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The Posttranslocational Chaperone Lipoprotein PrsA Is Involved in both Glycopeptide and Oxacillin Resistance in *Staphylococcus aureus*

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Understanding in detail the factors which permit *Staphylococcus aureus* to counteract cell wall-active antibiotics is a prerequisite to elaborating effective strategies to prolong the usefulness of these drugs and define new targets for pharmacological intervention. Methicillin-resistant *S. aureus* (MRSA) strains are major pathogens of hospital-acquired and community-acquired infections and are most often treated with glycopeptides (vancomycin and teicoplanin) because of their resistance to most penicillins and a limited arsenal of clinically proven alternatives. In this study, we examined PrsA, a lipid-anchored protein of the parvulin PPIase family (peptidyl-prolyl *cis/trans* isomerase) found ubiquitously in all Gram-positive species, in which it assists posttranslocational folding at the outer surface of the cytoplasmic membrane. We show by both genetic and biochemical assays that *prsA* is directly regulated by the VraRS two-component sentinel system of cell wall stress. Disruption of *prsA* is tolerated by *S. aureus*, and its loss results in no detectable overt macroscopic changes in cell wall architecture or growth rate under nonstressed growth conditions. Disruption of *prsA* leads, however, to notable alterations in the sensitivity to glycopeptides and dramatically decreases the resistance of COL (MRSA) to oxacillin. Quantitative transcriptional analysis reveals that *prsA* and *vraR* are coordinately upregulated in a panel of stable laboratory and clinical glycopeptide-intermediate *S. aureus* (GISA) strains compared to their susceptible parents. Collectively, our results point to a role for *prsA* as a facultative facilitator of protein secretion or extracellular folding and provide a framework for understanding why *prsA* is a key element of the VraRS-mediated cell wall stress response.

An impressive arsenal of virulence factors enables *Staphylococcus aureus* to promote diseases ranging from relatively minor soft tissue infections to life-threatening septicemia or endocarditis (42). Substantial antibiotic pressure has resulted in the emergence of multiresistant *S. aureus*, and this has led to a worldwide and alarming public health problem. Of particular importance and concern are infections caused by hospital- or community-acquired methicillin-resistant *S. aureus* (MRSA) (26, 35). These infections require prudent pharmacotherapy and often require considerable epidemiological and infection control measures.

Glycopeptide antibiotics (vancomycin and teicoplanin) are considered first-line drugs to combat MRSA. The first cases of clinical isolates with reduced susceptibility to vancomycin were reported in Japan in 1997 (28). These isolates show vancomycin intermediate resistance (VISA; MIC = 4 to 8 μg/ml) and are also designated glycopeptide-intermediate strains of *S. aureus* (GISA) because they frequently, but not invariably (20), display a reduced susceptibility to teicoplanin. The endogenous mechanism leading to low-level glycopeptide resistance is thought to result from stepwise accumulation of mutations and is not fully understood, although frequently mutations impact signaling pathways linked to the remodeling of cell wall architecture (29). A rare exogenous mechanism promoting fully vancomycin-resistant *S. aureus* results from the horizontal acquisition of the VanA-type resistance mechanism from *Enterococcus* spp. (51).

Compounding the rise of reduced susceptibility to glycopeptides is the recognized difficulty in detecting its onset. Detection of GISA strains is most often based on phenotypic detection of resistance by determination of vancomycin or teicoplanin MICs or laborious population analysis profiles. Alternative phenotypic detection methods exist; however, there is an important variability between the different methodologies used, and some underestimate MIC results, which compromises reliable detection of GISA isolates (65, 69).

Numerous transcriptomic studies conducted with *S. aureus* over the course of the last decade have defined the existence of a large gene set whose expression is altered upon exposure to a variety of antibiotic compounds that target steps in cell wall biosynthesis, or by conditional depletion of key cell wall biosynthetic enzymes such as MurF or PBP2. Collectively, the ensemble of these differentially regulated genes is termed the cell wall stress regulon (16, 24, 39, 44, 61, 62, 66). Careful analysis revealed that the gene sets induced are subject to considerable interstrain variation or to the exact nature of the inducing agent (15, 45). Thus, overall cell wall stress detection mediated by several two-component histidine kinase sensory systems (TCS) such as VraRS, WalKR, and GraRS, as well as the membrane-anchored PASTA domain containing ST/K kinase Stk1 and its cognate cytosolic phosphatase Stp, would be expected to result in complex aggregate transcriptional response profiles. Some progress to decipher genes under the control of these distinct sensory systems has been made, notably with WalKR and GraRS (18, 30, 34, 73), whereas genes directly regulated by VraRS or regulatory or biosynthetic proteins modulated by serine/threonine phosphorylation remain to be explored in detail.

Despite this overall variability underlying the cell wall stress

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response, certain genes appear to be strongly induced by many cell wall-active drugs or cationic peptides. Among these highly induced genes is *prsA*, which is considered to be part of the VraRS regulon solely on the basis of transcript profiling (16, 39, 45).

PrsA is a lipid-anchored protein located on the outer face of the plasma membrane and found ubiquitously in Gram-positive species (59, 64). By sequence homology, PrsA contains a parvulin-type peptidyl-prolyl *cis-trans* isomerase (PPIase) domain. PPIases catalyze *cis-trans* isomerization of peptide bonds preceding proline residues. By protease-coupled PPIase assay, PrsA proteins from *Bacillus subtilis* and *S. aureus* were both shown to function as prolyl isomerases (25, 70). By virtue of its location, PrsA is thought to assist folding of proteins destined for secretion. *B. subtilis prsA* was linked to secretion function by virtue of its effects on secretion of α-amylase (36). Initial attempts to inactivate *B. subtilis prsA* suggested that the gene was essential particularly under conditions of high-level protein secretion (37). However, growth defect and spherical cell morphology phenotype due to low *prsA* expression could be restored by the presence of a high concentration of magnesium (31) or by inactivation of biosynthetic genes governing the D-alanylation system of teichoic acids (33). Recently, PrsA was shown to be involved in lateral wall synthesis of *B. subtilis* by folding and stabilizing PBP2a (31).

prsA has been shown to affect virulence in a variety of organisms. The secretion of *Bacillus anthracis* protective antigen (PA), a component of the edema and lethal toxins, is dependent on PrsA (72). In *Listeria monocytogenes*, two copies of the *prsA* homologue (*prsA1* and *prsA2*) are present and *prsA2* is required for full virulence by controlling activity and stability of two secreted virulence factors: listeriolysin O (LLO) and a broad-specificity phospholipase (1). In group A *Streptococcus*, the posttranslational maturation of the extracellular cysteine protease SpeB is dependent on appropriate PrsA amounts (49).

In *S. aureus*, no functional role has been identified for *prsA*. In this study, we addressed whether *prsA* transcriptional induction was indeed primarily under the direct control of the VraSR TCS by both *in vivo* and *in vitro* methods. The observation that *prsA* is part of the *S. aureus* cell wall stress regulon led us to consider its role in cell wall-active antibiotic resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Phages φ11 and φ75 (48, 50) were used as transducing phages for *S. aureus* strains. *S. aureus* cells were grown at 37°C with aeration in Mueller-Hinton broth (MHB) supplemented with antibiotics when necessary. Luria-Bertani medium (LB) was used for cultivating *Escherichia coli*. Antibiotics were used at the following concentrations: for *S. aureus*, erythromycin at 5 μg/ml, tetracycline at 1.5 μg/ml, chloramphenicol at 30 μg/ml, and kanamycin at 50 μg/ml, and for *E. coli*, ampicillin at 100 μg/ml. Clinical strains named "non-GISA/GISA paired" (1–4) were isolated from a collection of clonally related MRSA isolates from independent episodes of unique patient bloodstream infections. Importantly, all susceptible strains from the paired strain sets were collected from the same patient prior to the onset of vancomycin therapy. Glycopeptide MICs were previously determined using modified macrodilution and agar testing methods. GISA strains are characterized by elevated teicoplanin and/or vancomycin MICs (≥4 mg/liter) (58).

Transcript mapping by 5′ RACE. The 5′ ends of *prsA* transcripts were mapped using the SMARTer RACE (random amplification of cDNA ends) cDNA amplification kit from Clontech (catalog no. 634923). Total RNA was extracted from an oxacillin-induced culture of strain ISP794, using a condition where levels of *prsA* transcription were strongly and reproducibly induced in pilot experiments (data not shown). Gene-specific cDNA with a SMARTer IIA tail was generated using total RNA (1 μg) and 5′ CDS_*prsA* specific primer (Table 2), according to the manufacturer's protocol. Rapid amplification of cDNA ends was accomplished using UPM and GSP5′race_*prsA* primers (Table 2) 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. PCR products were applied to 2% agarose gels and electrophoresed, and each gel band was extracted, purified, and directly sequenced. Primer-transcript junction sites permitted unambiguous assignment of the 5′ transcript ends.

Total RNA extraction. Overnight bacterial cultures were diluted 1:100 and grown at 37°C in MHB with vigorous shaking. When indicated, teicoplanin or vancomycin (conducted for 10 min at 10 μg/ml) was added. Bacteria were harvested, and RNA extraction and verification of the absence of contaminating DNA were performed as described previously (56).

Quantitative transcript analysis by qRT-PCR. mRNA levels were determined by quantitative reverse transcriptase PCR (qRT-PCR) using the one-step reverse transcriptase qPCR master mix kit (Eurogentec, Seraing, Belgium), as described previously (56). Appropriate *prsA* and *vraR* primers and probes were designed using PrimerExpress software (version 1.5; Applied Biosystems) and obtained from Eurogentec (see Table 2 and reference 22). The mRNA levels of target genes extracted from the different strains were normalized to 16S rRNA levels, which were assayed in each round of qRT-PCR as internal controls as described previously (68). The statistical significance of strain-specific differences in normalized cycle threshold (C_T) values of each transcript was evaluated by Student's paired *t* test, and data were considered significant when P was <0.05.

Cloning and purification of recombinant VraR protein. The open reading frame of the *vraR* gene was amplified by PCR using primers listed in Table 2. The PCR fragment was cloned into KpnI and PstI sites of pET28b to yield pET28b-*vraR*. After sequence verification, pET28b-*vraR* was transformed into *E. coli* BL21(λDE3). Single colony transformants were then grown in LB containing kanamycin to an optical density at 600 nm (OD_{600}) of 0.4. Induction was performed with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h at 30°C with vigorous shaking. Bacteria were harvested by low-speed centrifugation, and recombinant 6×His-tagged VraR (His_6-VraR) was purified by nickel affinity column chromatography as recommended by the manufacturer (His-Select nickel affinity gel; Sigma). Protein concentrations were determined by using the Bradford assay (Bio-Rad) with bovine serum albumin as a reference standard. Protein purity was greater than 99% as judged by SDS-PAGE analysis with Coomassie brilliant blue staining. VraR protein purified in this manner was stable for more than 6 months when stored at 4°C.

Electrophoretic mobility shift assay. Gel shift experiments were performed essentially as described in reference 3 by incubating increasing amounts of purified His_6-VraR with 5′-end-labeled *prsA* promoter probe prepared by PCR using primers listed in Table 2. Briefly, DNA probes were labeled with [γ-^{32}P]ATP (Hartmann Analytic, FP-301; 111 TBq/mmol) using T4 polynucleotide kinase (NEB). Unincorporated radioactivity was removed by microcentrifugation on a ProbeQuant G50 spin column (GE Healthcare). Aliquots (1 to 8 μg) of His_6-VraR were incubated with the radiolabeled probe (10 fmol) for 30 min at 25°C in binding buffer. Competition experiments were performed using as a negative control a 200-fold molar excess of a fragment from the *tst* promoter region (3) known for its undetectable specific affinity for VraR under any tested conditions (D. Andrey, unpublished observations). Specific competition used a 200-fold excess of unlabeled cold *prsA* probe. All reactions were electrophoresed in 5% nondenaturing polyacrylamide gels, dried, and autoradiographed (Amersham Hyperfilm). The apparent dissociation constant (K_d) for nonphosphorylated VraR binding to the *prsA* promoter was determined essentially as described previously (3).

Construction of AR916 containing the *vraSR*::*kan* allele. A chromosomal *vraSR* disruption mutant was constructed by deletion of the con-

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Strain name	Revelant genotype or characteristic	Source or reference
E. coli			
DH5α		Restriction-deficient DNA cloning strain	Gibco/BRL
BL21(λDE3)		IPTG-inducible T7 RNA polymerase	Novagen
S. aureus			
RN4220		8325-4, $r^- m^+$, restriction-defective strain which accepts foreign DNA	38
MRGR3		Teicoplanin-susceptible parent, pair strain 5	43
14-4		Teicoplanin-resistant derivative of MRGR3, pair strain 5	67
ISP794		8325 derivative, *rsbU* mutant	63
ISP4-2-1		ISP794-derived, teicoplanin-resistant strain	57
Newman		ATCC 25904, MSSA, $rsbU^+$	4
Newman Δ*prsA*	AJ3	Newman *prsA*::Kan^r	This study
Newman Δ*prsA*-C	AJ4	Newman *prsA*::Kan^r with *prsA* integrated into the *geh* locus	This study
CYL316		Derivative of RN4220 containing the integrase for insertion of pLC84 into the lipase gene	40
CYL316-*prsA*	AJ5	CYL316 with pCL84-*prsA* integrated into the lipase gene	This study
COL		MRSA, $rsbU^+$	17
COL Δ*prsA*	AJ6	COL Δ*prsA*::Kan^r; φ80α transductant of AJ3	This study
ISPΔ*vraSR*	AR916	ISP794 Δ*vraSR*::Kan^r	This study
ISP*vra*SH156A	AR769	ISP794 *vra*SH156A; Kan^r nearby	21
Pair 1 non-GISA		Clinical MRSA glycopeptide-susceptible strain of pair 1	69
Pair 1 GISA		Clinical MRSA glycopeptide-resistant strain of pair 1	69
Pair 2 non-GISA		Clinical MRSA glycopeptide-susceptible strain of pair 2	69
Pair 2 GISA		Clinical MRSA glycopeptide-resistant strain of pair 2	69
Pair 3 non-GISA		Clinical MRSA glycopeptide-susceptible strain of pair 3	69
Pair 3 GISA		Clinical MRSA glycopeptide-resistant strain of pair 3	69
Pair 4 non-GISA		Clinical MRSA glycopeptide-susceptible strain of pair 4	69
Pair 4 GISA		Clinical MRSA teicoplanin-resistant strain of pair 4	69
8325-4		MSSA strain	48
SH108		Autolysis-deficient *atl* mutant	19
Plasmids			
pBT2		*E. coli-S. aureus* thermosensitive shuttle vector; Amp^r Cam^r	12
pCL84		*tetK*; *S. aureus geh* locus-integrating plasmid	40
pET28b		*E. coli* T7 promoter-driven system	Novagen
pAM1315		pET28b-VraR	This study
pAR776		pBT2 for Δ*vraRS*::Kan^r mutant	This study
pAJ		pBT2 for Δ*prsA*::Kan^r mutant	This study

tiguous *vraRS* genes and their replacement by a kanamycin resistance gene inserted between N315 sequence coordinates 1946740 to 1948426, using the temperature-sensitive vector pBT2 (12). The resistance cassette transcription orientation was chosen in the same direction as the native VraR operon. Briefly, fragments 886 bp long upstream and 952 bp long downstream were separately amplified by PCR using primer pairs described in Table 2 and the products then reamplified for PCR fusion using the external driver primers. The resulting cloned KpnI/PstI fragment contained an engineered internal restriction site (BamHI) used to conveniently ligate a kanamycin resistance marker. The PCR fragment containing the kanamycin resistance marker was subsequently digested with KpnI/PstI and cloned into pBT2 to yield plasmid pAR776. Plasmid pAR776 was electroporated into the restriction-defective strain RN4220 and then transferred by electroporation into ISP794, selecting for kanamycin resistance at 30°C. ISP4-2-1 containing pAR776 was grown overnight at 30°C, followed by growth with applied marker selection for 6 days with dilution passages at 42°C, a nonpermissive temperature for pBT2 replication. Bacteria were plated on agar containing 50 μg/ml kanamycin and then replica streaked onto 15-μg/ml chloramphenicol plates to screen for chloramphenicol-sensitive colonies. Double-crossover events corresponding to the desired *vraRS* gene disruption were confirmed by PCR and sequence analysis. The resulting strain was named AR821. The *vraSR*::*kan* allele was finally transduced into ISP794 using bacteriophage φ80α to obtain AR916.

Construction of a *prsA*::*kan* gene disruption. A chromosomal *prsA* gene disruption mutant was constructed by deletion of *prsA* and insertion of a kanamycin resistance gene by using the temperature-sensitive vector pBT2 (12). Approximately 900 bp of the upstream and downstream flanking sequences of *prsA* from ISP794 were amplified with primers listed in Table 2. Upstream and downstream product fragments were reamplified with the external driver primers and then cloned into KpnI/PstI-digested pBT2. The kanamycin marker was subsequently inserted in the unique BamHI site between these two fragments and the transcription unit oriented as for the native *prsA* transcript. The recombinant plasmid pBT2 containing a 3.5-kb fragment was used to transform RN4220 strain, selecting for kanamycin-resistant colonies at 30°C. From RN4220, the recombinant plasmid was introduced into strain Newman by electroporation at 30°C. Single colony transformants harboring the recombinant plasmid were grown overnight at 30°C, followed by growth with applied marker selection for 6 days with dilution passages at 42°C, a nonpermissive temperature for pBT2 replication. Bacteria were plated onto agar containing 50 μg/ml kanamycin and were then replica streaked onto plates containing chloramphenicol at 15 μg/ml to screen for chloramphenicol-sensitive but kanamycin-resistant colonies. Double-crossover

TABLE 2 Primers used in this study

Name	Primer sequence (5′–3′)[a]	Use
5′PrsA-F-KpnI	ATGCGGTACCATATGCCCTGCCATATCCAT	*prsA*-targeted gene disruption
5′PrsA-R-BamHI	ATGCGGATCCAGTTGAAACTCCTTTGTAAG	
3′PrsA-F-BamHI	ATGCGGATCCCACAAAACCGAGCGACCGTGG	
3′PrsA-R-PstI	ATGCCTGCAGGCTACACAGGGTGTAACAGC	
KpnSA1699-446	GGGGTACCTGGTGGCTTATTATCTATCGG	*vraSR*-targeted gene disruption
SA1699-VraRBamH1R	CGGGATCCTCATATCGAATTAAGAAAAGTTAC	
BamH11702-674	CGGGATCCCGATAAATCACCTCTACGTCTC	
SA1703-147Pst1R	CAAAACTGCAGAAGTTAATTGACAGTCTAATGC	
Upstream_prsA_prom	ATGCGGTACCGAATTCTCCATATCATTTATAACAAAATAA	*prsA* complementation
3′PrsA-R-PstI	ATGCCTGCAGGCTACACAGGGTGTAACAGC	
PrsAProm_f	GGGGTACCGAATTCCCATATCATTTATAACAAAATAA	*prsA* promoter gel shift assay
PrsAProm_rev	AACTGCAGCTCGAGACTAGCTGTTACCGGAA	
*vraR*_forward	ATGCGGTACCATGACGATTAAAGTATTGTT	VraR recombinant protein cloning
*vraR*_reverse	GACTCTGCAGTCGATATGAACTATTGAAT	
5′CDS_*prsA*	TTATTGGCTCATGCCGGATTGTCCGCC	5′ RACE
GSP5′race_*prsA*	ACCGCCGTATTGCTTTTGCATTTTTTCAATTTGTTCGTCA	
prsA_F	AGTTAATGATAAGAAGATTGACGA	TaqMan assays
prsA_R	GAAGGGCCTTTTCAAATTTATCTTT	
prsA_P	TGAAAAAATGCAAAAGCAATACGGCGG	
vraR_F	TGCTTACAGAACGAGAAATGGAAA	
vraR_R	CCGTTTTAATAGTAATATGCGATGCA	
vraR_P	TGATTGCGAAAGGTTACTCAAATCAAGAAAT	
16S_F	GATAGAGCCTTCCCCTTCGG	
16S_R	CCGGCAGTCAACTTAGAGTGC	
16S_P	ACATCTCACGACACGAGCTGACGACA	

[a] Underlined regions represent restriction enzyme sites.

events corresponding to the desired gene disruptions were confirmed by PCR and sequence verified. For subsequent experiments, the *prsA*::*kan* allele was backcrossed to Newman, ISP794, COL, and ISP4-2-1 genetic backgrounds by transduction using bacteriophage φ75 or φ11 as appropriate. Transductants were verified by PCR-based assay and sequence analysis.

Construction of *prsA*$^+$::pCL84 for single-copy chromosomal complementation. To complement the *prsA*::*kan* mutation, we inserted the wild-type *prsA* gene under the control of approximately 400 bp of its upstream native promoter in the lipase *geh* chromosomal locus using pCL84 as previously described (40). Briefly, a 1,300-bp fragment derived from ISP794 was amplified using primers Upstream_*prsA*_prom and 3′PrsA-R-PstI listed in Table 2. The fragment was cloned into EcoRI and BamHI sites of pCL84 to yield pCL84-*prsA*. CYL316 harboring the bacteriophage L54a integrase was transformed with pCL84-*prsA* to select tetracycline-resistant colonies. The correct integration into the *geh* locus was verified by PCR and sequence analysis. The stably chromosomally integrated plasmid containing the complete *prsA* gene in CYL316 was then transduced into *prsA* deletion mutants as appropriate using phage φ11.

Transmission electron microscopy. Transmission electron microscopy was done as previously described (55). Briefly, *S. aureus* strains were grown on tryptic soy broth (TSB) at 37°C under shaking conditions until postexponential phase. The bacterial pellet was washed and fixed with 4% glutaraldehyde. Fixed cells were further treated with 2% osmium tetroxide and immersed in uranyl acetate to enhance membrane contrast. The pellets were dehydrated in increasing concentrations of ethanol followed by pure propylene oxide and then embedded in Epon resin. Thin sections for electron microscopy were stained with uranyl acetate and lead citrate and observed in a Technai 20 electron microscope (FEI Company, Eindhoven, Netherlands). Digital images were captured at ×37,000 magnification. Cell wall thickness was determined using equatorial sections and four independent measurements for each image from random placement of a cardinal point grid. The reported values represent the means of at least 25 independent images for each strain.

Triton X-100-induced autolysis assays. Overnight cultures of *S. aureus* were diluted to an OD_{600} of 0.05 and grown for 3 h with shaking at 37°C in MHB containing 1 M NaCl until the OD_{600} reached 0.7. Cells were harvested, washed twice with ice-cold water, and then resuspended in the same volume of 0.05 M Tris-HCl (pH 7.2) containing 0.05% Triton X-100. Cells were incubated at 37°C with shaking and checked for lysis by measuring the progressive decrease in absorbance (OD_{600}). Autolysis was quantified as a percentage of the initial OD_{600} remaining at each sampling point.

RESULTS

Transcriptional induction of *prsA* is dependent on *vraRS* TCS phosphosignaling. To examine the hypothesis whether *prsA* is indeed directly regulated by the VraRS TCS, we performed both detailed *in vivo* and *in vitro* analyses. As a first step to determine whether *prsA* transcription was VraR dependent *in vivo*, we constructed both a Δ*vraSR* disruption mutant and a phosphosignaling disruption mutant, VraS-H156A, in the ISP794 strain background used routinely in our laboratory for studies of low-level glycopeptide resistance mechanisms (63). *prsA* mRNA levels were subsequently examined by qRT-PCR in both the wild type and the Δ*vraRS* strain derivative, with or without exposure to teicoplanin or vancomycin as a cell wall stress inducer (Fig. 1). Approximately 8-fold and 10-fold inductions of *prsA* transcription were observed in ISP794 treated with teicoplanin and vancomycin, respectively. The Δ*vraRS* mutation led to a significant ($P < 0.05$) (2-fold) decrease in *prsA* mRNA basal levels compared to ISP794 and significantly prevented glycopeptide-evoked *prsA* transcriptional induction.

Next, we tested whether *prsA* glycopeptide induction was dependent on a functional VraRS phosphotransfer signaling. Previous work in our laboratory had established that H156 was the site

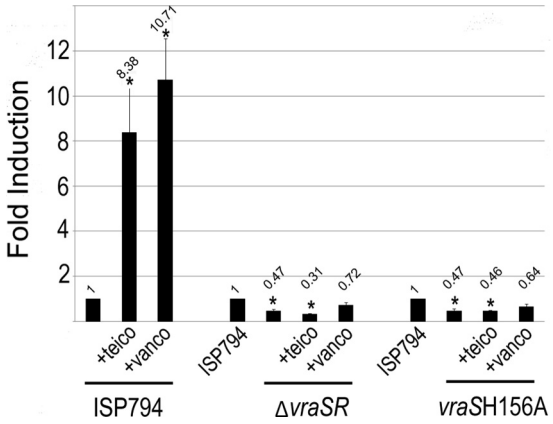

FIG 1 Genetic analysis of the VraRS TCS-dependent regulation of *prsA* transcription. Steady-state mRNA levels of *prsA* in glycopeptide-treated bacteria were compared to those in nontreated bacteria. The mRNA levels were determined by qRT-PCR and normalized to 16S rRNA. *prsA* mRNA levels of Δ*vraSR* and *vra*SH156A strains were compared to levels in the parental strain ISP794. Values represent the means ± standard errors of the means of at least three independent experiments. Asterisks indicate results significantly different ($P < 0.05$) from those for strain ISP794 in the absence of glycopeptide addition using Student's two-tailed *t* test.

of the VraS sensor kinase autophosphorylation and necessary for subsequent phosphotransfer to the carboxyl side chain of aspartate 56 of the VraR response regulator. Mutation of H156 resulted in a severe reduction in laboratory-derived low-level glycopeptide resistance in a variety of strain backgrounds, including ISP794, and permits analysis of cell wall stress by specifically eliminating this signal transduction pathway (21). Comparison of *prsA* transcription of wild-type ISP794 and its *vra*SH156A mutant derivative showed a significant ($P < 0.05$) (2-fold) decrease in *prsA* mRNA basal levels in the *vra*SH156A mutant (Fig. 1). Similar to our observations for the Δ*vraRS* mutant, *prsA* glycopeptide-mediated *prsA* transcription induction was abrogated in the *vra*SH156A mutant strain, suggesting that VraS-dependent phosphorylation of VraR and not just the presence of VraR and VraS was necessary for *prsA* induction (Fig. 1). Taken together, these findings lead us to conclude that VraS and VraR as well as the signal transfer between them are necessary for glycopeptide induction of *prsA* transcription.

***In vitro* binding of purified-recombinant VraR on the *prsA* gene promoter.** The aforementioned results strongly suggest direct regulation of the *prsA* promoter by the VraRS TCS. To determine whether VraR could bind specifically to the *prsA* promoter *in vitro*, we performed an electrophoretic mobility shift assay. A *prsA* promoter probe end labeled with γ-^{32}P and encompassing 290 bp was titrated with increasing amounts of purified-recombinant His_6-VraR. As shown in Fig. 2, a DNA-protein complex was observed with 3.9 to 10.4 μM VraR (nonphosphorylated) with an apparent K_d of 5.42 μM, consistent with the reported affinity of nonphosphorylated VraR for its own operon promoter, 5 ± 2 μM (6). The effect of phosphorylated VraR on DNA binding affinity using a small-molecule phosphate donor such as acetyl phosphate or cognate VraS kinase phosphorylation was not explored in these experiments. Complex formation was diffuse at the lowest concentration (3.9 μM) and was observed at higher concentrations as predominantly a single complex migrating with reduced mobility. These observations are most consistent with weak VraR monomer binding to the DNA probe followed by stable association of VraR dimers. To determine whether the observed binding was specific, we further performed a competition assay in the presence of a 200-fold molar excess of cold *prsA* promoter probe or a 200-fold molar excess of cold *tst* promoter region of similar length, known to have no detectable specific affinity for purified VraR (D. Andrey, unpublished). The specific complex VraR/*prsA* was significantly reduced in the presence of cold-specific *prsA* promoter, while addition of cold-unspecific probe did not appreciably affect complex formation (Fig. 2).

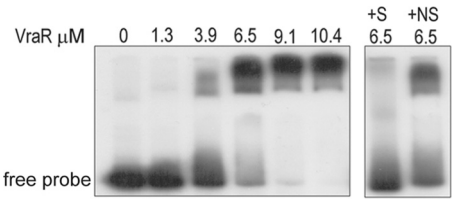

FIG 2 Autoradiogram of a 10% polyacrylamide gel shift analysis showing binding of purified recombinant VraR (nonphosphorylated) to the *prsA* promoter. Increasing amounts of VraR recombinant protein were incubated with a radiolabeled DNA *prsA* promoter probe. For determination of specific binding by competition, reactions used a 200-fold molar excess of a cold 100-bp DNA fragment far upstream of the *tst* promoter encoding toxic shock syndrome toxin 1 (TSST-1) as a nonspecific probe (NS), while the cold *prsA* promoter probe was used as a specific (S) competitor.

Inspection of the *prsA* promoter by T-Coffee alignment, together with analysis based on the palindromic VraR binding site proposed by Donaldson (in 2008) and later confirmed by Belcheva et al. (7) and in our laboratory by high-resolution contact site analysis (W. Kelley, unpublished observations), permitted us to identify a putative VraR box in the region located between −100 bp and −78 bp upstream of the PrsA ATG start codon (Fig. 3A). Modeling of the VraR binding site from structural analysis of the DNA binding domain originally predicted an invert hexamer separated by two nucleotides AAGACTNNAGTCTT, which is in close agreement with the core VraR site detected by footprint analysis on the VraR promoter AAGACTaaAGTatg (7). Uppercase letters correspond to identity with the predicted site. Refined analysis by mutational study extended the core regions to include additional nucleotides necessary for VraR binding (8). The VraR site in the *prsA* promoter shares considerable overall sequence identity with the VraR operon promoter site, with the notable exception of an imperfect downstream half-site and an enlarged (+6-bp) spacer region (Fig. 3B). Critical features of VraR binding sites which govern strong or weak binding and promoter activation strength are presently unknown.

To further verify that this putative VraR site within the *prsA* promoter bound VraR, we performed additional binding studies using three identical-length probes of comparable specific activities prepared by PCR, but carrying nucleotide replacement mutations that abolished either palindromic half-site or both. The results showed that either half-site mutation alone or the double mutation abolished specific complex formation with VraR in the range of the predetermined K_d described above (data not shown). We conclude from these studies that VraR is able to bind specifically to the *prsA* promoter *in vitro*. This represents the first demonstration of specific binding of VraR to a promoter of a gene within the cell wall stress regulon other than its own operon promoter.

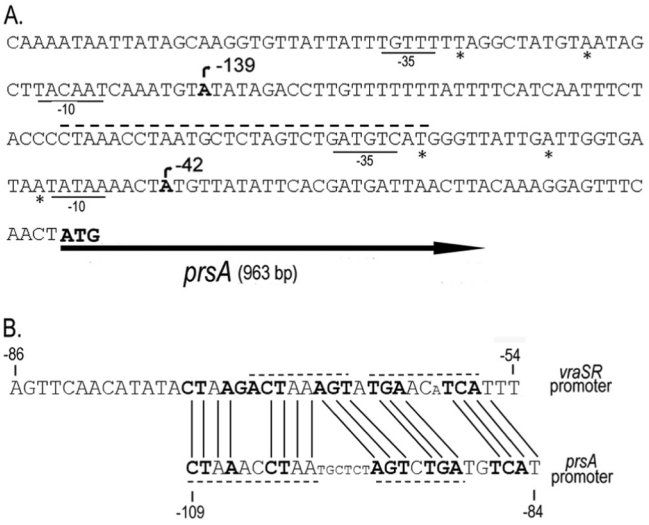

FIG 3 (A) Sequence of the *prsA* promoter region. The two different nucleotides corresponding to transcriptional start sites detected by 5′ RACE are shown in bold, and the −10 and −35 regions are underlined. The predicted VraR box is shown with dashed lines. (B) Schematic diagram comparing the *vraSR* operon promoter region located upstream of *SA1703* and upstream sequence corresponding to the *prsA* promoter region. Nucleotides in bold correspond to those described in reference 7 and essential for VraR binding. For the *prsA* promoter, nucleotide positions correspond to nucleotides upstream from the ATG start codon of the *prsA* gene. For the *vraSR* promoter, nucleotide positions correspond to nucleotides upstream from the transcription start site defined by Belcheva et al. (7).

Transcriptional analysis of *prsA* gene and 5′ RACE mapping. To further understand the nature of VraR-mediated regulation of *prsA* transcription and assign canonical promoter −10 and −35 elements, we next determined the transcript start site(s) for *prsA*.

As observed by our own laboratory (data not shown) and others (24), low-resolution Northern blot analysis of *prsA* shows a transcript of approximately 1,000 kb sufficient to encode the PrsA open reading frame (963 bp) and suggesting that *prsA* is expressed monocistronically. To extend these findings, we precisely mapped *prsA* 5′ transcript ends using 5′ RACE amplification. *prsA*-specific cDNA was generated using primer 5′CDS_*prsA* (Table 2). cDNA PCR amplification using primers GSP5′race_*prsA* and UPM generated two distinct DNA bands migrating in agarose gels in the range of 300 to 500 bp, suggesting that *prsA* is actually transcribed as two monocistronic transcripts, at least under our experimental conditions. Each band was gel extracted and directly sequenced. The deduced transcriptional starts of *prsA* were located 42 and 139 nucleotides upstream from the ATG start codon, generating predicted *prsA* transcripts of at least 1,005 and 1,102 bp, respectively, without taking into consideration the 3′ nontranslated region, which we did not map (Fig. 3A). The small size difference between the two transcripts is in close agreement with a single *prsA* transcript observed by Northern blot analysis. We cannot exclude at this time that other *S. aureus* strains used in the published studies where Northern blot data were shown display only a single transcript, or predominately one of several, or even whether alternative methods of *prsA* transcription induction result in differential promoter usage. The results we report above are from the only transcript mapping experiments performed to date using cell wall-active drug induction on this promoter (glycopeptide). Additional study will be required to address this issue.

As shown in Fig. 3A, the VraR box is located upstream of the −42 start site and is positioned in a region consistent with published models depicting a TCS activator binding site upstream of −35 and able to contact RNA polymerase (23). In contrast to the proximal promoter, the VraR box is positioned downstream of the distal −139 start site. We cannot rule out at this time whether additional VraR binding sites exist on the *prsA* promoter or whether the promoter architecture encountered *in vivo* favors differential *prsA* promoter usage. It is worthwhile noting that no canonical SigB promoter elements were detected in the *prsA* promoter and that *prsA* has not been reported to be differentially regulated by this alternative stress sigma factor (9). Collectively, we conclude from genetic analysis, protein-DNA interaction, site-specific mutagenesis of the VraR box, and transcript mapping that the *prsA* promoter is subject to positive regulation by the VraRS TCS.

***prsA* is not an essential gene in *S. aureus*.** Direct regulation of *prsA* by the VraRS TCS system and the inclusion of *prsA* in many defined cell wall stress studies strongly suggested that the PrsA PPIase plays an important role in the response to cell wall stress and, by extension, could contribute to low-level resistance to cell wall-active antibiotics. To explore these possibilities, we first constructed a Δ*prsA*::*kan* mutant in the Newman strain background (Table 1). Standard growth curves revealed that the mutant strain showed no clear defects under standard laboratory conditions compared to its isogenic wild-type parental strain (data not shown). Similar growth profile results were obtained when the *prsA*::*kan* allele was transferred by transduction into the unrelated ISP794 strain background, indicating that the tolerance for *prsA* disruption was not strain dependent. We conclude that *prsA* is nonessential in *S. aureus* and confirm results from transposon insertion libraries suggesting that *prsA* is nonessential in this organism (5, 13).

Loss of *prsA* does not affect cell wall thickness or Triton X-100 sensitivity. To extend our analysis of *prsA* disruption and its consequences, we reasoned that the loss of *prsA* could be associated with macroscopic alterations in cell wall morphology because of its probable function as an extracellular protein folding factor. Quantitative cell wall thickness analysis using transmission electron microscopy revealed no significant difference between Newman and its *prsA*::*kan*-derived strain grown under the same conditions as used for growth curve analysis (Fig. 4). Indeed, restoration of *prsA*$^+$ in the *prsA*::*kan* background by stable chromosomal insertion of *prsA* under the control of its own promoter at the *geh* locus (see Materials and Methods) showed cell wall thickness indistinguishable from that of Newman or the *prsA*::*kan* strain.

Perturbation of cell wall structure resulting from the disruption of *prsA* was also assessed by measuring the sensitivity of strains to Triton X-100 lysis. Whereas the control strain lacking the major autolysin (*atl*) gene (SH108) showed strong resistance to Triton lysis and 8325-4 showed strong sensitivity to Triton lysis, strain Newman, Newman *prsA*::*kan*, and the *prsA*$^+$ complemented strain showed indistinguishable time course lysis profiles (Fig. 4). These results strongly suggest that no major alteration in the cell wall arose from disruption of *prsA*, at least under our experimental conditions.

In a separate set of experiments, we next examined whether there was a detectable change in cell wall thickness or susceptibility to Triton X-100 lysis using cultures briefly exposed to teicoplanin

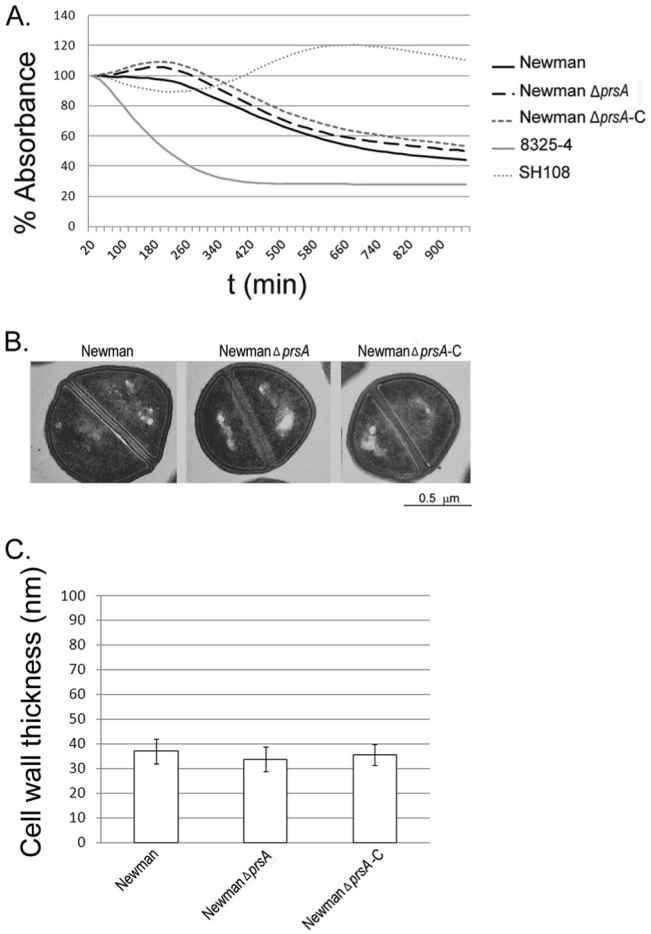

FIG 4 (A) Time course assay showing Triton X-100 lysis sensitivity. Newman and its derivatives were compared to a Triton X-100-sensitive strain (8325-4) and its isogenic derivative Triton X-100-insensitive strain SH108 lacking *atl* (19). Note that no significant difference in sensitivity to Triton X-100 was detected among any of the Newman strains. (B) Representative transmission electron microscope images of cells used for analysis in equatorial section. Digitized images were quantitated and cell wall thicknesses reported in nanometers (see Materials and Methods). Control images for enhanced or reference normal *S. aureus* cell walls were captured concurrently (ATCC 29213 and Mu50, respectively) on the same instrument and magnification and using the same preparation methodology (55). (C) Cell wall thickness analysis of strain Newman and its *prsA* disruption or complemented derivative. Note that no significant differences were observed.

at concentrations shown to promote strong induction of *prsA* (see Materials and Methods). Using this approach, where technically cell integrity must be maintained for a sufficient time after drug exposure to permit measurements, we were unable to detect any significant difference among the tested strains by either electron microscopy or Triton sensitivity (data not shown). Collectively, we conclude from these analyses that loss of *prsA* does not detectably alter cell wall structure under either of our drug stress or nonstressed growth conditions.

***prsA* modulates low-level glycopeptide resistance.** The preponderant role of the VraRS TCS in cell wall stress studies and our results showing direct *prsA* transcriptional regulation by VraR, as described above, prompted us to examine the effect of *prsA* disruption on the emergence of low-level glycopeptide resistance. The assay employed (spot population analysis profile [PAP] assay) measured viable CFU as a function of drug concentration (55) and is particularly well suited for detecting qualitative or quantitative changes in drug sensitivity. It is important to point out that in these assays viable colonies appear because they have acquired a nonheritable tolerant state (persister cells) or because they grow in the presence of the drug and often acquire first-step mutations at relatively high frequency, leading to changes in drug resistance profiles. The spot assay permits a rapid, sensitive, and reproducible test of low-level antibiotic resistance in genetic studies and is not limited by $\log_2$ dilutions commonly used in MIC assays or Etest strips. Control experiments revealed that the frequency of persister cells in our assays was 5% or less (although we have reported it to be as high as 20% in other strain backgrounds [21]).

We reproducibly observed a 2-$\log_{10}$ reduction of low-level teicoplanin resistance for Newman Δ*prsA*::*kan* compared to Newman on agar plates containing 1 μg/ml of teicoplanin (Fig. 5). To confirm that the enhanced sensitivity to teicoplanin was linked with inactivation of *prsA*, complementation experiments were performed. Introduction of a stable chromosomal copy of *prsA*$^+$ in the defective *prsA* strain (Newman Δ*prsA*-C) restored teicoplanin low-level resistance to those observed in the wild-type strain. The impact of Δ*prsA* on vancomycin resistance was also tested by spot PAP assays. In this case, an approximate 3-$\log_{10}$ reduction of low-level vancomycin resistance was reproducibly observed for Newman Δ*prsA* compared to wild-type Newman on agar plates containing 0.7 μg/ml vancomycin. Complementation assays confirmed that the enhanced sensitivity to vancomycin observed with the *prsA* deletion strain was the result of the loss of *prsA* (Fig. 5). Collectively, these results show that *prsA* deletion hypersensitizes *S. aureus* to low-level exposure (subinhibitory MIC) to glycopeptide antibiotics.

In order to examine next the effect of *prsA* disruption on the maintenance of the GISA phenotype, we transduced Δ*prsA*::*kan* into ISP4-2-1, a laboratory-derived GISA strain displaying intermediate glycopeptide resistance (MIC of teicoplanin, 16 μg/ml, and MIC of vancomycin, 4 μg/ml) (55). This strain has been extensively studied in our laboratory, and the GISA phenotype arose, including significantly enhanced cell wall thickness, by the combined effects of three single nucleotide changes occurring in three separate genes: *stp*, *vraS*, and *yjbH*. Using spot test viability assays, we reproducibly observed at least a 3-$\log_{10}$ reduction in viable colony formation on either teicoplanin- or vancomycin-containing agar plates for ISP4-2-1 Δ*prsA*::*kan* compared to its isogenic ISP4-2-1 parent (Fig. 5). The Δ*prsA*::*kan* disruption was also transduced into two additional and independent laboratory-derived GISA strains in the ISP794 genetic background and obtained using glycopeptide two-step selection conditions identical to those for ISP4-2-1. Our results showed that *prsA* disruption in these strains had no detectable growth reduction on either teicoplanin- or vancomycin-containing agar plates compared to their respective isogenic *prsA*$^+$ parental strains (data not shown). The precise nature of the mutations leading to the acquisition of a stable GISA phenotype in these strains is presently unknown. Taken together, the findings allow us to conclude that *prsA* is required to maintain the GISA phenotype in a subset of strains.

***prsA* is necessary for high-level oxacillin resistance in COL (MRSA).** Since *B. subtilis* PrsA has been shown to be involved in lateral wall synthesis by folding and stabilizing PBP2a, and a role for PrsA (31) in β-lactam resistance has recently been suggested (2), we also analyzed the effect of *prsA* deletion on oxacillin resis-

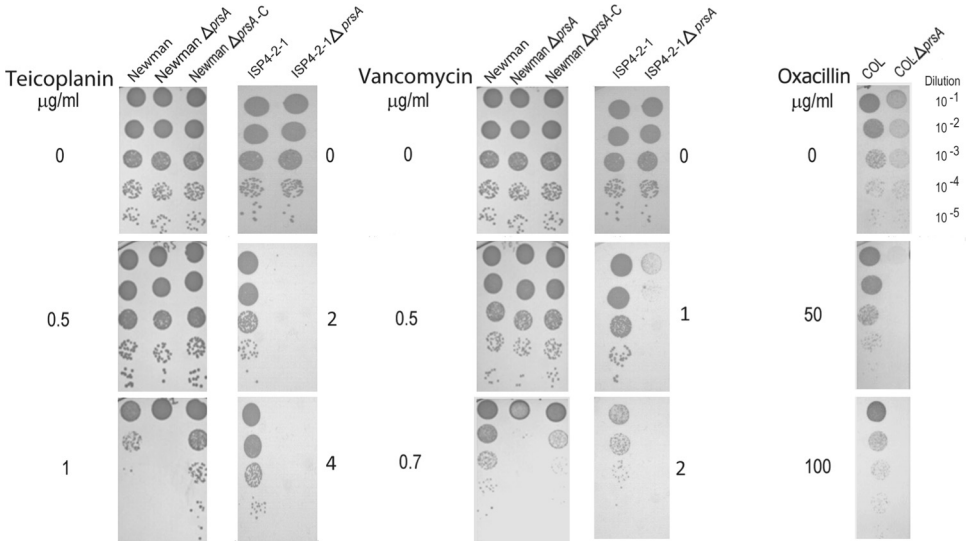

FIG 5 Effect of *prsA* gene disruption measured by agar plate assay in the presence of glycopeptides (strain Newman and its derivatives and GISA strain ISP4-2-1 and its derivative) or oxacillin (MRSA strain COL). Bacterial spot dilutions are indicated at the right. The first 10-μl spot dilution corresponds to 1×10^5 CFU.

tance. COL is a clinical MRSA strain that exhibits a high oxacillin resistance (MIC > 256 μg/ml). The effect of *prsA* deletion was therefore evaluated by transducing the *prsA* deletion from Newman into COL. As judged by spot PAP assays, the COL-*prsA*::*kan* strain showed a drastic reduction of oxacillin resistance compared to the parent COL strain (Fig. 5). Indeed, the >4-$\log_{10}$ reduction in viable colonies observed for COL-*prsA*::*kan* exposed to 50 μg/ml oxacillin suggests that the loss of *prsA* is much more severe with regard to oxacillin resistance than low-level glycopeptide resistance when considering the concentration range of drug tested in these assays. To exclude a general effect of *prsA* disruption on drug resistance, we also tested sensitivity to the non-cell-wall-active drug erythromycin. We observed no detectable change in sensitivity compared to the wild-type parental strain.

Transcript analysis of *prsA* in a panel of laboratory and clinical glycopeptide-resistant and -susceptible paired strain sets. Our finding that the *prsA* deletion affected low-level glycopeptide resistance prompted us to examine next the hypothesis whether steady-state *prsA* transcript levels were significantly and stably increased in a panel of stable GISA strains for which we had available the matched glycopeptide-sensitive precursor strains. The glycopeptide-susceptible and -resistant strains were derived from laboratory and clinical strains previously shown to be clonally related (69). Clinical paired sets came from a pediatric catheter infection case (MRGR3/14-4) and from a collection of MRSA isolates from episodes of bloodstream infection in four separate patients (see Materials and Methods). Importantly, all clinical susceptible strains were collected and archived before the onset of vancomycin therapy.

The *prsA* transcriptional levels were monitored by qRT-PCR for each strain set. As shown in Fig. 6, *prsA* transcription was significantly ($P < 0.05$) increased in GISA laboratory or clinical resistant strains compared to their susceptible parental counterparts. The increased *prsA* transcription was correlated with increased *vraR* transcription, as expected; however, the relative amplitude of *prsA* and *vraR*, or the measured fold induction, varied for each strain. Only the laboratory-derived strain ISP4-2-1 showed a nonsignificant (1.6-fold) increase of *prsA* expression even if *vraR* transcription was significantly increased in the same strain. These results show that in our panel of laboratory and clinical glycopeptide-resistant strains, *prsA* transcription is uniformly increased together with *vraR*, suggesting that constitutive increased *prsA* transcriptional levels could be considered a characteristic feature of some GISA strains. Of course, since genetic changes resulting in the emergence of GISA can be quite diverse and do not in all cases involve changes in the VraRS TCS that would be expected to drive transcriptional changes of its cell wall stress regulon (30), additional measurable transcriptional markers of the GISA phenotype will have to be identified to provide adequate coverage of all possible cases.

***prsA* and *vraR* are coordinately upregulated upon cell wall-**

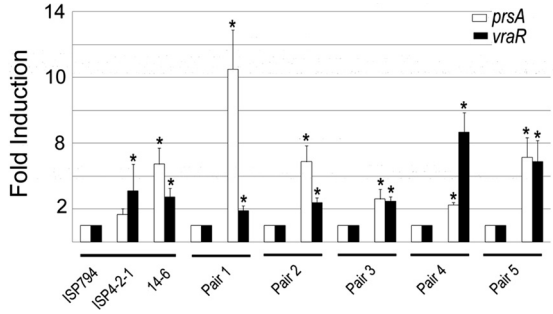

FIG 6 Analysis of steady-state transcriptional mRNA levels of *prsA* and *vraR* genes in glycopeptide-susceptible and stable low-level-resistant paired strains (see Materials and Methods). Transcript levels were determined by qRT-PCR of 16S rRNA-adjusted extracts. ISP794 and derivatives are laboratory paired strains. Pairs 1 to 4 correspond to clinical bacteremia MRSA: non-GISA and GISA paired strains, respectively. Pair 5 corresponds to the clinical MRGR3 and 14-4 paired set from a pediatric catheter infection. ISP4-2-1 and 14-6 are laboratory derivatives of ISP794 obtained by exposure to teicoplanin. ISP4-2-1 has been fully sequenced and genetic analysis of its three nucleotide changes reported (54). Values represent the means ± standard errors of the means of at least three independent experiments. Asterisks indicate results significantly different ($P < 0.05$) from those for the corresponding non-GISA strain.

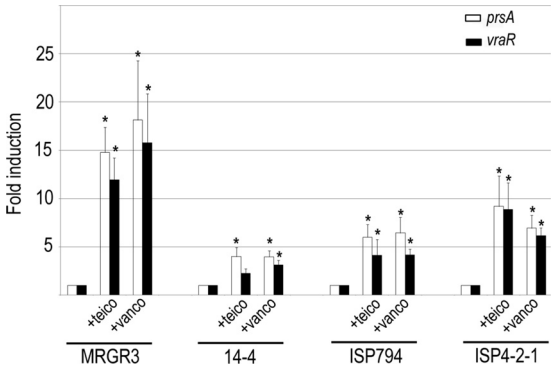

FIG 7 Effect of glycopeptides on transcriptional mRNA levels of *prsA* and *vraR* genes in glycopeptide-susceptible and derivative resistant strains. Steady-state levels of *prsA* and *vraR* transcripts were determined by qRT-PCR and normalized to 16S rRNA in strains MRSA-MRGR3 (teicoplanin susceptible), 14-4 (teicoplanin resistant), MSSA-ISP794 (teicoplanin susceptible), and ISP4-2-1 (teicoplanin resistant). The mRNA levels are compared to each corresponding strain in the absence of glycopeptide addition. Values represent the means ± standard errors of the means of at least three independent experiments. Asterisks indicate results significantly different (Student's two-tailed *t* test, $P < 0.05$) from those for the corresponding strain in the absence of glycopeptide addition.

active antibiotic treatment. A logical extension of our finding that steady-state *prsA* and *vraR* transcripts were upregulated in our panel of matched susceptible and GISA strains was to ask whether the observed transcription levels were saturated or whether they could be further induced by glycopeptide exposure. Heritable genetic changes that drive the emergence of glycopeptide resistance have been described to occur in a number of genes, most notably, genes involved in signal transduction. The impact of these mutations on gene expression and their impact on low-level glycopeptide resistance have, in most cases, not been examined systematically.

To address this issue, we examined two of our strain sets, one a well-studied MRSA/GISA strain, MRG3/14-4 (also called pair 5), and the other a laboratory methicillin-susceptible *S. aureus* (MSSA) strain, ISP794/ISP4-2-1. The latter strain set has the advantage of being fully sequenced, and the three point mutations mediating altered glycopeptide resistance are well characterized (55). Using qRT-PCR and probes for *vraR* and *prsA*, all strains were exposed to teicoplanin or vancomycin (see Materials and Methods) and compared to an uninduced control. The results are shown in Fig. 7.

Both *prsA* and *vraR* mRNA levels were significantly ($P < 0.05$) increased following glycopeptide addition in both susceptible and derived glycopeptide-resistant strains. For the teicoplanin-resistant strain 14-4, we observed a significant but reduced increase of both *prsA* and *vraR* expression following glycopeptide challenge compared to ISP4-2-1. The reduced glycopeptide-mediated induction in strain 14-4 is most likely explained by a higher constitutive expression of these genes in 14-4 than in ISP4-2-1 (Fig. 7). Nevertheless, our results suggest that saturating levels of gene expression for at least two markers of the cell wall stress regulon are not achieved in stable GISA strains. On the basis of our limited analysis, we conclude that exposure to cell wall-active drugs is thus a stronger stimulus signal than the changes engendered by genetic mutation underlying stable GISA strains. Consistent with our findings, it is worthwhile noting that the issue of maximal induction of cell wall stimulon was recently addressed by Dengler and colleagues (15). Induction by glycopeptides of *sas016*, part of the cell wall stimulon, was found to be higher at 5 times the MIC.

DISCUSSION

In this study, we attribute for the first time a role for the lipoprotein PPIase PrsA in *S. aureus* and describe its effects upon glycopeptide and oxacillin resistance in both MSSA and MRSA strains. Our results show that *prsA* expression is highly dependent upon the VraRS TCS as well as VraRS phosphotransfer signaling. To our knowledge, *prsA* is the first confirmed target directly regulated by the VraRS TCS outside its own four-gene operon.

Disruption of *prsA* resulted in measurable changes in the sensitivity to glycopeptides using agar plate assays and a dramatic increase in the sensitivity to oxacillin in the MRSA COL strain. By monitoring both steady-state *prsA* and *vraR* transcript levels in a panel of clinical and laboratory-derived GISA strains, we found significantly enhanced expression of both genes, suggesting that the underlying genetic changes that drive the emergence of the GISA or heterogeneous GISA (hGISA) phenotype could result in measurable and predictable transcriptional or protein markers for clinical laboratory detection. Since genetic changes in various pathways, apart from the VraRS TCS, have been described leading to GISA and hGISA, it is of course evident that multiple markers must ultimately be identified for each possible pathway to ensure coverage of transcriptional or proteomic alterations strongly correlated with these phenotypes. For example, and in contrast to our results, a recent study uncovered changes in the WalKR TCS in a panel of clinical GISA strains which displayed no demonstrable change in *prsA* or *vraRS* transcript levels (30).

In the course of this work, we have shown that the *prsA* deletion also significantly affects the maintenance of glycopeptide resistance in a laboratory-derived GISA strain. In contrast, two additional GISA strains isolated in our laboratory in the same ISP794 genetic background were not detectably affected by the loss of *prsA*, indicating that a requirement for *prsA* likely depends on the underlying mechanism driving the GISA phenotype.

The genetic basis of glycopeptide-intermediate resistance in *S. aureus* is incompletely understood. A growing inventory of mutations has been gathered by sequence analysis of clinical samples and, in some cases, detailed analysis of laboratory-derived strains. Results to date would suggest that there is no one genetic change, or perhaps even biochemical pathway, that underlies all reported GISA strains. To the contrary, it would appear that there are multiple mutations that can occur in order to drive the appearance of GISA, often in genes encoding components of signaling pathways, and that these mutations likely impact the expression of numerous downstream genes. Taken in this light, and considering the complex reprogramming of cell metabolism that is thought to underlie the maintenance of GISA, it is certainly plausible that *prsA* is necessary for some, but not all, pathways. In light of our findings reported herein, it would appear imperative to determine which mutations resulting in GISA require or do not require *prsA*. Such studies are under way and extend to the analysis of clinical GISA isolates amenable to genetic manipulation.

Upon encounter with cell wall-active antibiotics, what advantage does *S. aureus* gain by inducing *prsA*? A key corollary question arises as well: of the many genes thought to comprise the cell wall stress regulon, are some genes more important for *S. aureus*'s response to this form of stress than others? A response to the latter

question would require a more detailed and systematic examination of the cell wall stress regulon as a focus of future studies. In answer to the first question, and by borrowing clues from extensive research conducted with *B. subtilis*, PrsA could contribute to cell wall stress defense as a postsecretory protein folding factor. Gram-positive bacteria contain an unusual folding environment at the wall-membrane interface that has been likened to an ion-exchange resin (59). Unfolded proteins secreted via the Sec-dependent pathway must successfully refold in this environment, either by themselves or assisted by other proteins or metal cofactors. The distinction that *prsA* is essential in *B. subtilis* but nonessential in *S. aureus* suggests that under nonstressed conditions, some divergence has occurred in PrsA client substrates that render them less dependent upon PrsA in the latter organism. In contrast, under stressed conditions, PrsA may become limiting for some substrates because of either their enhanced production, secretion overload, or a combination of these factors.

A facultative need for PrsA is thus most consistent with its role as a foldase and/or protein secretion efficiency factor under conditions where only certain substrates must be synthesized and properly exported. Our findings support this notion, and we show evidence for no obvious macroscopic alteration in cell wall thickness, autolytic sensitivity, or growth defect under nonstressed laboratory culture conditions. Furthermore, disruption of *prsA* was not reported in the extensive *Caenorhabditis elegans* screen for *S. aureus* virulence factors, suggesting that its loss does not unduly perturb cell surface or exoprotein secretion contributing to cytopathology (5).

Important steps of cell wall assembly and turnover occur outside the cell. The terminal steps of *de novo* peptidoglycan biosynthesis are mediated by transglycosylase and transpeptidases. Autolysins, also called peptidoglycan hydrolases, act outside the cells to limit cell wall thickness and help split the cell wall at the division septum. Synthesis of the *S. aureus* cell wall requires the concerted action of both transglycosylase and transpeptidase enzymatic activities to assemble progressively substrate lipid II monomers to extend peptidoglycan layers. Transglycosylases catalyze polymerization of the amino sugar moieties, and transpeptidases cross-link the newly synthesized murein building block to the preexisting peptidoglycan (27). Transpeptidases are the targets of penicillin (β-lactam) antibiotics and are known as penicillin binding proteins (PBPs).

S. aureus encodes four PBPs: PBP1, PBP2, PBP3, and PBP4 (60). Only PBP2 is bifunctional and possesses both transpeptidase and transglycosylase activities. In MRSA strains, an additional PBP is present: PBP2a, encoded by *mecA* on one of several allotypes of the SCC*mec* mobile element. The acquisition and expression of PBP2a, which has low affinity for β-lactam antibiotics, render *S. aureus* (and *Staphylococcus epidermidis*) relatively insensitive to this class of antibiotics. MRSA strains catalyze peptidoglycan synthesis using PBP2 transglycosylase and PBP2a transpeptidase activities (53). Additional examples of collaboration between PBPs have been reported. Deletion of *pbp4* severely affects the transcription of *pbp2* in cells challenged with oxacillin (46), and it has recently been shown that SgtA and Mgt transglycosylases cooperate with PBP2, PBP1, and PBP2a in peptidoglycan biogenesis (54).

How could disruption of *prsA* affect both glycopeptide and oxacillin resistance? In *B. subtilis*, PrsA has recently been shown to be involved in both folding and stabilizing PBPs, and *prsA* deletion negatively impacts peptidoglycan cross-linking (31). Thus, similarly to *B. subtilis*, *S. aureus* PrsA may conceivably affect cell wall metabolism by affecting stability or folding of PBPs. Further clues emerge from glycopeptide-resistant or glycopeptide-treated *S. aureus* strains, in which concomitant increases in *pbp2* and *vraR* expression have been observed (10, 47, 74). Increased *pbp2* expression could trigger a demand for enhanced production of PrsA driven by VraRS.

Apparently, oxacillin resistance does not exclusively rely on both PBP2 and PBP2a. Indeed, it has been reported that oxacillin susceptibility in a mutant *vraS* strain cannot be restored by overexpression of PBP2 even if PBP2a protein abundance was reported to be similar to that of the wild type (11). In this study, the authors suggested that additional VraRS-dependent factors were implicated in oxacillin resistance besides PBP2 and PBP2a. In this context, it is tempting to speculate that PrsA could be a candidate for one of these additional factors. In this scenario, the lack of PrsA induction explains the absence of oxacillin restoration upon overexpression of PBP2 in the *vraS* mutant. Whereas the PBP2 protein is produced, it is unable to reach or acquire a functional conformation in the wall-membrane interface compartment, where its activity is normally performed. Ongoing studies are focusing on the potential PrsA dependence of *S. aureus* PBPs and/or monofunctional glycosyltransferases.

Cell surface negative net charge has been described as a crucial element influencing vancomycin binding capacity, and regulating surface charge is known to influence susceptibility to cationic peptides. The highly charged teichoic acids confer a global negative net charge to the bacterial surface. In an *S. aureus* strain with a *dlt* disruption, in which teichoic acids are no longer D-alanylated, the cell wall negative net charge is increased and the strain is more susceptible to cationic peptides (52). This is also a feature of disruption of the GraRS TCS, which is responsible for transcriptional activation of the *dlt* operon (18, 41, 73).

In a particularly provocative discovery, a *B. subtilis* secretion-defective mutant caused by *prsA3* mutation (D268N) could recover its secretion profile through insertional inactivation of the *dlt* operon. The authors suggested that increasing bacterial surface negative net charge increased the rate of posttranslocational folding of exported proteins, possibly by trapping divalent cations at the membrane-wall interface (33). Second, a *dlt* mutant of *Streptococcus gordonii* shows an overexpression of PrsA (14) and a concomitant increase in the secreted fusion protein Spa-S1. In both cases, *prsA* and *dlt* appear to cocompensate their roles in posttranslocational folding events. Finally, PrsA was shown to be a limiting factor for high-level secretion of active α-amylase and subtilisin, but only in the presence of the bacterial cell wall (71). Such observations underscore the importance of the extracellular microenvironment, and especially the net charge of the cell wall, in the exoprotein folding process. Clearly, it would be of great interest to investigate the impact of altered teichoic acid D-alanylation in the absence of *prsA* and cell wall-active antibiotic resistance.

Bacterial adaptation to cell wall-active antibiotics such as glycopeptides or penicillins relies on an intricate combination of cellular processes: stress sensing, signal transduction, microenvironment, protein secretion, and posttranslocational folding. The potential link between genes strongly induced by secretion stress in *B. subtilis* and those induced by cell wall-active antibiotics in *S. aureus* warrants pursuing in detail. Among the genes identified by

severe secretion stress in *B. subtilis* by the *prsA3* mutation are genes of the *dlt* operon, *pbpA*, *clpC*, *htrA*, *htrB*, and genes within the *lia* operon, which shows high overall similarity to the VraRS TCS (32). Inspection of differential gene expression profiles reported from cell wall stress transcriptomic studies in *S. aureus* consistently revealed significant upregulation of *clpC*, *htrA*, *prsA*, and the *vraR* operon, among others (39, 44, 45, 66). It is certainly conceivable that a protein secretion quality control network evolved long before medical intervention with antimicrobial pharmacotherapy and that *S. aureus* responds to cell wall-active drug challenge by calling upon elements of this ancient system.

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ADDENDUM IN PROOF

Mu50 *prsA*::*kan* also shows enhanced susceptibility to glycopeptides and oxacillin compared to Mu50 (a MRSA/GISA strain).

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