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The *Staphylococcus aureus* Thiol/Oxidative Stress Global Regulator Spx Controls *trfA*, a Gene Implicated in Cell Wall Antibiotic Resistance

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***S. aureus* combats cell wall antibiotic stress by altered gene expression mediated by various environmental signal sensors. In this study, we examined the transcriptional regulation of *trfA*, a gene related to *mecA* of *Bacillus subtilis* encoding an adaptor protein implicated in multiple roles, notably, proteolysis and genetic competence. Despite strong sequence similarity to *B. subtilis mecA*, the function of *S. aureus trfA* remains largely unexplored; however, its deletion leads to almost complete loss of resistance to oxacillin and glycopeptide antibiotics in glycopeptide-intermediate *S. aureus* (GISA) derivatives of methicillin-susceptible or methicillin-resistant *S. aureus* (MRSA) clinical or laboratory isolates. Northern blot analysis and 5′ rapid amplification of cDNA ends (RACE) mapping revealed that *trfA* was expressed monocistronically by three promoters. Cell wall-active antibiotic exposure led to both increased *trfA* transcription and enhanced steady-state TrfA levels. *trfA* promoter regulation was not dependent upon the cell wall stress sentinel VraSR and other sensory stress systems, such as GraRS, WalkRK, Stk1/Stp1, and SigB. Notably, we discovered that the global oxidative-stress regulator Spx controlled *trfA* transcription. This finding was also confirmed using a strain with enhanced Spx levels resulting from a defect in *yjbH*, encoding a Spx-interacting protein governing Spx proteolytic degradation. A cohort of clinical GISA strains revealed significant steady-state upregulation of *trfA* compared to corresponding susceptible parental strains, further supporting a role for *trfA* in antibiotic resistance. These data provide strong evidence for a link between cell wall antibiotic stress and evoked responses mediated by an oxidative-stress sensor.**

Diseases caused by *Staphylococcus aureus* range from relatively benign soft tissue infections to life-threatening invasive illness (1, 2). Of particular concern are infections arising from encounters with strains with altered susceptibility to antibiotics, such as methicillin-resistant *S. aureus* (MRSA). Glycopeptide antibiotics (vancomycin and teicoplanin) are frequently considered the mainstay for therapy of MRSA infections. Recent studies suggest, however, that relatively minor increases in MIC levels of glycopeptides, even at the upper range of glycopeptide susceptibility, are correlated with higher rates of therapeutic failure (3–6). This troubling issue has prompted recent changes in glycopeptide susceptibility breakpoints and underscores the need for alternative pharmacotherapeutic agents.

High-level resistance to glycopeptides in *S. aureus*, termed VRSA (vancomycin-resistant *S. aureus*), arises from infrequent horizontal acquisition of Tn*1546* encoding the multiprotein VanA complex from *Enterococcus faecalis*. Mechanistically, the Van complex enzymes alter the stem peptide of cell wall precursor molecules so that glycopeptides no longer bind efficiently. Worldwide, less than a dozen examples of VRSA strains have occurred since the first outbreak was reported (7, 8). In contrast to high-level resistance, clinical *S. aureus* isolates showing low-level glycopeptide resistance (MIC range, 4 to 8 μg/ml) have been reported since 1997 and are referred to as glycopeptide-intermediate *S. aureus* (GISA). Low-level glycopeptide resistance is much more prevalent, and mechanistically, it is thought to occur by stepwise acquisition of mutations that confer survival advantage in the face of drug encounters (2, 9, 10). A complete understanding of the mechanism of acquisition of low-level resistance is currently lacking, although genetic studies to date have identified mutations in genes such as *graRS*, *tcaA*, *stp1*, *vraRS*, *yjbH*, *walKR*, and *trfAB* that contribute to the acquisition or loss of the resistance phenotype (10–14). The two-component histidine kinase sensor genes *graRS* and *vraRS*, as well as the serine/threonine kinase stk1-stp phosphatase, are phosphosignaling systems controlling a large number of downstream genes, suggesting that the mechanism of low-level glycopeptide resistance is complex. This is perhaps not surprising in light of the fact that glycopeptides inhibit end-stage cell wall assembly steps occurring outside the plasma membrane, and thus, for topological reasons, initiating a response to drug encounters must involve transmembrane-signaling steps.

Previous studies of a unique set of clinical isolates in our laboratory led to the discovery of two adjacent genes linked with teicoplanin resistance (Tei^r) that we named *trfA* and *trfB* for teicoplanin-resistant factors A and B (14). Detailed analysis showed that individual or combined deletion of *trfA* and/or *trfB* led to the loss of glycopeptide or oxacillin resistance in an *in vitro*-selected teicoplanin-resistant derivative of ISP794, as well as in the clinical GISA strain NRS3 (14). A clear functional role of the *trfA* or *trfB* gene in *S. aureus* remains undefined. Conceptual translation of *trfA* indicates that its product most closely resembles the MecA adaptor protein of *Listeria monocytogenes* and *Bacillus subtilis*

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TABLE 1 Bacterial strains and plasmids used in this study

Strain/plasmid	Revelant genotype	Characteristics	Source/reference
Strains			
RN4220	8325-4; r^- m^+; restriction-defective laboratory strain		72
ISP794	8325 *pig* 131; *rsbU* mutant		73
AR852	ISP794; *rsbU*$^+$	ISP794; (*rsbUVW-sigB*)$^+$ *tetL* nearby	24
AR376	ISP4-2-1	GISA *in vitro*-derived strain	14
AR774	*vraS* (G45R)	ISP794; *vraS* (G45R); Kan^r nearby	10
AR826	*stp1* (Q12stop)	ISP794, *stp1* (Q12stop); Kan^r nearby	10
AR1079	*yjbH* (K23stop)	ISP794, *yjbH* (K23stop); Ery^r nearby	10
Δ*spx*	8325-4-derived strain	*spx*-deleted strain	30
spx$^+$	8325-4 Δ*spx* P_{spx}-*spx*::*geh*	*spx*-deleted strain chromosomally complemented with the intact copy of *spx* inserted into the *geh* locus	30
Δ*spx rsbU*$^+$	8325-4-derived strain	*spx*-deleted strain; (rsbUVW-sigB)$^+$; *tetL* nearby	This study
AR612	ISP4-2-1; Δ*trfA*	ISP4-2-1; *trfA*::*tetK*	14
AR916	ISPΔ*vraSR*	ISP794; Δ*vraSR*::Kan^r	74
Pair 1 non-GISA		Clinical MRSA glycopeptide-susceptible strain of pair 1	27
Pair 1 GISA		Clinical MRSA glycopeptide-resistant strain of pair 1	27
Pair 2 non-GISA		Clinical MRSA glycopeptide-susceptible strain of pair 2	27
Pair 2 GISA		Clinical MRSA glycopeptide-resistant strain of pair 2	27
Pair 3 non-GISA		Clinical MRSA glycopeptide-susceptible strain of pair 3	27
Pair 3 GISA		Clinical MRSA glycopeptide-resistant strain of pair 3	27
Pair 4 non-GISA		Clinical MRSA glycopeptide-susceptible strain of pair 4	27
Pair 4 GISA		Clinical MRSA glycopeptide-resistant strain of pair 4	27
Pair 5 non-GISA		Clinical MRSA glycopeptide-susceptible strain of pair 5	27
Pair 5 GISA		Clinical MRSA glycopeptide-resistant strain of pair 5	27
BL2 α(DE3)	*E. coli*	IPTG-inducible T7 RNA polymerase	New England Biolabs
Plasmids			
pBluescript II KS(+)	Cloning vector; Amp^r		
pAM845	pKS$^+$ containing a Kpn-Pst fragment coding for TrfA		This study
pTYB12	N-terminal fusion IMPACT intein and chitin binding domain plasmid		New England Biolabs
pAM873	pTYB12 containing *trfA* cloned into NdeI-PstI restriction sites		This study

(14), whereas the conceptual translation of *trfB* shows strong similarity with YjbF of *B. subtilis*, also called CoiA in *Streptococcus pneumoniae* (14). Studies with both organisms suggest that YjbF/CoiA contributes to competence for genetic transformation (15).

Importantly, the MecA adaptor protein has no known functional relation to the *S. aureus mecA* encoding the PBP2′ enzyme, which confers the MRSA phenotype on strains acquiring any of several allotypes of the horizontally transmitted SCC*mec* element. In *B. subtilis*, the MecA adaptor has been extensively studied and plays a regulatory role in genetic competence development, motility, and autolysis (16, 17). Notably, *B. subtilis* MecA serves dual functions as an assembly factor/chaperone for the AAA$^+$ Hsp100/Clp ATPase family member ClpC and as a substrate specificity factor for regulated proteolysis (18).

A few substrates bound by MecA in *B. subtilis* and fed to proteolytic machinery are ComK, CtsR, and MurAA, the enzyme controlling the first committed step in cell wall biosynthesis (19–22). By virtue of strong overall sequence similarity, *S. aureus* TrfA is most likely a MecA ortholog, although this awaits experimental confirmation. MecA-dependent control of regulated proteolysis, and especially MurAA turnover, naturally suggests a link between MecA/TrfA function and biological mechanisms that exist to combat cell wall-active antibiotics.

In order to further our understanding of pathways that lead to altered sensitivity to cell wall-active antibiotics in *S. aureus*, we report in this study the detailed transcriptional regulation of *trfA*. Our results surprisingly reveal that *trfA* is a previously unrecognized member of the cell wall stress regulon, and we present evidence that it is under the transcriptional control of the global thiol/oxidative-stress regulator Spx. These findings are discussed in light of the growing body of evidence linking the bactericidal activities of various antibiotics to the production of reactive oxygen species (ROS).

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. The *rsbU*-defective NCTC8325 strain ISP794 (MIC = 1 μg/ml) and its teicoplanin derivative AR376 (MIC = 8 μg/ml) were described previously (10, 14). All *S. aureus* strains were grown in Mueller-Hinton broth (MHB) and *E. coli* strains in Luria-Bertani medium supplemented with 100 μg/ml of ampicillin or carbenicillin when required. All antibiotics were obtained from Sigma-Aldrich, except teicoplanin (Sanofi-Aventis) and vancomycin (Sandoz). Diamide(1,1′-azobis(*N*,*N*-dimethylformamide)) was obtained from Sigma-Aldrich.

Total RNA extraction. Overnight bacterial cultures were diluted in MHB (1/100) and grown at 37°C with agitation to an optical density at 600 nm (OD_{600}) of 0.6. When indicated, oxacillin (1 μg/ml), teicoplanin (10 μg/ml), vancomycin (10 μg/ml), D-cycloserine (10 μg/ml), ciprofloxacin (1 μg/ml), or diamide (5 mM) was added and incubated for an additional

hour (for oxacillin), 10 min (for vancomycin, teicoplanin, and D-cycloserine), or 30 min (for ciprofloxacin and diamide). Bacteria were harvested, and RNA extraction was performed as previously described (14). The absence of contaminating DNA was always verified for every experiment by PCR using quantitative real-time PCR (qRT-PCR) probes in the absence of reverse transcription.

qRT-PCR. The mRNA levels were determined by qRT-PCR using the one-step reverse transcriptase qPCR Master Mix Kit (Eurogentec, Seraing, Belgium) as described previously (23). Primers and probes for *trfA*, *spx*, and *hu* were designed using PrimerExpress software (version 1.5; Applied Biosystems) and obtained from Eurogentec (see Table S1 in the supplemental material). Primers and probes for 16S, *vraR*, and *asp23* genes were previously described (23–25). Reverse transcription and PCR were performed using primers and probes at a concentration of 0.2 and 0.1 μM, respectively. For Hu gene detection, primers and probes were all used at a concentration of 0.1 μM. All mRNA levels were normalized on the basis of their 16S rRNA levels, which were assayed in each round of qRT-PCR as internal controls, as described previously (23).

Expression of recombinant TrfA protein. The open reading frame of the *trfA* gene (N315 SA0857) was PCR amplified with primers indicated in Table S1 in the supplemental material and cloned in pBluescriptII KS(+). A sequence-verified *trfA* fragment was next subcloned into *E. coli* expression vector pTYB12 (New England Biolabs) using NdeI-PstI sites, generating plasmid pAM873. *E. coli* strain BL21 λ(DE3) (New England Biolabs) containing pTYB12-TrfA protein was grown in Luria-Bertani medium containing carbenicillin at 100 μg/ml to an OD_{600} of 0.7 and induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for an additional 150 min at room temperature with vigorous shaking. Bacteria were harvested by low-speed centrifugation and resuspended in Laemmli buffer, and whole-cell extracts were used in Western blot analysis as described below.

Anti-TrfA antibody production. Rabbit polyclonal antibodies were raised in specific-pathogen-free (SPF) New Zealand White rabbits against a 15-amino-acid synthetic peptide (FSREDLWTNRKRGEE, corresponding to amino acids 25 to 39 of SA0857) with MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester)-conjugated keyhole limpet hemocyanin (KLH) carrier protein using the 87-day protocol of Eurogentec (Seraing, Belgium). Specific antiserum was further affinity purified against the immunizing peptide. The peptide was affinity coupled using AF-amino Toyopearl 650 M (TOSOH Bioscience GmbH, Germany) and an equal mixture of ACH (Na-[e-aminocaproyl]-DL-homoarginine hexylester) Sepharose and CNBr Sepharose. The antibody was eluted with 0.1 M glycine, pH 2.5. The specificity of the antibody was assessed by Western blot analysis using *E. coli* whole-cell extracts expressing inducible recombinant TrfA (see below and Fig. 3).

Western blot analysis. Western blot analyses of protein extracts from *S. aureus* were performed as follows. Overnight cultures of strains in MHB growing at 37°C with agitation were diluted (1/100) in MHB and grown at 37°C with agitation to an OD_{600} of 0.5. When indicated, oxacillin (1 μg/ml) was added, and bacteria were grown for an additional hour. After centrifugation, the cell pellets were washed and resuspended in 500 μl TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA). Bacterial cells were disrupted by adding 500 μl of acid-washed glass beads (100 to 200 μm; Sigma) and using a FastPrep cell disrupter (MP Biomedicals). The cell debris was separated from soluble protein extracts by centrifugation at 14,000 rpm (10 min at 4°C). The supernatant was concentrated on Amicon spin columns (10-kDa cutoff; Milian, Geneva, Switzerland). Protein concentrations were determined by Bradford assay (Bio-Rad) using bovine serum albumin standards. Aliquots of proteins (75 μg) were loaded on 15% SDS-PAGE gels and blot transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). After blocking using 5% low-fat milk in phosphate-buffered saline, TrfA was probed with anti-TrfA antibody at a 1/5,000 dilution, followed by incubation with a secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody at a 1/50,000 dilution. Chemiluminescence was detected using the Western Pico Super Signal reagent (Pierce).

Northern blotting. TrfA transcript analysis was essentially performed as previously described (24). Total RNA (6 μg) was separated in 1% agarose formaldehyde gels and blotted to nylon membranes (Hybond-N; Amersham). An [α-^{32}P]UTP (Hartmann Analytics; FP-110; 15 TBq/mmol)-labeled *trfA* riboprobe was generated from pAM845 (Table 1; see Table S1 in the supplemental material). After plasmid linearization with Acc65I and gel purification, an [α-^{32}P]UTP-labeled complementary antisense transcript was produced by *in vitro* transcription using T7 polymerase essentially as described previously (26). Unincorporated nucleotide was removed by passage over a microspin ProbeQuant G-50 column (GE Healthcare). The riboprobe mixture was treated with DNase I (Promega; RQ1) to eliminate the template DNA, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated with ethanol in the presence of 16 μg glycogen carrier. The pellet was washed with ice-cold 70% ethanol, dried, and resuspended in a minimal volume of TE. An aliquot was tested for probe purity on a 6% polyacrylamide, 8 M urea sequencing gel. The membrane was prehybridized with QuikHyb (Stratagene) buffer and incubated overnight with the *trfA* riboprobe at 65°C. Washes were done as follows: the first wash at 55°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS for 15 min and a second wash with 1× SSC, 0.1% SDS for 10 min at 55°C, followed by one wash with 0.1× SSC, 0.1% SDS for 10 min at 55°C. The membrane was transferred to 3MM paper (Amersham Hyperfilms) without drying, sealed, and autoradiographed.

Mapping of the *trfA* transcriptional start site. The 5′ ends of *trfA* transcripts were mapped using a Smarter RACE cDNA Amplification Kit from Clontech (catalog no. 634923). Total RNA was extracted from an oxacillin-induced culture of strain ISP794, conditions under which levels of *trfA* transcription were shown to be high in pilot experiments. Gene-specific cDNA with a Smarter IIA tail was generated using 1 μg of total RNA and a *trfA* PstI-specific primer (see Table S1 in the supplemental material), according to the manufacturer's protocol. Rapid amplification of cDNA ends (RACE) was next generated using UPM (Clontech kit) and *trfA* (see Table S1 in the supplemental material) nested primers, with the following specific PCR program: 5 cycles of 94°C for 30 s, 94°C for 30 s, and 72°C for 5 min; 5 cycles of 94°C for 30 s, 70°C for 30 s, and 72°C for 5 min; and, finally, 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 5 min. The PCR products were run in a 2% agarose gel, and each gel band was extracted and sequenced using a *trfA* nested primer. Primer-transcript junction sites revealed by direct sequencing permitted unambiguous assignment of the 5′ transcript ends.

RESULTS

Quantitative *trfA* transcription analysis in a cohort of clinical GISA strains. Our previously published work revealed that *trfA* deletion significantly reduced glycopeptide and oxacillin resistance levels in both GISA clinical and laboratory-derived isolates (14). This result led us to the hypothesis that *trfA* plays an important role in modulating resistance levels to these antibiotics and, in addition, led us to predict that (i) *trfA* mRNA levels are altered upon addition of cell wall-active antibiotics and (ii) *trfA* steady-state mRNA levels were significantly higher in MRSA strains displaying stable reduced susceptibility to glycopeptides than in their susceptible counterparts. As a first step to test these possibilities, we addressed the hypothesis that steady-state *trfA* transcript levels were altered in a set of five isogenic clinical-strain pairs consisting of a pretherapy susceptible MRSA isolate and its corresponding posttherapy GISA derivative (27). We observed that *trfA* transcript levels analyzed by qRT-PCR were indeed significantly ($P < 0.05$) increased by 2- to 3-fold in four of the five GISA derivatives tested compared to their non-GISA parents (Fig. 1). Identical transcriptional *trfA* alterations are not expected in all GISA

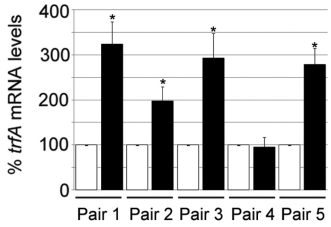

FIG 1 Analysis of *trfA* mRNA levels in glycopeptide-susceptible and GISA paired clinical strains. Steady-state levels of *trfA* transcripts were determined by qRT-PCR and normalized to 16S rRNA. All GISA mRNA levels (black bars) were compared to the corresponding glycopeptide-susceptible strains (white bars). The values represent the means and standard errors of the mean (SEM) of three independent experiments. *, results significantly different by Student's two-tailed *t* test ($P < 0.05$).

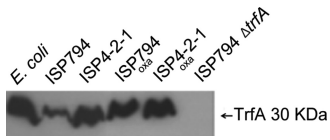

FIG 3 Western blot analysis of TrfA. Total soluble protein extracts (75 μg) from *E. coli* and *S. aureus* strains were loaded in SDS 15% acrylamide gels. TrfA protein (30 kDa) was detected using rabbit-polyclonal anti-peptide-TrfA antibodies (see Materials and Methods), and a typical Western blot is shown. As reference controls, an aliquot of a whole-cell extract of IPTG-induced *S. aureus* TrfA produced in *E. coli* (lane 1 from left) and an extract from an ISP794 control strain lacking *trfA* (lane 6) were included. Lanes 2 and 3, respectively, compare extracts derived from ISP794 or it isogenic Tei^r derivative. Lanes 4 and 5 show the results for ISP794 and ISP4-2-1, each exposed to 1 μg/ml of oxacillin (lane 4 and 5). The position of the 30-kDa protein marker is shown on the right.

strains, since different genetic changes could drive emergence of the GISA phenotype. We conclude from these results that the GISA phenotype could be correlated with increased *trfA* transcription and that *trfA* levels could be considered a characteristic feature of some GISA strains.

Cell wall-active antibiotics induce *trfA* gene transcription. We next explored how *trfA* transcription was affected by exposure of cells to cell wall-active antibiotics. We chose to use the 8325-derived strain ISP794 for these studies because of the extensive use of the strain in our and other laboratories for genetic analysis of antibiotic resistance mechanisms. We observed a significant 3-fold induction ($P < 0.01$) of *trfA* following a 1-h exposure of strain ISP794 to 1 μg/ml oxacillin (Fig. 2). These conditions were chosen because oxacillin has been widely used to induce cell wall stress in a variety of *S. aureus* strains (28, 29). In contrast, we observed that control mRNA levels of a housekeeping gene encoding the nucleoid protein Hu were not significantly affected by oxacillin treatment (data not shown).

To extend these findings, we further tested the effects of other cell wall-active antibiotics: vancomycin, teicoplanin, and D-cycloserine. We observed that *trfA* transcription was significantly ($P < 0.05$) induced in every case compared to an untreated control, by 2-, 2.8-, and 2.4-fold following brief exposure to 10 μg/ml vancomycin, teicoplanin, and D-cycloserine, respectively. In contrast, no significant transcriptional alteration (exceeding ±25% of the untreated control) of the housekeeping control gene *hu* mRNA levels was observed with the same treated samples (data not shown). To address the question of whether *trfA* transcriptional induction was obtained exclusively with cell wall-active antibiotics, we exposed cells to ciprofloxacin, an inhibitor of DNA gyrase and type IV topoisomerase, and observed no induction of *trfA* transcription (Fig. 2). Collectively, we conclude from these results that *trfA* transcription is induced by four antibiotics encompassing three distinct classes and known to induce cell wall stress.

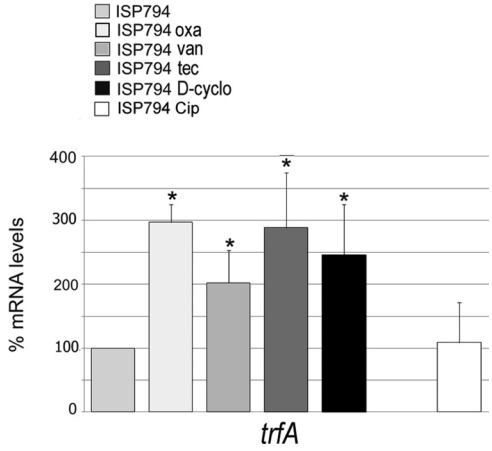

FIG 2 Induction of *trfA* mRNA levels by cell wall-active antibiotics. Steady-state mRNA levels of *trfA* and *hu* (control) were determined by qRT-PCR and normalized to 16S rRNA. The mRNA levels of antibiotic-treated bacteria were compared to that of strain ISP794 in the absence of antibiotic addition. oxa, oxacillin; van, vancomycin; tec, teicoplanin; D-cyclo, D-cycloserine; Cip, ciprofloxacin. The values reported represent the means and SEM of at least three independent experiments. *, results significantly different by Student's two-tailed *t* test ($P < 0.05$). Note the absence of *trfA* induction by the non-cell-wall-targeting drug ciprofloxacin.

Steady-state TrfA protein levels rise in response to oxacillin challenge. Affinity-purified rabbit polyclonal anti-TrfA antibody was prepared against an amino-terminal TrfA peptide (see Materials and Methods). The antibody specificity was first confirmed using recombinant *S. aureus* TrfA produced in *Escherichia coli* (Fig. 3, lane 1, and data not shown). Western blot analysis consistently detected a band apparently migrating at 30 kDa, consistent with the predicted 28.3-kDa TrfA molecular mass. TrfA migrating with the same apparent molecular mass was detected by Western blotting of whole-cell protein extracts derived from *S. aureus* Tei^s strain ISP794 and its Tei^r (GISA) derivative, ISP4-2-1 (Fig. 3, lanes 2 and 3). In contrast, no TrfA was detected in an *S. aureus* strain containing an internal disruption of *trfA* (Fig. 3, compare lanes 2, 3, and 6). Notably, we consistently observed higher steady-state TrfA levels in Tei^r ISP4-2-1 than in its Tei^s parent (Fig. 3, compare lanes 2 and 3). TrfA levels were also significantly increased in ISP794 exposed to oxacillin (under the same conditions as for Fig. 2) compared to the untreated control (Fig. 3, lanes 2 and 4). Oxacillin did not result in a significant increase in steady-state levels of TrfA in strain ISP4-2-1. It is worth mentioning that we consistently observed TrfA levels that were comparable between extracts from strain ISP4-2-1 and ISP794 treated with oxacillin.

Taken together, we conclude from these results that transcriptional induction of *trfA* by various stimuli is mirrored by comparable increased production of the TrfA protein.

Transcriptional analysis of *trfA*. The induction of *trfA* by antibiotics targeting cell wall biosynthesis led us to examine *trfA* transcriptional regulation in detail. As a first step to dissect the regulatory pathways controlling *trfA* expression in *S. aureus*, *trfA*

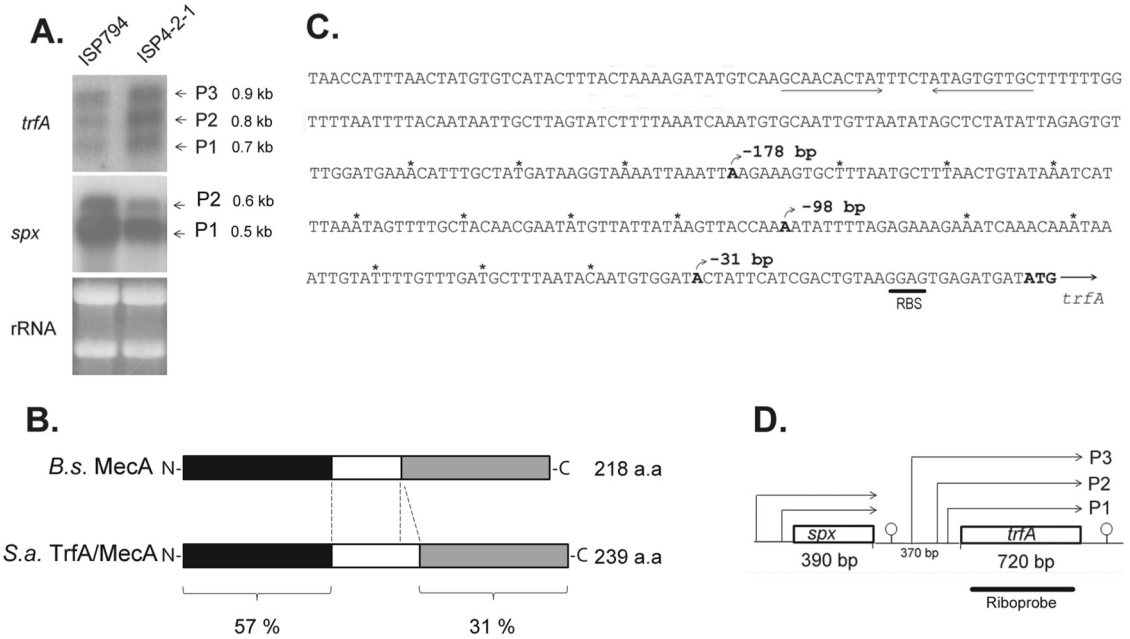

FIG 4 Transcriptional analysis of *trfA*. (A) Northern blot analysis of *trfA* and *spx* in strains ISP794 and ISP4-2-1, using ^{32}P-radiolabeled RNA *trfA*- and *spx*-specific probes. The arrows indicate the *trfA* (0.7-kb, 0.8-kb, and 0.9-kb) and *spx* (0.6-kb and 0.5-kb) transcripts. Ethidium bromide-stained rRNA from the agarose gel prior to blot transfer is shown as a loading control. (B) Schematic representation of *B. subtilis* (*B.s.*) MecA and *S. aureus* (*S.a.*) TrfA/MecA proteins. Throughout the text, we refer to *S. aureus* TrfA to avoid confusion with *S. aureus mecA*, a gene unrelated to the MecA adaptor protein of *B. subtilis* and encoding an alternative penicillin binding protein responsible for the MRSA phenotype in the organism. The N-terminal and C-terminal protein regions are depicted in black and gray and show 57% and 31% protein identity, respectively. The linker region (white) is smaller in *B. subtilis* MecA. (C) Sequence of the *trfA* promoter region. The three different nucleotides corresponding to transcriptional start sites detected by 5′RACE are shown in boldface, and the rho-independent transcriptional terminator of *spx* is underlined. RBS, ribosome binding site. Asterisks mark each 10-nucleotide region. (D) Schematic diagram showing *spx* and *trfA* gene transcription organization. The arrows indicate *spx* or *trfA* transcripts produced from the corresponding promoters. Predicted rho-independent transcriptional terminators are shown.

transcription was first examined by both Northern analysis and 5′-RACE mapping. Northern blots consistently detected three transcripts of approximately 0.9, 0.8, and 0.75 kb in both strains ISP794 and ISP4-2-1, using a strand-specific *trfA* riboprobe spanning only *trfA* coding sequence (Fig. 4A). Under the conditions of the assay, we consistently noted that the three transcripts were of comparable intensities, suggesting equivalent promoter usage. The transcripts detected were sufficient to encode full-length TrfA (predicted 239 amino acids) (Fig. 4B), and furthermore, Northern hybridization with a specific *spx* probe confirmed the presence of two monocistronic upstream *spx* transcripts of 0.6 and 0.5 kb, as previously described (30) (Fig. 4A). Characterization by 5′-RACE amplification of the 5′ ends of each *trfA* transcript of *S. aureus* allowed unambiguous identification of 3 distinct start sites, located at coordinates −178, −98, and −31 nucleotides upstream of the TrfA ATG translation initiation codon, respectively (Fig. 4C and D). Taking into consideration the length of the *trfA* coding sequence, but not 3′ untranslated regions, the three start sites produced calculated *trfA* transcripts of 898, 818, and 751 bp, corroborating the *trfA* monocistronic transcripts observed by Northern blotting.

Collectively, from these results, we conclude that *trfA* is transcribed monocistronically from three promoters. This finding contrasts with a previously published prediction placing *trfA* within a large multigene operon (31).

Searching for regulators of *trfA*. Previous studies had established that many members of the cell wall stress regulon were under the control of the cell wall stress sentinel two-component system VraRS (29, 32). In light of the aforementioned induction of *trfA* by various cell wall-active antibiotics, the impact of VraRS on transcriptional regulation of *trfA* was tested in the presence or absence of oxacillin. We observed an identical pattern of *trfA* transcription by Northern blotting in a Δ*vraSR* disruption mutant of ISP794 compared with its isogenic wild-type parent when incubated in antibiotic-free medium (see Fig. S1A in the supplemental material). Importantly, incubation of ISP794 and its Δ*vraSR* mutant in an oxacillin-containing medium resulted in identical antibiotic-triggered upregulation of *trfA* transcription for both strains (see Fig. S1B in the supplemental material). We also detected no impact on *trfA* transcription by disruption of GraRS, another two-component regulator of cell wall antibiotic stress (data not shown). A third two-component phosphosignaling system (TCS), WalKR is essential in *S. aureus*, precluding direct examination of its genetic disruption upon *trfA* transcription. However, recent comprehensive mapping of WalRK-regulated genes and determination of a WalR consensus binding site failed to provide any evidence for its role in *trfA* regulation (33). Collectively, we conclude that none of the TCSs implicated in cell wall sensing play a detectable role in the transcriptional regulation of *trfA*.

The alternative sigma factor σ^B mediates many responses to diverse environmental stresses in *S. aureus*, so we next examined whether it played any role in *trfA* transcriptional regulation. Many laboratory strains derived from 8325 (including ISP794) show a defective σ^B stress response because of constitutive sequestration of σ^B by an anti-sigma factor resulting from a defective RsbU phosphatase (34).

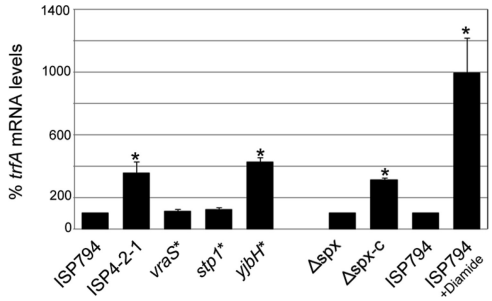

FIG 5 Analysis of *vraS** (vraSG45R), *stp1** (stp1Q12stop), *yjbH** (yjbHK23stop), and *spx* on *trfA* mRNA levels. Strains harboring each of the three nucleotide changes detected in strain ISP4-2-1 compared to ISP794 (10) were used to determine which mutation(s) conferred enhanced *trfA* expression in strain ISP4-2-1. Steady-state levels of *trfA* transcripts were determined by qRT-PCR and normalized to 16S rRNA. Steady-state levels of *trfA* were also compared between the Δ*spx* mutant and its *spx*$^+$ restored derivative (Δ*spx*-c), as well as between ISP794 treated with the thiol-specific oxidant diamide and untreated ISP794. The values represent the means and SEM of three independent experiments. *, results significantly different by Student's two-tailed *t* test ($P < 0.05$).

We performed qRT-PCR using both ISP794 (*rsbU* mutant) and its corresponding *rsbU*$^+$ restored derivative strain ISP794 (*rsbU*$^+$) (see Fig. S1C in the supplemental material) (24). As expected, the restoration of *rsbU*$^+$ in ISP794 strongly restored σ^B activity, since significant ($P < 0.05$) 7-fold-increased mRNA levels were observed for *asp23*, a gene known to be exclusively σ^B dependent (35). In contrast, no difference was observed for *vraR* mRNA levels known to be regulated in a σ^B-independent manner (36). We observed only a minor change in basal *trfA* transcription (<1.5-fold) in the *rsbU*$^+$ restored strain compared to ISP794 (see Fig. S1C in the supplemental material). The σ^B regulon has been extensively studied in *S. aureus*, and consistent with our findings, no σ^B consensus promoter motif or altered *trfA* mRNA levels were reported by transcriptome analysis (36). Since the addition of various cell wall-active antibiotics results in a robust induction of *trfA* transcription in ISP794 in the absence of significant σ^B activity in this strain background (Fig. 2), we conclude from these experiments that *trfA* is not part of the σ^B regulon.

***trfA* transcription is modulated by stabilization of Spx, a global regulator of thiol/oxidative stress.** The strong constitutive upregulation of *trfA* transcription observed in the Tei^r strain ISP4-2-1 compared to Tei^s ISP794 (Fig. 4A) prompted us to examine in detail which of the three previously studied mutations discovered in ISP4-2-1 (10) could account for this observation. ISP4-2-1 harbors two nonsense mutations: one in *stp1* (Q12stop), encoding a serine/threonine phosphatase, and one in *yjbH* (K23stop), encoding a negative regulator of the thiol/oxidative-stress global regulator Spx. The third mutation is a nonconservative missense mutation (G45R) in VraS, the sensor histidine kinase of the VraRS two-component system. In our previous study, we had reconstructed each mutation found in ISP4-2-1 and prepared all possible single, double, and triple mutations in the ISP794 genetic background (10).

The results of qRT-PCR analysis (Fig. 5) using the three single-mutation derivative strains of ISP794 shows that neither *vraS*G45R nor *stp1*Q12stop had any significant impact on *trfA* transcription. In contrast, we observed that *yjbH*K23stop significantly increased *trfA* transcription, by 4.2-fold compared with wild-type ISP794. Interestingly, this transcriptional upregulation of *trfA* was at least equivalent to the 3.5-fold increase observed in ISP4-2-1 compared with its parent, ISP794 (Fig. 5). We conclude that the loss of *yjbH* most likely fully accounts for the observed altered regulation of *trfA* transcription in strain ISP4-2-1.

Disruption of *yjbH* in *S. aureus*, as well as in *B. subtilis*, is known to result in stabilization of Spx (10, 37, 38). Thiol/oxidative stress triggers a release of YjbH from Spx, permitting it to interact with the α-C-terminal domain (αCTD) of RNA polymerase to direct expression of Spx-regulated genes (38–40). In order to determine whether *trfA* was regulated by Spx, we performed qRT-PCR analysis on RNA obtained from a strain lacking *spx* or a derivative strain where *spx* had been restored by chromosomal insertion of cloned *spx*$^+$ under the control of its own promoter (30). The data in Fig. 5 revealed a significant (3.1-fold) increase in *trfA* transcription in the complemented *spx* deletion strain compared to the *spx* deletion mutant. To further demonstrate Spx-dependent transcription of *trfA*, we hypothesized that induction conditions known to increase Spx protein levels via thiol stress (37, 41) would enhance transcription of the *trfA* gene. The addition of the thiol-specific oxidant diamide (5 mM) to strain ISP794 strongly induced *trfA* transcription by approximately 9-fold compared to the untreated control (Fig. 5). Taken together, these results led us to conclude that *trfA* is regulated by Spx.

Moreover, similar *spx*-dependent transcription of *trfA* in both *rsbU* mutant and *rsbU*$^+$ strain backgrounds was observed (see Fig. S2 in the supplemental material). Restoration of the defective *rsbU* mutant gene present in the Δ*spx* strain by phage transduction from donor strain AR852 (24) carrying the *rsbU*$^+$*VW-sigB* operon tetracycline inserted nearby shows identical patterns of *trfA* expression in both backgrounds, with or without oxacillin administration.

Effect of oxacillin on *spx* expression. Our finding that *trfA* transcription could be induced by a variety of antibiotics targeting various steps in cell wall biosynthesis led us to ask next whether cell wall antibiotic stress altered *spx* transcription. Using qRT-PCR, we measured *spx* transcription in oxacillin-treated compared to untreated bacteria, together with *hu*, encoding a nucleoid protein not known to be significantly altered by cell wall antibiotic stress (Fig. 6A). The results showed that neither *spx* nor *hu* transcription was detectably altered by the addition of oxacillin under conditions where *trfA* was otherwise strongly induced by this treatment (Fig. 2 and 5B). In contrast to these results, Western blot analysis performed with extracts from the same samples used for RNA extraction revealed strongly enhanced Spx protein levels in oxacillin-treated extracts compared to the untreated control (Fig. 6B). These data strongly suggest that posttranscriptional regulation of Spx accounts for the induction of *trfA* transcription following oxacillin exposure. Taken together with our findings with *yjbH* noted above, our results further suggest a model whereby exposure to cell wall-active antibiotics results in signals that disrupt the negative regulation of Spx by YjbH by an as-yet-unknown mechanism in *S. aureus*.

DISCUSSION

The mechanisms underlying low-level glycopeptide resistance are multifactorial and still poorly understood. Since glycopeptides are considered to be among first-line drugs for the treatment of MRSA, there is considerable research devoted to understanding these mechanisms, as well as interest in identifying target genes or

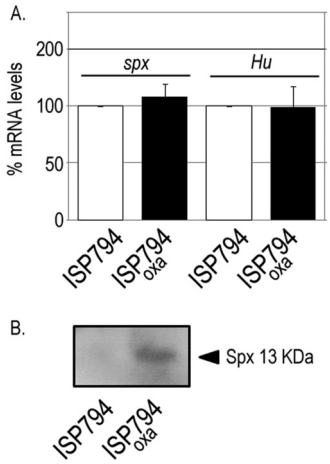

FIG 6 Effect of oxacillin on *spx* mRNA levels. (A) Steady-state levels of *spx* and *hu* transcripts were determined by qRT-PCR and normalized to 16S rRNA in strain ISP794 compared to oxacillin-treated (oxa) ISP794 cells. The values represent the means and SEM of three independent experiments. (B) Western blot analysis of total soluble protein extracts (75 μg) of *S. aureus* ISP794 or the same strain treated with oxacillin loaded in SDS-15% acrylamide gels. Spx protein (13 kDa) was detected using rabbit polyclonal anti-*S. aureus* Spx antibody as previously described (10).

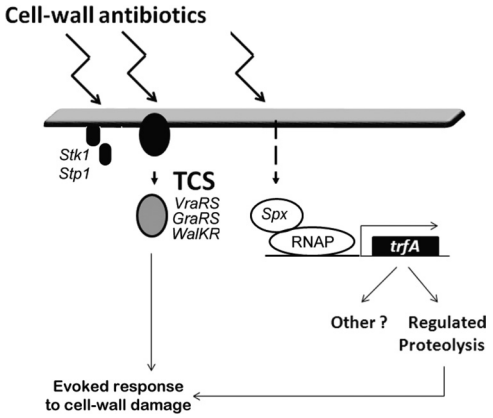

FIG 7 Model of proposed pathways regulating the evoked response to cell wall antibiotic encounter. Stk1/Stp, serine/threonine kinase-phosphatase sensor; RNAP, RNA polymerase. The dashed arrow denotes the presumptive pathway leading to Spx protein stabilization.

lead compounds that would restore sensitivity of MRSA strains to drugs such as β-lactams.

In previous work from our laboratory using both GISA and MRSA strains, we discovered that *S. aureus trfA* played an important role in both glycopeptide and β-lactam resistance in the organism (14). Study of TrfA/MecA in other organisms, notably *B. subtilis*, suggests that it has numerous biological functions, including as an assembly chaperone for ClpC, as an adaptor protein for regulated proteolysis of bound substrates, and as a regulator of transcription factor function (42–45). Precisely how *trfA* contributes to drug resistance in *S. aureus* is unknown.

In the present study, we report that a panel of four cell wall-active antibiotics leads to induction of the *S. aureus trfA* promoter and that transcription is dependent upon Spx, a global regulator of oxidative-stress defense. We did not find evidence that linked *trfA* transcription to other known sensory systems related to cell wall stress or cell wall antibiotic resistance, such as VraRS or GraRS (32, 46), or the global alternative stress sigma factor SigB (36). Detailed transcriptome and/or binding site analysis also failed to reveal that *trfA* was controlled by WalKR/YycFG (33, 47) or detectably altered by disruption of the Stk1 kinase (48, 49). Our present findings, together with other published studies, indicate that encounters with cell wall-active antibiotics not only can trigger the induction of multiple sensory pathways that rely, for example, upon transmembrane phosphosignaling mechanisms, but also can trigger pathways that lead to posttranscriptional stabilization of Spx and concomitant changes in the expression of Spx-dependent genes. Figure 7 depicts a model summarizing these various sensory systems and their collective roles in mediating responses to antibiotic-induced cell wall damage.

A central role for *trfA* as a modulator of multiple stress defenses is underscored by other published reports. A survey of global transcription profiling studies in *S. aureus* using various strains uncovered conditions showing that *trfA* is induced as part of the stringent response triggered by exposure to mupirocin (50), is upregulated following nitrosative stress and exposure to subinhibitory sodium nitrite (51), and responds to the proton ionophore carbonyl cyanide *m*-chloromethyl hydrazone (52). A microarray-based transcriptome study also revealed *trfA* transcriptional induction by both daptomycin (a calcium-dependent membrane-active lipopeptide antibiotic) and oxacillin, but it was not explored in detail (52).

In *B. subtilis*, supporting our results presented here, induction of a proteolytically stabilized Spx variant (Spx-DD) leads to upregulation of *mecA* and *trfA* (53), and recent work using chromatin immunoprecipitation methods revealed Spx occupancy of the *trfA* promoter under basal conditions that becomes strongly enhanced following exposure to diamide (54). Additional studies in *B. subtilis* have established that disulfide stress triggers the stringent response and that most major oxidative-stress genes were induced by disulfide stress (55). Loss of YjbH, a negative regulator and interacting partner of Spx, has been linked to reduced sensitivity to diamide (38) in *B. subtilis*, as well as nitrosative stress via altered susceptibility to sodium nitroprusside (56). In *S. aureus*, disruption of the corresponding YjbH ortholog results in pleiotropic effects that include altered sensitivity to β-lactam and glycopeptide antibiotics (10, 37, 57), as well as enhanced peptidoglycan cross-linking and overproduction of penicillin binding protein PBP4 (57).

The diversity of stress stimuli channeled through Spx comes primarily from studies in *B. subtilis*. The five *spx* promoters are controlled by four different sigma factors; at least two stress-sensitive repressors, PerR and YodB, binding to the RNA polymerase αCTD governed by a redox-sensitive CXXC switch; and protein levels modulated by ClpXP directed by the Spx partner protein and negative regulator YjbH (40, 58–60). The recently discovered YjbH-interacting protein YirB acts as an antiadaptor by inhibiting YjbH-mediated proteolysis of Spx (39). Many of these regulatory features are likely preserved in *S. aureus* (30, 38), with the exception of multiple sigma factor control, the fact that *spx* is bicistronic in *B. subtilis* but monocistronic in *S. aureus*, and the apparent lack of a protein with similarity to YirB (W. L. Kelley, unpublished observations).

Of the cell wall-active drugs tested in this study that lead to

upregulation of *trfA*, three have sites of action outside the cell membrane (vancomycin, teicoplanin, and oxacillin), while one targets a cytosolic enzyme (D-cycloserine). The mechanism leading to *spx*-dependent upregulation of *trfA* in response to these various agents targeting cell wall biosynthesis must ultimately take into consideration how the various drug-induced stresses are sensed. Our observation that Spx protein levels were dramatically stabilized using oxacillin as a stimulus whereas *spx* transcription was unaffected strongly suggests that cell wall antibiotic stress acts on Spx primarily at the posttranscriptional level. Redox regulation of cysteine residues in YjbH has been proposed as a mechanism governing the proteolytic turnover of Spx (40, 57). In this scenario, oxidation of cysteines would have the dual effect of disrupting YjbH-Spx interaction, as well as possibly promoting Spx-αCTD interaction through oxidation of the Spx cysteine switch (58). The discovery of YirB in *B. subtilis* raises the additional possibility that competitor proteins can also disrupt YjbH-Spx interaction. In this regard, it is tempting to speculate that cell wall antibiotic stress in *S. aureus* results in the production of ROS, triggers induction of hypothetical YjbH antiadaptor protein expression, or modulates YjbH expression or turnover, resulting in altered YjbH-Spx stoichiometry. These mechanisms are not necessarily mutually exclusive. Evidence exists that *S. aureus* encounters with certain bactericidal antibiotics, including β-lactams and vancomycin, can trigger production of ROS, resulting in bacterial killing (61, 62). However, recent studies have challenged this model, and thus, the role of ROS production linked to antibiotic killing is controversial (63, 64).

A key question is what does a cell gain by stabilizing Spx in response to cell wall antibiotic stress and among the ensuing consequences driving *trfA* transcription? Among the genes included in the Spx regulon are those dedicated to oxidative-stress defense and redox homeostasis, which are clearly beneficial (41). In addition, Spx is thought to mediate both positive and negative regulation of genes that impact intermediary metabolism and has been proposed to exert a metabolic brake to attenuate growth and production of endogenous ROS until damage is repaired and the noxious stimulus is removed (41, 60). This notion is reminiscent of the SOS-response-mediated inhibition of cell division or the growth arrest mediated by PBP inhibition and the DpiAB TCS in *E. coli* in response to β-lactams (65). Finally, previous work from our laboratory (10) revealed that loss of *spx* resulted in a significant decrease in the frequency of emergence of low-level glycopeptide mutants, suggesting that Spx-dependent gene regulation impacts antibiotic resistance at many levels.

The understanding of glycopeptide resistance in *S. aureus* is far from complete. Mutations in numerous distinct genes, either individually or collectively, can contribute to altered susceptibilities. Since signaling systems often appear mutated, it is clear that effects mediated by genes under the control of these signaling systems will ultimately have an effect on drug resistance. Our present results add to this evolving story and, further, suggest a role for TrfA in some, but perhaps not all, pathways that govern glycopeptide and other cell wall-active antibiotic resistance mechanisms in *S. aureus*.

A role for TrfA as an adaptor and assembly factor for ClpC opens numerous possibilities for regulated proteolysis and cellular processes controlled by ClpCP (66–69). Global studies of ClpC reveal this network to be quite extensive (70, 71), and preliminary work shows that deletion of *clpC* in *S. aureus* closely mirrors the effect of *trfA* deletion with respect to glycopeptide resistance (A. Renzoni, unpublished data). Furthermore, cell wall antibiotic resistance is often correlated with changes in cell wall thickness, peptidoglycan cross-linking, or decreased autolysis (10). As an adaptor protein linked with proteolysis, TrfA could conceivably contribute to the regulation of any of these steps (17, 42, 43). Preliminary data indeed show that *trfA* deletion significantly affects cell wall thickness and morphology (Renzoni, unpublished). Clearly, identifying TrfA-interacting proteins and elucidating its role as a ClpCP adaptor will be of paramount importance in future studies.

The cell wall stress regulon/stimulon includes genes induced by certain antibiotics and subject to VraR-dependent regulation (29, 52). Although *trfA* is not formally part of the cell wall stress regulon by these criteria, *trfA* nevertheless is clearly upregulated in response to multiple cell wall-active antibiotics. Our study therefore highlights a previously unrecognized link between cell wall antibiotic stress and gene expression governed by an RNA polymerase-interacting factor responding to oxidative stress. These findings clearly reveal that the cell wall stress regulon is more complex than previously imagined.

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