpoB*-A477D allele modulates reduced susceptibility to daptomycin, vancomycin, and β -lactam antibiotics and contributes to a variety of pleiotropic effects, including modulation of Spx levels (61). Spx has been linked with both glycopeptide and β -lactam resistance in *S. aureus* in our own laboratory by virtue of the implication in drug resistance of its negative regulator *yjbH* (35) and an adaptor gene, *trfA*, that was shown to be Spx regulated (76). Glycopeptide resistance is multifactorial and often involves mutation of global regulators, such as the two-component systems VraRST, GraRS, and WalKR or the serine threonine kinase/phosphatase Stk/Stp. In this regard, it is noteworthy that *rpoB* mutations which do not always confer rifampin resistance, are now frequently associated with the intermediate vancomycin resistance phenotype in MRSA strains (77–82).

Collectively, the evidence suggests that certain rifampin alleles often confer new properties upon RNAP that affect gene expression and physiology, probably by several mechanisms. Pinpointing the precise mechanism that dictates how particular *rpoB* mutations and not others can suppress the otherwise lethal disruption of *spx* in *S. aureus* may therefore prove challenging.

In the absence of Spx in *S. aureus*, it is reasonable to consider that the suppression mechanism must account for the loss of Spx-dependent transcription regulation, and by extension, postulate a role for compensatory transcription modulation. Regulators like Spx that act through the UP element are thought to direct their promoter specificity by communicating with the sigma factor and core promoter elements via the α -CTD (19, 21, 22, 26, 83, 84). Indeed, a detailed study of Spx-dependent promoter selection in *B. subtilis* (22) revealed that Spx binds to an upstream sequence in the *trxB* promoter and helps SigA engage canonical promoter elements that otherwise are poorly recognized (22).

Structural studies of transcription initiation have revealed new insights into promoter recognition and the steps leading to open

promoter formation (18, 23, 87, 89). Promoter recognition is thought to be mediated by sigma factor-DNA interaction and core promoter elements comprising -35 , -10 , and extended -10 regions (12, 23); however, a high-resolution study of an open promoter complex with *Thermus thermophilus* holoenzyme recently revealed the presence of a new determinant called the core recognition element (CRE) that is thought to provide sequence-specific promoter recognition of the -4 to $+2$ region of the nontemplate DNA strand (89). Additional studies in *E. coli* have extended these findings and shown that the CRE can modulate transcription start site selection, elongation, and pausing and that mutation of the CRE impairs transcription (88, 97).

The binding specificity originally attributed to the CRE is thought to be mediated via contacts provided by amino acids within RpoB, particularly those comprising van der Waals contacts that define a pocket for interaction with $+2G$ in the RNA template used for cocrystal formation (89). It awaits further experimentation to ascertain whether determinants throughout the -4 to $+2$ region are truly sequence specific.

The CRE motif is composed of two discontinuous polypeptide segments of the RpoB β subunit (positions 440 to 455 and 535 to 555 in *E. coli* coordinates) (88, 89, 91). This motif therefore flanks domain I of region D, comprising the rifampin pocket fold (16). In light of our finding that specific rifampin alleles in a highly conserved region of RpoB are able to compensate for the loss of an essential gene, itself an RNAP-interacting protein mediating promoter selection (22, 27), it is tempting to speculate that the proximity of certain rifampin resistance mutations to the CRE could perturb the local polypeptide chain configurations sufficiently to alter promoter recognition determinants and/or other parts of the transcription initiation process. Experiments are underway to address this hypothesis.

The second mechanism of Δ *spx* suppression we describe in this study was uncovered by considering the possibility that bypass by acquisition of particular rifampin resistance alleles in some way mimics what Spx itself accomplishes when contacting the RNAP α -CTD. In this respect, an *spx*-dependent gene(s), most likely essential itself, displayed insufficient transcription in the absence of *spx* to support viability. Since both *trxA* and *trxB* are transcriptionally regulated by Spx, indeed the most reasonable scenario explaining the suppression mechanism is that heterologous promoter control of *trxA* or of *trxB* compensates for insufficient transcription in the absence of Spx. Although it is conceptually clear that multicopy expression using a heterologous promoter could create conditions whereby *spx* deletion could be introduced into cells without *rpoB* mutation by augmenting the gene dosage of certain *spx*-regulated genes, we cannot at this time definitively say that the two mechanisms are related simply by compensatory transcription levels. For example, *S. aureus trxB* is known to be regulated both negatively, by SarA and PerR (45, 93, 94), and positively, by Spx (36), and so one possible alternative scenario would be that a particular rifampin resistance mutation perturbs the expression of SarA or PerR such that it relieves negative regulation, thus leading to adequate *trxB* expression.

The full extent of *spx*-regulated genes in *S. aureus* is incompletely understood, although proteomics has shown differential regulation of dozens of proteins (36) and the results of a global ChIP assay in *B. subtilis* indicate that perhaps several hundred promoter regions bind Spx (27). Furthermore, it cannot be for-

mally excluded that Spx interacts with RNAP at sites outside the α -CTD (22, 98). Future experiments will investigate this.

Our examination of phenotypes arising with *spx* disruption underscores a critical role for Spx in several cellular processes, particularly low-temperature survival and osmotolerance. Recent work has highlighted an important role for potassium uptake systems in counteracting hypersalinity, mediated by the *kdp* and *ktr* systems, together with the finding that cyclic di-AMP is an apparent negative regulator of potassium uptake by virtue of its interaction with KdpD (99–101). These findings suggest important contributions of Spx and Spx-regulated genes in survival in diverse microenvironments. Furthermore, rifampin resistance can have a profound influence on gene expression in both Gram-negative and Gram-positive organisms, including a significant impact upon antibiotic resistance mechanisms. This suggests a particularly fruitful avenue of research to discover how the acquisition of rifampin resistance globally affects cellular physiology.

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