The Staphylococcus aureus thiol/oxidative stress global regulator Spx controls trfA, a gene implicated in cell-wall antibiotic resistance

Ambre Jousselin, William L. Kelley#, Christine Barras, Daniel P. Lew,
and Adriana Renzoni#

Service of Infectious Diseases, University Hospital and Medical School of Geneva, CH-1211 Geneva 14

Running title: S. aureus TrfA transcription regulation

# Corresponding authors. Mailing address: Service of Infectious Diseases, University Hospital and Medical School of Geneva, CH 1211 Geneva 14, Switzerland.
Phone: (4122) 3795651. Fax: (4122) 3795702. E-mail: Adriana.Renzoni@hcuge.ch
William.Kelley@unige.ch
ABSTRACT

*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 *B. subtilis* encoding an adaptor protein implicated in multiple roles, notably proteolysis and genetic competence. Despite strong sequence similarity with *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 (MRSA) clinical or laboratory isolates. Northern blot analysis and 5’ 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. The *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.
INTRODUCTION

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 encounter with strains with altered susceptibility to antibiotics, such as methicillin-resistant *S. aureus* (MRSA). Glycopeptide antibiotics (vancomycin and teicoplanin) are frequently considered as the mainstay for therapy of MRSA infections. Recent studies suggest, however, that relatively minor increases in minimal inhibitory concentration (MIC) levels to 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, termed VRSA (vancomycin-resistant *S. aureus*), arises from infrequent horizontal acquisition of Tn1546 encoding the multigene 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 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 4-8 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 encounter (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* which contribute to the acquisition or loss of the resistance phenotype (10-14). The two component histidine kinase sensors *graRS* and *vraRS*, as well as the serine/threonine kinase...
Stk1/stp phosphatase encode 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 encounter 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 that we named trfA and trfB for teicoplanin-resistant factor 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 trfA or trfB genes in S. aureus remains undefined. Conceptual translation of trfA indicates that it most closely resembles the MecA adaptor protein of Listeria monocytogenes and Bacillus subtilis (14) whereas the conceptual translation of trfB shows strong similarity with YjbF of B. subtilis, also called CoiA in Streptococcus pneumonia (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 to strains acquiring any of several allotypes of the horizontally transmitted SCCmec 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 handful of substrates bound by MecA in *B. subtilis* and fed to proteolytic machinery includes 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 activity of various antibiotics to the production of reactive oxygen species.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** 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* were grown in Mueller-Hinton broth (MHB) and *E. coli* strains in Luria-Bertani medium supplemented 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 until O.D.₆₀₀ = 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 (5mM) were 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 qRT-PCR probes in the absence of reverse transcription.

### Quantitative real-time qRT-PCR.
The mRNA levels were determined by quantitative RT-PCR (qRT-PCR) using the one-step reverse transcriptase qPCR Master Mix Kit (Eurogentec, Seraing, Belgium) as described (23). Primers and probes for *trfA*, *spx* and *hu* were designed using PrimerExpress software (version 1.5; Applied Biosystems) and obtained from Eurogentec (Table S1). 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 (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 and cloned in pBluescriptII KS+. A sequenced verified *trfA* fragment was next subcloned into *E. coli* expression vector pTYB12 (New England Biolabs) using Nde1-Pst1 sites, generating plasmid pAM873. *E. coli* strain BL21 ADE3 (New England Biolabs) containing pTYB12-TrfA protein was grown in Luria-Bertani media containing carbenicillin at 100 µg/ml until an OD₆₀₀ of 0.7, induced with 0.5 mM isopropyl-b-D-1-thiogalactopyranoside (IPTG) for an additional 150 min at room temperature.
with vigorous shaking. Bacteria were harvested by low speed centrifugation, resuspended in Laemmli buffer and whole cell extracts used in western blot analysis as described below.

**Anti-TrfA antibody production.** Rabbit polyclonal antibodies were raised in SPF New Zealand white rabbits against a 15 amino acid synthetic peptide (FSREDLWTNRKRGEE-CONH2 corresponding to amino acids 25-39 of SA0857) with MBS (m-maleimidobenzoyl-N-hydroxysuccinimide ester) conjugated KLH carrier protein using the 87 day protocol of Eurogentec (Seraing, Belgium). Specific antiserum was further affinity purified against the immunizing peptide. Peptide was affinity coupled using AF-amino Toyopearl 650M and an equal mixture of ACH Sepharose and CN-Br Sepharose. 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.** 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 growth at 37°C with agitation until an O.D.$_{600}$ = 0.5. When indicated, oxacillin (1 µg/ml) was added and bacteria were grown for an additional hour. After centrifugation, cell pellets were washed and resuspended in 500 µL TE buffer (10mM Tris pH7.5, 1mM EDTA). Bacterial cells were disrupted by adding 500 µl of acid washed glass beads (100-200 micron, Sigma) and using FastPrep cell disrupter (MP Biomedicals). The cell debris was separated from soluble protein extracts by centrifugation at 14000 rpm (10 min at 4°C). Supernatant was concentrated on Amicon spin columns (10 kDa cut-off, 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 a polyvinylidene difluoride membranes (PVDF Bio-Rad). After blocking using 5% low fat milk in phosphate buffered saline, TrfA was probed with anti-TrfA antibody at a 1/5000 dilution.
followed by incubation with a secondary HRP-conjugated goat anti-rabbit antibody at a 1/50000 dilution. Chemiluminescence was detected using the Western Pico Super Signal reagent (Pierce).

**Northern blot.** 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). A $\alpha^{32}$P-UTP (Hartmann Analytics, FP-110 15Tbq/mmol) labeled trfA riboprobe was generated from pAM845 (Table 1 and S1). After plasmid linearization with Acc65I and gel purification, an $\alpha$-$P^{32}$-UTP labeled complementary antisense transcript was produced by in vitro transcription using T7 polymerase essentially as described (26). Unincorporated nucleotide was removed by passage over a microspin ProbeQuant G-50 column (GE Healthcare). The riboprobe mixture was treated with DNasel (Promega RQ1) to eliminate the template DNA, extracted with phenol:chloroform:isoamylalcohol (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 8M 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: first wash at 55°C with 2×SSC, 0.1%SDS for 15min, 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 without drying, sealed and autoradiographed (Amersham Hyperfilms).

**Mapping of trfA transcriptional start site.** The 5' end of trfA transcripts were mapped using SMARTer RACE cDNA Amplification Kit from Clontech (Cat. No. 634923). Total RNA was extracted from an oxacillin-induced culture of ISP794 strain, a condition where levels of trfA transcription was shown to be high in pilot experiments. Gene specific cDNA with a Smarter IIA
tail was generated using 1 µg of total RNA and trfA Pst1 specific primer (Table S1), according to the manufacturer’s protocol. Rapid amplification of cDNA ends was next generated using UPM and trfA nested primers (Clontech Kit and table S1, respectively) with the following specific PCR program: 5 cycles (94°C 30sec, 94°C 30 sec, 72°C 5 min), 5 cycles (94°C 30sec, 70°C 30 sec, 72°C 5 min) and finally 30 cycles (94°C 30sec, 60°C 30 sec, 72°C 5 min). PCR products were run in a 2% agarose gel, each gel band was extracted and sequenced using 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 previous 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 compared to 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 pre-therapy susceptible MRSA isolate and its corresponding post-therapy 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 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 \textit{trfA} transcription, and that \textit{trfA} levels could be considered as a characteristic feature of some GISA strains.

Cell-wall active antibiotics induce \textit{trfA} gene transcription. We next explored how \textit{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 our extensive use of this strain in our and other laboratories for genetic analysis of antibiotic resistance mechanisms. We observed a significant 3-fold induction ($p < 0.01$) of \textit{trfA} following a 1 h exposure of ISP794 strain 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 \textit{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 effect of other cell wall active antibiotics: vancomycin, teicoplanin and D-cycloserine. We observed that \textit{trfA} transcription was significantly ($p < 0.05$) induced in every case compared to 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 untreated control) of the housekeeping control \textit{hu} mRNA levels was observed with the same treated samples (data not shown). To address the question whether \textit{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 \textit{trfA} transcription (Fig. 2). Collectively, we conclude from these results that \textit{trfA} transcription is induced by four antibiotics encompassing three distinct classes and known to induce cell wall stress.
Steady-state TrfA protein rises in response to oxacillin challenge. Affinity-purified rabbit polyclonal anti-TrfA antibody was prepared against an amino terminal TrfA peptide (Materials and Methods). The antibody specificity was first confirmed using recombinant *S. aureus* TrfA produced in *E. Coli* (Fig. 3, lane 1 and data not shown). Western blot analysis consistently detected a band migrating with an apparent 30 kDa consistent with the predicted 28.3 kDa TrfA molecular weight. TrfA migrating with the same apparent molecular weight was detected by Western blot analysis of whole cell protein extracts derived from *S. aureus* Tei strain ISP794 and its Tei (GISA) derivative ISP4-2-1 (Fig. 3, lanes 2 and 3). In contrast, no TrfA was detected in a *S. aureus* strain containing an internal disruption of *trfA* (compare lanes 2, 3 and 6, respectively). Notably, we consistently observed higher steady state TrfA levels in Tei ISP4-2-1 compared to its Tei parent (Fig. 3, compare lanes 2 and 3). TrfA levels were also significantly increased in ISP794 exposed to oxacillin (using the same conditions as for figure 2) compared to 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 worthwhile 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 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* 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 ISP794 and ISP4-2-1 strains, 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 intensity 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 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 blot.

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 blot, in a ΔvraSR disruption mutant of ISP794 compared with its isogenic wild-type parent when incubated in antibiotic-free medium (Fig. S1A). Importantly, incubation of ISP794 and its ΔvraSR mutant in an oxacillin-containing medium resulted in identical antibiotic-triggered upregulation of trfA transcription for both strains (Fig. S1B). 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 TCS system, 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 TCS systems 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* and 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).

We performed qRT-PCR using both ISP794 (rsbU^-) and its corresponding rsbU^+ restored derivative ISP794 (rsbU^+) strain (Fig. S1C) (24). As expected, the restoration of rsbU^+ in ISP794 strongly restored σ^B^ activity since a 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 (Fig. S1C). The σ^B^ regulon has been extensively studied in *S. aureus* and consistent with our findings, we found no σ^B^ consensus promoter motif nor detection of altered *trfA* mRNA levels 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 a 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 strain Tei ISP4-2-1 compared to Tei ISP794 (Fig. 4A) prompted us to examine in detail which of the previously studied three 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 regular Spx. The third mutation is a non-conservative 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 (α-CTD) 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.
The data in Figure 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 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 untreated control (Fig. 5). Taken together, these results led us to conclude that trfA is regulated by Spx.

Moreover, a similar spx-dependent transcription of trfA in both rsbU- and rsbU+ strains backgrounds was observed (Fig. S2). Restoration of the defective rsbU- gene present in Δspx strain by phage transduction from donor strain AR852 (24) carrying the rsbU+VWsigB operon tetracycline marked nearby shows an identical pattern of trfA expression in both backgrounds, with or without oxacillin administration.

The 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 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 control 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 untreated control (Fig. 6B). These data strongly suggest that
posttranscriptional regulation of Spx accounts for the induction of \textit{trfA} transcription following oxacillin exposure. Taken together with our findings with \textit{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 \textit{S. aureus}.

**DISCUSSION**

The mechanisms underlying low-level glycopeptide resistance are multifactorial and still poorly understood. Since glycopeptides are considered 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 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 \textit{S. aureus} \textit{trfA} played an important role in both glycopeptide and β-lactam resistance in this organism (14). Study of TrfA/MecA in other organisms, notably \textit{B. subtilis}, suggests that it has numerous biological functions including an assembly chaperone for ClpC, an adaptor protein for regulated proteolysis of bound substrates, and a regulator of transcription factor function (42-45). Precisely how \textit{trfA} contributes to drug resistance in \textit{S. aureus} is unknown.

In the present study, we report that a panel of four cell wall active antibiotics leads to induction of the \textit{S. aureus} \textit{trfA/mecA} promoter and transcription is dependent upon Spx, a global regulator of oxidative stress defense. We did not find evidence that linked \textit{trfA} transcription to other known sensory systems linked 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 \textit{trfA} was controlled by \textit{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 encounter with cell wall active antibiotics can trigger not only the induction of multiple sensory pathways that rely, for example, upon transmembrane phosphosignaling mechanisms, but can also trigger pathways that lead to post-transcriptional 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 mediating responses to antibiotic-induced cell wall damage.

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

In \textit{B. subtilis}, and in support of our results presented herein, induction of a proteolytically stabilized Spx variant (Spx-DD) leads to upregulation of \textit{mecA}/\textit{trfA} (53) and recent work using chromatin immunoprecipitation methods reveal Spx occupancy of the \textit{trfA} promoter under basal conditions that becomes strongly enhanced following exposure to diamide (54). Additional studies in \textit{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 \textit{B. subtilis} as well as nitrosative stress via altered susceptibility to sodium nitroprusside (56).
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 crosslinking 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. 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 post-transcriptional 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 reactive oxygen species (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* encounter 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 removed (41, 60). This notion is reminiscent of the SOS-response mediated inhibition of cell division, or the growth arrest mediated by the 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 often signaling systems 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 (Renzoni, unpublished data). Furthermore, cell wall antibiotic resistance is often correlated with changes in cell wall thickness, peptidoglycan crosslinking 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 (A. Renzoni, unpublished data). 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 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.

ACKNOWLEDGEMENTS

This work was supported by the Swiss National Science Foundation grants 310030-125109 (DL) and 3100A0-120428 (WLK) and the Canton of Geneva. We thank Pierre Vaudaux for critical comments and Dorte Frees and Hanne Ingmer (Royal Veterinary and Agricultural University, Denmark) for strains and helpful comments.

REFERENCES


FIGURE LEGENDS

FIG. 1. Analysis of trfA mRNA levels in glycopeptide-susceptible and glycopeptide-intermediate (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) were compared to the corresponding glycopeptide-susceptible strain (white). Values represent the mean ± SEM of three independent experiments. (*) Represents results significantly different by student’s two-tailed t-test (p <0.05).

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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 antipeptide-TrfA antibodies (Materials and Methods) and a typical western blot is shown. As reference controls, an aliquot a whole cell extract of IPTG-induced S. aureus TrfA produced in E. coli (lane 1) together with an extract from an ISP794 control strain lacking trfA (lane 6) were included. Lanes 2 and 3 compare, respectively, extracts derived from ISP794 or it isogenic Tei7 resistant derivative. Lanes 4 and 5 show the result of ISP794 and ISP4-
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**FIG. 4. Transcriptional analysis of trfA.** A) Northern blot analysis of trfA and spx in ISP794 and ISP4-2-1 strains, using radiolabeled 32P RNA trfA and spx-specific probes. Arrows indicate the trfA (P1: 0.7 kb, P2: 0.8 kb and P3: 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 this organism. The N-terminal and C-terminal protein regions are depicted in black and gray and show 57 % and 31 % of protein identity, respectively. The linker region (white) is smaller in B.s. MecA. C) Sequence of the trfA promoter region. The three different nucleotides corresponding to transcriptional start sites detected by 5’RACE are shown in bold and the rho-independent transcriptional terminator of spx is underlined. D) Schematic diagram showing spx and trfA gene transcription organization. Arrows indicate spx or trfA transcripts produced from the corresponding promoters. Predicted rho-independent transcriptional terminators are shown.

**FIG. 5. Analysis of vraS*, stp1*, yjbH* and spx on trfA mRNA levels.** A) Strains harboring each of the three nucleotide changes detected in strain ISP4-2-1 compared to ISP794 (Renzoni, 2011) 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 mutant Δspx and its spx+
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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 ISP794 cells. Values represent the mean ± 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. Abbreviations: TCS (Two-component phospho-signaling systems); Stk1/STp (serine/threonine kinase-phosphatase sensor); RNAP (RNA polymerase). A dashed arrow denotes the presumptive pathway leading to Spx protein stabilization.
TABLE 1. Bacterial strains and plasmids used in this study

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