Site-Specific Mutation of \textit{Staphylococcus aureus} VraS Reveals a Crucial Role for the VraR-VraS Sensor in the Emergence of Glycopeptide Resistance\(^\text{\textsuperscript{\textcopyright}}\)

Elena Galbusera,\(^1\)† Adriana Renzoni,\(^2\)† Diego O. Andrey,\(^2\) Antoinette Monod,\(^2\) Christine Barras,\(^2\) Paolo Tortora,\(^1\) Alessandra Polissi,\(^1\) and William L. Kelley\(^2\)\(^*,\)

Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, Piazza della Scienza 2, 20126 Milan, Italy,\(^1\) and Service of Infectious Diseases, University Hospital and Medical School of Geneva, 4 Rue Gabrielle-Perret-Gentil, CH-1211 Geneva 14, Switzerland\(^2\)

Received 26 May 2010/Returned for modification 22 August 2010/Accepted 13 December 2010

An initial response of \textit{Staphylococcus aureus} to encounter with cell wall-active antibiotics occurs by transmembrane signaling systems that orchestrate changes in gene expression to promote survival. Histidine kinase two-component sensor-response regulators such as VraRS contribute to this response. In this study, we examined VraS membrane sensor phosphotransfer signal transduction and explored the genetic consequences of disrupting signaling by engineering a site-specific vraS chromosomal mutation. We have used \textit{in vitro} autophosphorylation assay with purified VraS[64-347] lacking its transmembrane anchor region and tested site-specific kinase domain histidine mutants. We identified VraS H156 as the probable site of autophosphorylation and showed phosphotransfer \textit{in vitro} using purified VraR. Genetic studies show that the vraS(H156D) mutation in three strain backgrounds (ISP794, Newman, and COL) fails to generate detectable first-step reduced susceptibility teicoplanin mutants and severely reduces first-step vancomycin mutants. The emergence of low-level glycopeptide resistance in strain ISP794, derived from strain 8325 (\Delta rsbU), did not require a functional \(\sigma^B\), but \(\text{rsbU}\) restoration could enhance the emergence frequency supporting a role for this alternative sigma factor in promoting glycopeptide resistance. Transcriptional analysis of vraS(H156D) strains revealed a pronounced reduction but not complete abrogation of the vraRS operon after exposure to cell wall-active antibiotics, suggesting that additional factors independent of VraS-driven phosphotransfer, or \(\sigma^B\), exist for this promoter. Collectively, our results reveal important details of the VraRS signaling system and predict that pharmacologic blockade of the VraS sensor kinase will have profound effects on blocking emergence of cell wall-active antibiotic resistance in \textit{S. aureus}.

\textit{Staphylococcus aureus} is a major human pathogen that causes a variety of diseases ranging from relatively minor skin infections to invasive and systemic disease with significant morbidity and mortality (17, 44). Of particular concern are \textit{S. aureus} strains carrying one of several allotypes of a mobile genetic element, the SCCmec cassette, which encodes \textit{mecA}, and a low-affinity penicillin-binding protein variant, PBP2A (12). The expression of PBP2A renders \textit{S. aureus} insensitive to a broad range of \(\beta\)-lactams, including methicillin, hence the name MRSA (for methicillin-resistant \textit{S. aureus}). Various MRSA strains have become endemic in hospitals and in the community prompting worldwide efforts for detection and infection control.

Glycopeptide antibiotics (teicoplanin and vancomycin) are considered first-line drugs for the treatment of infections due to MRSA. The emergence of resistance (most often termed reduced sensitivity) to glycopeptides poses a major challenge to the treatment of MRSA infections since few clinically proven and effective alternative therapies exist (29).

Altered sensitivity to glycopeptides occurs by two mechanisms termed exogenous and endogenous. The exogenous mechanism (VanA type) conferring high-level resistance (vancomycin MIC \(\geq 16 \mu g/ml\)) occurs by the horizontal acquisition of the \textit{vanA} multigene complex from \textit{Enterococcus faecalis} encoded on \textit{Tn1546} and results in the alteration of peptidoglycan terminal stem peptide from D-Ala–D-Ala to D-Ala–D-Lac, a structure to which glycopeptides no longer bind efficiently and therefore fail to block transglycosylase and transpeptidase cell wall cross-linking (47, 57). Worldwide, only a few examples of VRSA have been reported (55).

In contrast, endogenous resistance to glycopolypeptides is much more prevalent. \textit{S. aureus} displaying intermediate glycopeptide resistance (termed VISA if referring to vancomycin-intermediate \textit{S. aureus} and GISA for glycopeptide-intermediate \textit{S. aureus} encompassing both vancomycin and teicoplanin) are thought to arise stepwise from so-called heterogenous (hVISA and hGISA) precursor populations through selection of mutation(s) during the course of exposure to glycopeptides (28, 29, 43, 47). Rare subpopulations of bacteria displaying higher levels of resistance presumably serve as a reservoir driving the eventual emergence of glycopeptide resistance. Subpopulations of this type are difficult to detect, and no routine clinical laboratory tests exist that are standardized and reliable for their detection (29, 61).

The MIC breakpoint defining the transition from sensitive to intermediate resistant for glycopeptide intermediate-resistant \textit{S. aureus} (GISA and VISA) is not universally agreed upon;
However, relatively minor alterations in reduced sensitivity to glycopeptides (minor changes in MIC) are now frequently associated with clinical failure, requiring recourse to alternative pharmacotherapy (29).

The genetic basis of endogenous glycopeptide resistance is poorly understood. Mutation in genes such as tcaA, vraRS, and vraRS have been described and are known to be causal or strongly correlated to the emergence of VISA and GISA (15, 30, 45, 48, 53). In some, but not all cases, morphological changes associated with the emergence of glycopeptide resistance include a thickened cell wall, reduced cross-linking, and decreased autolytic activity, suggesting that complex alterations in cell wall biosynthesis and turnover underlie the resistance mechanism (27, 47, 57).

Transcriptomic studies demonstrated that an encounter with cell wall-active antibiotics elicits a cell wall stress response in S. aureus (22, 40, 46, 49, 51, 73). The precise mechanisms that are responsible for the detection of cell wall damage are also poorly understood, and there are known significant interstrain variations (49).

In several studies, the transcriptional induction of the vraRS two-component sensor (TCS) system, which is part of a four-gene operon, is significantly induced after encounter with cell wall-active drugs such as oxacillin, vancomycin, teicoplanin, and n-cycloserine (22, 40, 49, 69, 77). The vraRS operon is also strongly induced after reduced transcription of pbp2, which encodes the bifunctional penicillin-binding protein PBP2 (22). The VraRS TCS is highly conserved in the low-percent G+C Gram-positive family Firmicutes. In S. aureus, the VraRS TCS is thought to regulate numerous genes, some necessary for cell wall biosynthesis and proteolytic quality control (40). At least two S. aureus TCS systems—WalKR (YycFG) and GraRS—have been implicated in modulating resistance to cell wall-active antibiotics (16, 18, 30, 53, 63).

TCS systems are widespread in bacteria and represent environmental sensing systems that integrate a broad range of input stimuli to effector proteins, often including transcription factors (20, 21). A typical TCS system is composed of a membrane sensor histidine kinase and a cognate response regulator. Environmental signal captured by the receptor kinase results first in histidine autophosphorylation. In a second step, phosphotransfer from the histidine kinase to a conserved aspartate in the receiver domain of the cognate response regulator ultimately culminates in alterations in downstream gene expression, or altered enzymatic activity, appropriate to the applied stimulus.

In the present study, we dissected the VraRS phosphotransfer sensing mechanism. We identified the key elements of VraS-VraR phosphotransfer in vitro and examined genetic consequences in vivo by using a site-specifically engineered vraS autophosphorylation-defective chromosomal point mutation. Our results reveal a crucial role for VraS-VraR signaling in mediating the emergence of endogenous glycopeptide resistance.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The strains and plasmids used in the present study are listed in Table 1. Antibiotics and their suppliers were as follows: teicoplanin (Sanofi Aventis), vancomycin (Sandoz), and oxacillin, n-cycloserine, and carbenicillin (Sigma).

Cloning and purification of recombinant VraR and VraS proteins. The open reading frame of the vraS gene (SA170 using the N315 ordered sequence tag numbering [41]) and the nucleotide region encoding the cytoplasmic domain of the vraS gene (corresponding to amino acids 65 to 347), hereafter referred to as VraS<sub>cyt</sub>, were amplified by the PCR using S. aureus genomic DNA and primers indicated in Table 2. Fragments of 884 bp (vraS) and 650 bp (vraR) were cleaved with KpnI and PstI and cloned into the KpnI and PstI sites of pKS111-Bluescript, respectively. After sequence verification, the fragments were excised with NdeI-PstI and cloned into the Escherichia coli expression vector pTYB12 (New England Biolabs). Recombinant VraR and VraS<sub>cyt</sub> proteins were then purified by using an N-terminal chitin affinity tag (Impact System; New England BioLabs). E. coli strain ER2566 containing pTYB12-VraR and pTYB12-VraScyt was grown in Luria-Bertani medium containing carbenicillin at 100 μg/ml until reaching an optical density at 600 nm of 0.4, induced with 0.5 mM IPTG (isopropyl-β-thiogalactopyranoside), and incubated for an additional 6 h at 30°C with vigorous shaking. Bacteria were harvested by low-speed centrifugation and resuspended in buffer containing 0.1% Tween 20, followed by affinity chromatography and in-tein-mediated proteolytic cleavage of the affinity tag with 50 m dithiotreitol (DTT) according to the manufacturer’s recommendations. Thiol-induced in-tein cleavage resulted in the N-terminal attachment of three additional amino acids (Ala-Gly-His) for VraR and four amino acids (Ala-Gly-His-Met) for VraS<sub>cyt</sub>, upstream of Ser65. Cleaved protein was eluted from the chitin beads, and the supernatant was concentrated and dialyzed using Centricon-10 spin columns. Protein concentrations assuming monomeric molecular weights predicted from the amino acid composition were determined using Bradford reagent (Bio-Rad) and a bovine serum albumin standard. Recombinant proteins were stored at 4°C and were stable for at least 6 months.

Quantitative PCR mutagenesis (Stratagene) was used to create additional protein expression vectors by a similar strategy as described above for mutants VraR-D55A, VraS<sub>cyt</sub>-H156A, and VraS<sub>cyt</sub>-H238A (Table 2). All protein expression vector constructs were sequenced prior to use. This purification strategy was used for the preparation of each mutant recombinant protein.

**Autophosphorylation and phosphotransfer assay.** To assess histidine autophosphorylation, 5 μM concentrations of either VraS<sub>cyt</sub>, VraS<sub>cyt</sub>-H156A, or VraS<sub>cyt</sub>-H238P (Hartmann And with 20 μC of [γ-32P]ATP (111 TBq/mmol) were incubated with 25 mM Tris-HCl (pH 7.0), 2.5 mM MgCl<sub>2</sub>, 100 mM KCl, 0.1 mM DTT, 5% glycerol, and 20 μg cold ATP. Aliquots (10 μl) were removed after 5, 15, and 45 min and transferred to an equal volume of 2% sodium dodecyl sulfate (SDS) sample loading buffer and applied to 12% polyacrylamide-SDS protein gels (without heating). Protein 11 minigels (Bio-Rad) were electrophoresed at 11 W for 2 to 3 h. The gels were dried and autoradiographed (Amersham Hyperfilms). For phosphotransfer reactions between VraS and VraR, an VraS<sub>cyt</sub>- or VraS<sub>cyt</sub> labeling protocol identical to that described above was used. After 45 min of VraS autophosphorylation, 40 μl was mixed with either purified VraR or with VraR-D55A to a final concentration of 17.5 μM. Aliquots (10 μl) were removed and mixed with an equal volume of 2% SDS sample buffer and applied to SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels as described above. Control phosphotransfer reactions run in parallel and stained with Coomassie brilliant blue showed that all recombinant proteins were stable under the conditions of the in vitro assay.

**Total RNA extraction.** Overnight bacterial cultures were diluted 1:100 and grown at 37°C for 4 h in Mueller-Hinton broth (MHB) with shaking. When indicated, oxacillin (1 μg/ml; ISP794 MIC = 2 μg/ml; thus, conducted at 0.5 MIC) was added, and bacteria were grown for an additional hour. Bacteria were harvested, and RNA extraction and verification of the absence of contaminating DNA was performed as described previously (59). Purified RNA samples were analyzed by using the RNA NanoLab chip on a 2100 Bioanalyzer (Agilent, Palo Alto, CA).

**Real-time RT-PCR.** mRNA levels were determined by quantitative RT-PCR (qRT-PCR) by using a one-step reverse transcriptase qPCR master mix kit (Eurogentec, Seraing, Belgium), as described previously (59). Appropriate vraR and vraS primers and probes were designed by using Primer Express software (version 1.5; Applied Biosystems) and obtained from Eurogentec. 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 (74). The statistical significance of strain-specific differences in normalized cycle threshold ([C<sub>T</sub>] values of each transcript was evaluated by using a Student paired t test, and data were considered significant when P was < 0.05.

**Northern blotting.** For transcript analysis, 6 μg of total RNA was separated in a 1% agarose formaldehyde gel and blotted onto a nylon membrane (Hybond-N Amersham) using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer (Quantum Biotechnologies) with a semidyblot apparatus (Bio-Rad).
were then mixed and reamplified with the outside primers to create the 

products were then mixed and reamplified with the outside primers to create the

Washes were done as follows: a first wash at 50°C with 2× SSC–0.1% SDS for 15 min, a second wash with 1× SSC–0.1% SDS for 15 min at 65°C, and then three washes with 0.1× SSC–0.1% SDS for 15 min at 70°C. The membrane was transferred to 3MM paper without drying, sealed, and autoradiographed (Amersham Hyperfilms).

**Construction of AR756 containing a vraS disruption.** To facilitate the construction of a site-specific codon change VraS H156A by allelic exchange, a chromosomal vraS disruption mutant was first constructed by deletion of the vraS coding sequence corresponding to amino acids 2 to 160 (inclusive) and insertion of an erythromycin resistance gene using the temperature-sensitive vector pBT2 (11). Briefly, a 1,076-bp upstream fragment and a 1,185-bp downstream fragment were separately amplified by using the primer pairs presented in Table 2; the products were then mixed and reamplified with the outside primers to create the fusion PCR-generated internal deletion of vraS and cloned into pUC18-digested

TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or characteristics</th>
<th>Source or reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER2566</td>
<td>IPTG-inducible T7 RNA polymerase restriction-deficient DNA cloning strain</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>DH5α</td>
<td></td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COL</td>
<td>MRSA, <em>rsbU</em>°</td>
<td>24</td>
</tr>
<tr>
<td>RN4220</td>
<td>8325-4, r° m°, restriction-defective laboratory strain</td>
<td>38</td>
</tr>
<tr>
<td>Newman</td>
<td>ATCC 25904, MSSA, <em>rsbU</em>°</td>
<td>1</td>
</tr>
<tr>
<td>MB211</td>
<td>8325, (rsbUVW-sigB)°, tetL nearby</td>
<td>23</td>
</tr>
<tr>
<td>MB212</td>
<td>8325, ∆rsbUW-sigB::ermB</td>
<td>39</td>
</tr>
<tr>
<td>ISP794</td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>ALC1001</strong></td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>ALC2057</strong></td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>AR850</strong></td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>AR851</strong></td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>AR852</strong></td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>AR76</strong></td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>AR769</strong></td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>AR758</strong></td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>AR943</strong></td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>AR828</strong></td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>AR878</strong></td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>AR872</strong></td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>AR848</strong></td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>AR868</strong></td>
<td>8325, pig-131 <em>rsbU</em> mutant</td>
<td>60, 67</td>
</tr>
<tr>
<td><strong>pCL84</strong></td>
<td>tetr, <em>S. aureus geh</em> locus integrating plasmid</td>
<td>42</td>
</tr>
<tr>
<td><strong>pUC18</strong></td>
<td>Multicopy E. coli cloning vector</td>
<td>76</td>
</tr>
<tr>
<td><strong>pUC19-MuSupF</strong></td>
<td>Amp′ mini-Mu transposon</td>
<td>26</td>
</tr>
<tr>
<td><strong>pEC2</strong></td>
<td>ermB cassette</td>
<td>11</td>
</tr>
<tr>
<td><strong>pMK4</strong></td>
<td>E. coli S. aureus shuttle vector; Amp′ Cam′</td>
<td>70</td>
</tr>
<tr>
<td><strong>pBT2</strong></td>
<td>E. coli S. aureus thermostable shuttle vector; Amp′ Cam′</td>
<td>11</td>
</tr>
<tr>
<td><strong>pTYB12</strong></td>
<td>N-terminal fusion Impact intein and chitin-binding domain plasmid</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td><strong>pKSII+BlueScript</strong></td>
<td>Routine multicopy E. coli cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>pAM1118</strong></td>
<td>pKS′-II VraS (NdeI-PstI)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pAM1158</strong></td>
<td>pKS′-II VraR (NdeI-PstI)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pEG1129</strong></td>
<td>pTYB12-VraS (NdeI-PstI)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pEG1</strong></td>
<td>pTYB12-VraS (NdeI-PstI)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pEG2</strong></td>
<td>pTYB12-VraS (NdeI-PstI)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pEG1055</strong></td>
<td>pTYB12-VraR (Nde-Pst)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pEG3</strong></td>
<td>pTYB12-VraR (Nde-Pst)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pAR712</strong></td>
<td>pBT2, vraS-Kan′-SA1699 intergenic ts shuttle vector</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pAR907</strong></td>
<td>pBT2, vraS-tetK- SA1699 intergenic ts shuttle vector</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pAM1284</strong></td>
<td>pBT2, vraS(1-160)::ermB is shuttle vector</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pAR747</strong></td>
<td>pBT2, vraS(H156A), Kan′ nearby ts shuttle vector</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pAM1483</strong></td>
<td>pMK4, 3.3-kb entire vraR operon and upstream promoter region, Kan′-PstI</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pAM1246</strong></td>
<td>pKS′-II, 3.3-kb entire vraR operon and upstream promoter region, Kan′-PstI</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pAM902</strong></td>
<td>pMK4, pGlyS gfpuv4</td>
<td>72</td>
</tr>
</tbody>
</table>

*Cam′, chloramphenicol resistance; Amp′, ampicillin resistance; Kan′, kanamycin resistance.
TABLE 2. Oligonucleotide PCR primers used in this study

<table>
<thead>
<tr>
<th>Gene, mutant, protein, or method</th>
<th>Primer or probe</th>
<th>Primer sequence (5′-3′)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>VraR wild-type protein</td>
<td>VraR-Kpn-Nde</td>
<td>GGGGTACCAAAGGAAACACATATGACAGTATTTAAAGTATTTG</td>
</tr>
<tr>
<td></td>
<td>VraR-Pst</td>
<td>CAAACCTGCAGCTATTGGAATTTATGTTGGAATGCT</td>
</tr>
<tr>
<td>VraR-D55A protein</td>
<td>VraR D55A-A</td>
<td>GATTTAATTATATGGCTTACTTATTGGAAG</td>
</tr>
<tr>
<td></td>
<td>VraR D55A-B</td>
<td>CTTCATTAAGTAACGCAATTATATATATATC</td>
</tr>
<tr>
<td>VraS&lt;sub&gt;SY&lt;/sub&gt; wild-type protein</td>
<td>VraS&lt;sub&gt;SY&lt;/sub&gt;-KpnNde PCR</td>
<td>GGGGTACCAAAGGAAACACATATGACAGTATTTAAAGTATTTG</td>
</tr>
<tr>
<td></td>
<td>VraS&lt;sub&gt;SY&lt;/sub&gt;-Pst PCR</td>
<td>CAAACCTGCAGCTATTGGAATTTATGTTGGAATGCT</td>
</tr>
<tr>
<td>VraS-H156A protein</td>
<td>VraS H156A-A</td>
<td>GCTCGAGAAGACTGCGATTTGTTAGTC</td>
</tr>
<tr>
<td></td>
<td>VraS H156A-B</td>
<td>GACATACAGAAGTGGGAAAGTCTGAGG</td>
</tr>
<tr>
<td>VraS-H238A protein</td>
<td>VraS H238A-A</td>
<td>ATGGAAGGTTGGGCTGAATACAAGATTAAAG</td>
</tr>
<tr>
<td></td>
<td>VraS H238A-B</td>
<td>CTTCATTAAGTAACGCAATTATATATATC</td>
</tr>
<tr>
<td>VraSΔ[2-160]:ermB</td>
<td>SA1703 Kpn-Bam</td>
<td>GGGGTACCGGATCAGAATCTGTTGGAACCGTTATATTGGAAC</td>
</tr>
<tr>
<td>Primer 2 Bam</td>
<td>CAAACCTGCAGCTATTGGAATTTATGTTGGAATGCT</td>
<td></td>
</tr>
<tr>
<td>Primer 3 Bam</td>
<td>SA1699 staEco-Pst</td>
<td>GGGGTACCAAAGGAAACACATATGACAGTATTTAAAGTATTTG</td>
</tr>
<tr>
<td>VraS-H156A chromosomal mutant</td>
<td>SA1703KpnBamHI</td>
<td>GGGGTACCGGATCAGAATCTGTTGGAACCGTTATATTGGAAC</td>
</tr>
<tr>
<td></td>
<td>SA1699EcoPstI</td>
<td>CAAACCTGCAGCTATTGGAATTTATGTTGGAATGCT</td>
</tr>
<tr>
<td>Kan marked vraS-SA1699 intergenic</td>
<td>3BKnBamHI</td>
<td>GGGGTACCGGATCAGAATCTGTTGGAACCGTTATATTGGAAC</td>
</tr>
<tr>
<td></td>
<td>12ABlg2</td>
<td>GAAGATCTGGTAGAIAACATTTTTGCTTATTGGAACCGTTATATTGGAAC</td>
</tr>
<tr>
<td></td>
<td>12BBgl2</td>
<td>GAAGATCTGGTAGAIAACATTTTTGCTTATTGGAACCGTTATATTGGAAC</td>
</tr>
<tr>
<td></td>
<td>SA1699EcoPstI</td>
<td>CAAACCTGCAGCTATTGGAATTTATGTTGGAATGCT</td>
</tr>
<tr>
<td>Upstream vraR operon promoter</td>
<td>EYKpn</td>
<td>GGGGTACCGGATCAGAATCTGTTGGAACCGTTATATTGGAAC</td>
</tr>
<tr>
<td></td>
<td>Tet marker upF</td>
<td>GGGGTACCGGATCAGAATCTGTTGGAACCGTTATATTGGAAC</td>
</tr>
<tr>
<td></td>
<td>Tet marker downR</td>
<td>CAAACCTGCAGCTATTGGAATTTATGTTGGAATGCT</td>
</tr>
<tr>
<td></td>
<td>Kan marker upF</td>
<td>GGGGTACCGGATCAGAATCTGTTGGAACCGTTATATTGGAAC</td>
</tr>
<tr>
<td></td>
<td>Kan marker downR</td>
<td>CAAACCTGCAGCTATTGGAATTTATGTTGGAATGCT</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>VraR-450 forward</td>
<td>TGCTTACAGAAGGAAAGTAAAAAG</td>
</tr>
<tr>
<td></td>
<td>VraR-535 reverse</td>
<td>CGGTTTTAAATATATATATATGCGATG</td>
</tr>
<tr>
<td></td>
<td>VraR-473T (TAMRA-FAM)</td>
<td>TGATTGGGAAAGTGTTTACCTAATCAAGAAAT</td>
</tr>
</tbody>
</table>

* Underlined regions represent restriction enzyme sequences.

by using KpnI and PstI. The erythromycin resistance ermB cassette was obtained by BamHI digestion of a mini-Mu transposon derivative of pUC19-MuSupF (26) harboring the ermB gene subcloned with linkers from pEC2 (11). The BamHI ermB fragment was cloned into the unique BglII site. A plasmid with the transcriptional orientation of ermB oriented in the same sense as the native vra operon transcript was chosen, digested with KpnI, and partially digested with PstI, and then the 3.7-kb fragment was cloned into pBT2 to yield pAM1284. Plasmid pAM1284 was electroporated into the restriction-defective strain Rm4220 and then transferred by electroporation into ISP794, selecting for erythromycin resistance at 30°C. ISP794 containing pAM1284 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 5 μg/ml chloramphenicol plates to screen for chloramphenicol resistance at 30°C. ISP794 containing pAM1284 was grown overnight at 30°C, followed by growth with applied marker selection for 6 days with dilution passages at 42°C. Bacteria were plated on agar containing 5 μg/ml kanamycin/ml and then replica streaked on 15-μg/ml chloramphenicol plates to screen for chloramphenicol-sensitive colonies. Double-crossover events corresponding to vraS gene disruption was confirmed by PCR. The resulting strain was named AR756.

Generation of AR756 containing the vraS(H156A) mutant. The site-specific chromosomal two-nucleotide change for the creation of vraS(H156A) was constructed using the thermosensitive plasmid pBT2 as follows. A KpnI-BglII overlap PCR fragment of ~2.700 bp was amplified from ISP794 using the primers SA1703KpnBamHI, H156A-B, H156A-A, and 12ABgl2 (Table 2). The overlap PCR fragment spanning the region from SA1703 gene to the vraS-SA1699 intergenic region contains the nucleotide changes in positions 466 (C to G) and 467 (A to C) of the vraS open reading frame, resulting in the H156A codon change. A second downstream PCR fragment was amplified by using the primer pair 12BBgl2 and SA1699EcoPst (Table 2) incorporating BglII and PstI restriction sites covering ~1.300 bp from the vraR-SA1699 intergenic region and the adjacent SA1699 gene. The fragments were cloned together in a three-piece ligation with KpnI-PstI-digested pBT2. The unique BglII restriction site was engineered in the vraR-SA1699 intergenic region was used to insert a kanamycin resistance marker obtained by PCR amplification from strain ALC2057 and incorporating functional BamHI restriction sites (60). The site-specific vraS(H156A) codon change gene targeting plasmid was named pAR747 and was fully sequence confirmed.

Plasmid pAR747 was passed through the nonrestricting strain RN4220, followed by recovery and electroporation into AR756, selecting for kanamycin resistance (Kanr) at 30°C. A single colony was isolated and subjected to kanamycin selection for 6 days with regular dilution and subculture passages at 42°C. Bacteria were plated on agar containing 40 μg of kanamycin/ml and then replica streaked on 15-μg/ml chloramphenicol and 5-μg/ml erythromycin plates to screen for the Kanr and erythromycin-sensitive (Erys) double-crossover events corresponding to the expected vraS allele exchange. A single colony was grown and confirmed by PCR and sequencing. The resulting strain harboring vraS(H156A) and its nearby linked Kanr marker to facilitate bacteriophage-mediated cotransduction was named AR769. Bacteriophage φ80s was used to backcross the kanamycin-linked vraS(H156A) allele to ISP794 to yield AR828 (>95% cotransduction). For both AR769 and AR828, the entire chromosomal 3.3-kb vra four-gene operon, including 600 bp of upstream promoter sequence from the SA1703 ATG start codon, was sequence verified. Both strains had only the desired vraS gene (H156A) codon change. Bacteriophage φ80s and φ11 were used to backcross the kanamycin-linked vraS(H156A) allele to strains COL and New...
man, yielding strains AR868 and AR848, respectively. The presence of the vraS(H156A) allele in each derivative strain was verified by sequencing.

Construction of AR758 and AR943 containing a kanamycin- or tetracycline-marked wild-type vraR operon. Identical targeted insertions of only the kanamyacin resistance gene, or a tetracycline resistance gene, inserted near the vraR operon in the integron region between vraR and SA1699, were obtained using pBT2 as described above. Briefly, the regions 1,200 bp downstream and 1,300 bp upstream from chromosomal location 1946712 (using sequence coordinates based on N315 annotation [41]) were amplified by using the primer pairs described in Table 2. The kanamycin resistance marker was obtained by PCR amplification as described above. A BglII tetracycline resistance marker cassette was obtained as described previously (60). The desired double-crossover events were identified by screening for kanamycin/tetracycline-resistant but chloramphenicol-sensitive colonies. Correct insertion of the markers into the integron region was confirmed by PCR and sequencing.

Constructions of rsbU* and sigB mutant derivatives of ISP794. The wild-type rsbU* allele was restored in ISP794 by 480bs bacteriophage transduction of the tetL-linked (rsbUVW-sigB*) operon from donor strain MB211 and selection for tetracycline to yield AR852. ISP794 derivatives lacking a functional sigB gene (AR851) or disrupted for the entire rsbUVW-sigB operon (AR850) were constructed by 480bs bacteriophage transduction using the donor strain ALC1001 or MB212, respectively. The restoration of rsbU* in strain ISP794 was monitored both by the reappearance of yellow-orange pigment and by the presence of a functional σ^ph^ monitored by using a qRT-PCR assay of the σ^ph^ dependent transcription of aspG (23, 39, 59).

Genetic assays for the emergence of glycopeptide resistance. Tests for the emergence of glycopeptide resistance were performed essentially as described previously (60). Briefly, overnight cultures of each strain to be tested were grown in MHB at 37°C with vigorous agitation. Each bacterial culture was subsequently normalized using sterile 0.9% (wt/vol) NaCl to McFarland standard 2 by using a Densimat apparatus (bioMérieux, France). Aliquots (500 µL, 1.5 × 10^8 CFU) were spread on MHB agar containing various concentrations of freshly prepared teicoplanin or vancomycin, followed by incubation for 48 h at 37°C. To determine the relative efficiency of colony formation, serial dilutions of each culture were also plated on MHB plates without drug. For each strain background—ISP794, Newman, or COL—an emergence assay was performed using drug concentrations determined from pilot experiments to be at or above the broth macrodilution MIC. (Specifically, for teicoplanin these consisted of an ISP794 MIC of 1 to 2 µg/ml with an emergence assay performed at 2 µg/ml; a Newman MIC of 1 to 2 µg/ml [although the emergence assay was performed at 4 µg/ml because pilot experiments showed that plates were nearly confluent if selection at 2 µg/ml was applied]; and a COL MIC of 8 µg/ml with an emergence assay performed at 8 µg/ml. For vancomycin, they were an ISP794 MIC of 1 to 2 µg/ml with an emergence assay performed at 2 µg/ml; a Newman MIC of 2 to 4 µg/ml, with an emergence assay performed at 2 µg/ml; and a COL MIC of 2 to 4 µg/ml with an emergence assay performed at 2 µg/ml.) Broth macrodilution MIC assays were performed as described previously (60). The data were tabulated as the number of viable colonies at each drug concentration tested and normalized per McFarland unit. The sum of CFU obtained on selective media divided by the number of CFU obtained on nonspecific media. A subset of colonies was retested by replica plating on selective agar plates to select for growth on selective media.

Newman, or COL—an emergence assay was performed using drug concentrations determined from pilot experiments to be at or above the broth macrodilution MIC. (Specifically, for teicoplanin these consisted of an ISP794 MIC of 1 to 2 µg/ml with an emergence assay performed at 2 µg/ml; a Newman MIC of 1 to 2 µg/ml [although the emergence assay was performed at 4 µg/ml because pilot experiments showed that plates were nearly confluent if selection at 2 µg/ml was applied]; and a COL MIC of 8 µg/ml with an emergence assay performed at 8 µg/ml. For vancomycin, they were an ISP794 MIC of 1 to 2 µg/ml with an emergence assay performed at 2 µg/ml; a Newman MIC of 2 to 4 µg/ml, with an emergence assay performed at 2 µg/ml; and a COL MIC of 2 to 4 µg/ml with an emergence assay performed at 2 µg/ml.) Broth macrodilution MIC assays were performed as described previously (60). The data were tabulated as the number of viable colonies at each drug concentration tested and normalized per McFarland unit. The sum of CFU obtained on selective media divided by the number of CFU obtained on nonspecific media. A subset of colonies was retested by replica plating on selective agar plates to select for growth on selective media.

A subset of colonies was retested by replica plating on selective agar plates to select for growth on selective media.

RESULTS

Prediction of the VraS H-box region and protein purification. Phylogenetic and functional studies of histidine kinase sensors have revealed several highly conserved motifs within the cytoplasmic kinase domain (21). The motifs comprise two distinct domains: a HisKA domain containing the H-box and a conserved histidine residue that serves as the site of autophosphorylation, as well as a C-terminal domain termed HATPase within which are found boxes named N1, G, F, G2, and G3 that comprise the ATP-binding pocket (54).

Multiple sequence alignment using T-Coffee and CLUSTAL W2 algorithms (http://www.expasy.org/tools/proteome) were performed using the S. aureus VraS sequence and a panel of bacterial HK sensors. We identified one region, ARELH^156^DSVSQ, which was similar to the H-box region of eight HK sensors subclassified as type III (36). In addition, sequence contest inspection of all other histidines revealed a second potential histidine autophosphorylation site, VVH^238^EI, which was highly similar to the VSH^239^EI found in the S. aureus TCS HssR5 system (68). A schematic diagram summarizing the predicted domain architecture of VraS is shown in Fig. 1.

We cloned the region comprising the predicted cytoplasmic domain of VraS, amino acids 65 to 347, hereafter referred to as VraS^Cyt,. The N-terminal 64 amino acids of VraS are very rich in hydrophobic residues, and this region is thought to comprise the transmembrane anchor sequence (Fig. 1). The precise topological configuration of the transmembrane region is unknown, as are the amino acids responsible for sensing external signals. To purify VraS^Cyt, we designed PCR primers (Table 2) that incorporated both NdeI and PstI restriction sites appropriately positioned to permit cloning in plTBY12 (NEB). A recombinant hybrid VraS^Cyt, protein was produced in E. coli as an N-terminal intein fusion. Protein was purified by chitin affinity chromatography, cleaved in situ using DTT, eluted, and concentrated (see Materials and Methods). Thiol-induced cleavage resulted in the release of VraS^Cyt, containing four additional N-terminal amino acids: Ala, Gly, and His due to the intein self-cleavage recognition sequence, together with a Met introduced by the NdeI restriction site. A similar strategy was used to purify two variants of VraS^Cyt, in which histidines H136 and H238 had been separately replaced by alanine and named H156A and H238A (Fig. 1).

As a substrate for the detection of phosphotransfer by VraS^Cyt, we used the same intein fusion protein strategy to purify wild-type VraR and a point mutant, VraR-D55A. Purified VraR and VraRD55A also possessed an additional N-terminal three amino acids, Ala-Gly-His, after thiol cleavage.

Aliquots of purified proteins were examined by Coomassie blue staining after electrophoresis in 12% polyacrylamide-SDS gels and judged to be >99% pure (Fig. 2). Purified wild-type and mutant VraS^Cyt, proteins all comigrated with an apparent mass of 34 kDa, which is consistent with the predicted 34-kDa molecular mass for this fragment. Purified wild-type VraR and VraRD55A both comigrated in SDS-PAGE with an apparent molecular mass of 22 kDa, which is slightly smaller than the predicted 24 kDa for this fragment. Purified proteins were stable for at least several months at 4°C.

Determination of the site of VraS autophosphorylation. To examine the ability of each VraS^Cyt, protein to undergo autophosphorylation in vitro, samples were incubated in the presence of [γ-^32^P]ATP, and aliquots were removed at various times, resolved on 12% polyacrylamide SDS gels, dried, and autoradiographed. The results are shown in Fig. 3A.

We observed a progressive increase in the intensity of radiolabeled wild-type VraS^Cyt, indicating autophosphorylation that
reached an apparent plateau at 45 min. We observed a similar autophosphorylation of VraScyt H238A, which also showed a plateau after 45 min of incubation. The relative intensity of VraScyt and H238A autophosphorylation seen in the representative autoradiogram is most likely due to experimental conditions, or perhaps to altered protein specific activity. We did not further explore the exact cause of this discrepancy. In contrast, to the results obtained with VraScyt and H238A, we observed no detectable autophosphorylation of the H156A variant under identical conditions and replicate experiments.

We conclude from this analysis that VraS H156 is essential for autophosphorylation of VraScyt H238A, which also showed a plateau after 45 min of incubation. The relative intensity of VraScyt and H238A autophosphorylation seen in the representative autoradiogram is most likely due to experimental conditions, or perhaps to altered protein specific activity. We did not further explore the exact cause of this discrepancy. In contrast, to the results obtained with VraScyt and H238A, we observed no detectable autophosphorylation of the H156A variant under identical conditions and replicate experiments.

We conclude from this analysis that VraS H156 is essential for autophosphorylation of the purified VraScyt fragment in vitro and that this site most probably represents the site of autophosphorylation. The arrangement and position of conserved amino acid residues in the N, G1, G2, and G3 regions of the VraS cytoplasmic domain fragment are coincident when comparing VraS alignment with other sensor kinase proteins such as Escherichia coli NarX and UhpB or Haemophilus influenzae NarQ (Fig. 1). The absence of an F region, together with the 108-amino-acid spacing between H156 and N strongly
suggests the classification of VraS as HK subtype type III and kinase type unorthodox (36).

**VraS histidine kinase phosphotransfer assay.** Alignment of response regulators from multiple bacteria has consistently revealed a highly conserved acidic pocket positioned at the top of the characteristic αβ5 protein fold of the receiver domain. An aspartate side chain within the pocket serves as an acceptor for phosphotransfer reaction from the cognate histidine kinase receiver domain. Inspection of the VraR domain revealed that D55A was most likely the site of phosphorylation. Using purified proteins and *in vitro* phosphotransfer assay, we demonstrated that phosphorylated VraS_H156 can transfer its phosphate to VraR but not to VraR-D55A (Fig. 3B). While the present study was in progress, VraR-D55A was independently identified by mass spectrometry as the site of VraR phosphorylation (3), thus confirming these results. Collectively, these findings define key molecular details of the essential amino acid residues, VraS-H156 and VraR-D55, comprising the phosphorelay network of the VraRS TCS system.

**Targeted engineering of vraS(H156A) point mutation in the S. aureus chromosome.** To examine the functional consequences of uncoupling VraS-VraR phosho-signaling *in vivo*, we next constructed *S. aureus* strains (AR769 and AR828) in which the *vraS*(H156A) mutation tagged with a nearby kanamycin resistance marker was stably introduced into the bacterial chromosome. Another strain, AR758, containing only the kanamycin marker within the VraR-SA1699 intergenic region, but otherwise wild type for the entire *vraSR* operon, was designed in parallel. The two-step strategy used for the genetic engineering by allelic exchange of this targeted codon mutation is depicted in Fig. 4.

A design feature that permitted efficient screening for the kanamycin-linked *vraS*(H156A) mutation in strain AR769 was the prior construction of an intermediate strain, AR756, that contained an internal deletion within *vraS* (Fig. 4B). Kanamycin selection with the thermosensitive pAR747 targeting plasmid resulted in a subset of colonies having lost the *ermB* marker by allelic exchange, thus guaranteeing the
chromosomal insertion of the desired vraS(H156A) mutation (Fig. 4B).

Growth curves for ISP794, AR758, and the mutant strains AR769 and AR828 were identical and indicated that under our routine laboratory conditions, neither the introduction of a kanamycin marker in the VraR-SA1699 intergenic region nor the H156A codon change in VraS resulted in detectable altered fitness (data not shown). Strains AR769 and AR828 were both used interchangeably for subsequent in vivo experiments.

**Northern blot analysis.** To assess the effect of VraS-VraR phosphotransfer uncoupling by the H156A mutation in vivo, transcriptional induction of the vraR operon was monitored by Northern blot and qRT-PCR analysis for strains ISP794 and AR828 after challenge with subinhibitory amounts of cell wall-active antibiotics.

Using a radiolabeled vraR probe, we observed a strong transcriptional induction of the vraR operon in the presence of 0.5 MIC oxacillin as an inducer (Fig. 5A). We detected multiple distinct bands by this Northern blot analysis, including the longest transcript (2.7 to 3.0 kb) sufficient to encode the entire operon (22, 40, 77). In contrast, transcriptional induction was severely reduced in the mutant strain AR828. Similar results were obtained with subinhibitory concentrations of n-cycloserine and teicoplanin as cell wall stress inducers (data not shown).

Quantification of vraR mRNA levels by qRT-PCR analysis confirmed the oxacillin-stimulated transcriptional induction of the vraR operon. A small, but significant (P < 0.05) residual level of transcriptional induction (~2-fold) was observed in the vraS(H156A) mutant strain compared to its uninduced control level (Fig. 5B). This result suggests the possibility that additional transcriptional regulatory circuits exist for this operon that do not depend upon VraS-mediated signal transduction.

We next repeated the qRT-PCR assay using RNA extracted from strains harboring the multicopy plasmid pMK4, or pMK4 containing the entire 3.3-kb vraR operon including native upstream promoter sequences (pAM1483). We observed strong transcriptional induction of the vraR operon in the mutant strain harboring pAM1483, but not pMK4 vector alone, in the presence of subinhibitory oxacillin as described above (data not shown). We conclude that the vraS(H156A) mutation is recessive and that only the vraS(H156A) mutation accounts for the observed loss of transcriptional induction in the experiments described above.

**Effect of vraS(H156A) on the frequency of emergence of reduced sensitivity first step glycopeptide mutants.** To test the functional consequences in vivo of uncoupling VraS-VraR phospho-signaling, we next examined whether the mutation detectably altered the MIC, or the frequency with which resistant colonies appeared on agar plates supplemented with various amounts of glycopeptide antibiotics. We reasoned that uncoupling the VraS-VraR phosphotransfer sensory system would significantly affect the detection of cell wall stress and thus alter or abolish the emergence of glycopeptide-resistant mutants.

We addressed this hypothesis using three S. aureus strain backgrounds: ISP794 (an 8325 derivative defective in rsbU and consequently lacking a normal alternative stress sigma factor σH pathway), Newman (rsbU'), and COL (an rsbU' MRSA strain). The teicoplanin and vancomycin MICs were determined for ISP794, Newman, and COL, and the emergence assay was designed to detect the number of viable colonies arising on plates containing drug at the MIC determined for each parental strain (see Materials and Methods). We observed a significant MIC reduction in every case (Table 3). Measurement of the oxacillin MIC for the MRSA strain COL (MIC = 400 μg/ml) and AR868 (MIC 100 μg/ml) indicated that the vraS(H156A) mutation also significantly reduced me-thicillin resistance in this strain background.

It is worthwhile to emphasize that the MIC values we report...
TABLE 3. Effect of vraS(H156A) mutation on glycopeptide MIC

<table>
<thead>
<tr>
<th>Strain</th>
<th>Teicoplanin (µg/ml)</th>
<th>Vancomycin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISP794</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>AR828</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Newman</td>
<td>1-2</td>
<td>2-4</td>
</tr>
<tr>
<td>AR848</td>
<td>0.5</td>
<td>0.5-1</td>
</tr>
<tr>
<td>COL</td>
<td>4-8</td>
<td>2-4</td>
</tr>
<tr>
<td>AR868</td>
<td>0.5</td>
<td>0.5-2</td>
</tr>
</tbody>
</table>

* MIC assays were performed by using broth microdilution. Note that discrepancies (ranges) have been indicated for glycopeptide MICs that are method dependent (75).

throughout our studies here were determined using broth microdilution; this method may give higher values than other susceptibility testing methods. Indeed, a recent study conducted in our laboratory indicates that microdilution methods tend to underestimate glycopeptide resistance (75). Glycopeptide MIC values for both COL and Newman have also been shown to be sensitive to experimental conditions (7, 66). Some caution is warranted, therefore, for cross-comparison of MIC values in the published literature since it is becoming clear that inoculum size, growth medium, time of incubation, and method are important parameters that collectively impact reported MIC values.

Next, for each parental strain tested, we observed the consistent appearance of viable colonies on agar plates containing various amounts of teicoplanin or vancomycin in the emergence assay. The frequency of emergence was computed for each strain and defined as the ratio of the number of colonies appearing using a standardized bacterial inoculum applied to each plate (1.5 × 10⁸ CFU) under selective (T MIC or V MIC) or nonselective (T₀ or V₀) conditions. We measured a frequency of viable colonies (defined hereafter as emergence) by this method predominantly in a range from 1.1 × 10⁻⁶ to 5.0 × 10⁻⁵, depending upon the strain and applied selection (Table 4). Similar results were observed when vancomycin was used, although we noted that the emergence frequency was lower for this drug than for teicoplanin under our standard laboratory conditions.

Colonies that arise in our emergence assay are either false positives and do not grow under selective conditions upon replating or represent stable mutants that do grow at or above the original selective conditions upon replating. We estimated the false-positive rate in multiple experiments by sampling subsets of colonies and determined it to be in the range of 5 to 20%. The tabular data we report, however, reflect the summed raw data from multiple independent experiments without correction for false-positive rate, which was not computed for every experiment. Thus, the resulting calculation of resistance frequencies yields an upper limit. In some cases, we also examined the spread of MIC values of colonies that arose during selection and which were classed as stable. For example, ISP794 selected for growth on MH agar plates in the presence of 2 µg of teicoplanin/ml gave rise to a range of MICs from 2 to 8 µg/ml (data not shown). Thus, colonies that grow at the applied selection concentration and that are not false positive show MIC values at or above the drug concentration used for the initial applied selection.

In striking contrast to our results obtained with strains harboring a wild-type vraS allele, we never observed colonies arising in multiple independent trials using teicoplanin selection for any of the three strains tested containing the vraS(H156A) mutation. When using vancomycin selection, we did not observe the appearance of resistant colonies with strain ISP794; however, we did observe the appearance of several viable colonies derived from strains Newman or COL (Table 4). The appearance of these infrequent colonies, whose vancomycin resistance was confirmed by replating, indicated that during selection with vancomycin the vraS(H156A) mutation drastically reduced (by >2 log₁₀) but did not entirely abolish the emergence of resistant colonies in these strain backgrounds. Control experiments with strains carrying only the kanamycin resistance marker in the vraR-SA1699 intergenic region showed equivalent emergence frequencies of drug-resistant colonies compared to parental strains, thus ruling out any deleterious role for this marker that could account for the observed abolition or significant reduction in viable CFU for strains AR828, AR848, and AR868 exposed to glycopeptides (data not shown).

To confirm that the abolition of the emergence of glycopeptide-resistant mutants was indeed the result of the vraS(H156A) mutation, we performed a complementation analysis using strains AR878 and AR872 containing multicopy plasmid pM1483 encoding the entire four-gene vraR operon or containing pM902 control vector harboring green fluorescent

TABLE 4. Effect of vraS(H156A) mutation on emergence of glycopeptide resistance

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Relevant genotype</th>
<th>Teicoplanin (CFU Σ n (n))</th>
<th>FOE (T MIC/T₀)</th>
<th>Vancomycin (CFU Σ n (n))</th>
<th>FOE (V MIC/V₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISP794</td>
<td></td>
<td>MSSA, ΔrsbU</td>
<td>5591 (9)</td>
<td>1.1 × 10⁻⁶</td>
<td>37 (3)</td>
<td>5.0 × 10⁻⁸</td>
</tr>
<tr>
<td>AR828</td>
<td>ISP794, vraS(H156A)</td>
<td>MSSA, ΔrsbU</td>
<td>0 (9)</td>
<td>NC</td>
<td>0 (3)</td>
<td>NC</td>
</tr>
<tr>
<td>AR878</td>
<td>ISP794, vraS(H156A), pAM1483</td>
<td>MSSA, ΔrsbU</td>
<td>238 (3)</td>
<td>6.6 × 10⁻⁷</td>
<td>30 (3)</td>
<td>6.7 × 10⁻⁸</td>
</tr>
<tr>
<td>AR872</td>
<td>ISP794, vraS(H156A), pAM902</td>
<td>MSSA, ΔrsbU</td>
<td>0 (3)</td>
<td>NC</td>
<td>0 (3)</td>
<td>NC</td>
</tr>
<tr>
<td>Newman</td>
<td></td>
<td>MSSA, rsbU⁺</td>
<td>504 (6)</td>
<td>4.0 × 10⁻⁷</td>
<td>200 (3)</td>
<td>2.0 × 10⁻⁷</td>
</tr>
<tr>
<td>AR848</td>
<td>Newman, vraS(H156A)</td>
<td>MSSA, rsbU⁺</td>
<td>0 (6)</td>
<td>NC</td>
<td>1 (3)</td>
<td>7.6 × 10⁻¹⁰</td>
</tr>
<tr>
<td>COL</td>
<td></td>
<td>MRSA, rsbU⁺</td>
<td>626 (4)</td>
<td>8.0 × 10⁻⁷</td>
<td>456 (3)</td>
<td>3.5 × 10⁻⁶</td>
</tr>
<tr>
<td>AR868</td>
<td>COL, vraS(H156A)</td>
<td>MRSA, rsbU⁺</td>
<td>0 (4)</td>
<td>NC</td>
<td>4 (3)</td>
<td>3.1 × 10⁻⁸</td>
</tr>
</tbody>
</table>

* The frequency of emergence (FOE) is expressed as the ratio of CFU under selective (T MIC or V MIC) and nonselective (T₀ or V₀) conditions computed as the mean from the indicated number (n) of independent experiments. Each emergence assay was performed using MH agar and 1.5 × 10⁶ applied bacteria per plate. CFU were counted at 48 h and 37°C. False-positive rates of CFU detected in the emergence assay were found to be 5 to 20% depending upon the experiment. NC, not computed.
The results revealed that restoration of wild-type vraS coding sequence concomitantly restored emergence of glycopeptide resistant mutants using either teicoplanin or vancomycin selection (Table 4). Similar results were obtained in a second type of complementation analysis without multicopy plasmid where we restored wild-type vraS in the ISP794 genetic background by allelic exchange using bacteriophage-mediated transduction from AR943 (data not shown).

Collectively, we conclude from these results that the VraS-VraR phosphotransfer uncoupling mutation vraS(H156A) abolished the detectable emergence of teicoplanin-resistant colonies and severely reduced the frequency of emergence of vancomycin resistant colonies. Since the vraS(H156A) mutation also alters COL resistance to oxacillin, we conclude that uncoupling VraS-VraR phosphotransfer impacts S. aureus response to at least two classes of antibiotics and can act both at the level of blocking emergence or altering the sensitivity profile of preexisting drug resistance.

**Role of the alternative sigma factor σB in the emergence of glycopeptide resistance.** The ISP794 strain and its derivatives used routinely in our laboratory (8, 60) and also extensively for studies of fluoroquinolone resistance (19) is a derivative of 8325 and carries an 11-bp deletion in rsbU. Because of this mutation, ISP794 possesses impaired transcriptional responses mediated by the alternative stress sigma factor σB. A functional σB is thought to play a role in glycopeptide resistance (5, 7), but its role in the early steps of emergence of drug resistance have not been previously examined. Therefore, to test what role, if any, σB played in the emergence of glycopeptide resistance in this strain background, we constructed three isogenic derivatives of ISP794: AR850, AR851, and AR852, which were characterized by deletion of the entire four-gene rsbUW-sigB operon, disruption of the sigB alone, or restoration of rsbU+, respectively. Each strain was tested in multiple independent assays as described above. The results are shown in Table 5.

We observed that the deletion of the entire rsbUW-sigB operon, or sigB alone, did not abolish the emergence of teicoplanin-resistant colonies. In contrast, when rsbU+ was restored in ISP794, we noted dramatic enhanced emergence frequency compared to ISP794 (ΔrsbU) conducted under identical conditions. We conclude that a functional σB markedly enhances the emergence of glycopeptide resistance in this strain background but that σB is not absolutely required for resistant colonies to arise.

### DISCUSSION

In this study, we have identified the amino acids, VraS H156 and VraR D55, involved in phosphotransfer signaling in the VraRS TCS, a sentinel system for cell wall stress in *S. aureus*. Importantly, we have found that three distinct strains—ISP794, Newman, and COL, each genetically engineered to harbor the mutation encoding autophosphorylation-defective VraS H156A—are unable to generate first step low-level teicoplanin resistance mutants at a detectable frequency. The mutation also abolished detectable first-step low-level vancomycin-resistant mutants in strain ISP794 and severely reduced the frequency of mutants in Newman and COL strains. Disruption of VraS-mediated signaling in these strains also significantly altered the MICs of both teicoplanin and vancomycin as measured by broth macrodilution and also significantly altered the oxacillin MIC in the MRSA strain COL.

A broad range of antibiotics provoke cell wall stress in *S. aureus* and transcriptional upregulation of the vraSR four-gene operon is a feature commonly observed (22, 40, 77). Recent studies provide a mechanistic explanation since VraR binds directly to its own promoter and thus autoregulates itself (4).

Besides extensive evidence from transcriptome and sequence analysis of clinical isolates that highlight the importance of VraRS for glycopeptide resistance (9, 16, 22, 32, 34, 40, 46, 49, 52, 56, 73), additional mutations in the graRS TCS or tcaA disruption promote glycopeptide resistance (10, 15, 30, 45, 48, 53).

Transcriptional upregulation of the WalKR TCS is also associated with altered glycopeptide resistance (31). Considering our inability to isolate first-step mutants using strains carrying the vraS(H156A) mutation, it is tempting to speculate that a functional VraRS phosphotransfer signaling system is necessary for all, or certainly a majority, of pathways leading to the emergence of glycopeptide resistance. It is important to stress, nevertheless, that the emergence assay that we describe in the present study used selection at very low drug concentrations, and therefore the role of VraRS has not been systematically assessed under other selection conditions using, for example, higher drug concentrations.

Several recent studies also provide evidence for glycopeptide resistance arising as a consequence of genetic selection with antibiotics other than glycopeptides. For example, selection of *S. aureus* growth in the presence of imipenem or daptomycin has been shown to produce strains with altered glycopeptide resistance profiles (32, 33, 50). These results are also supported.
by clinical case reports suggesting that the mechanisms responsible for altered sensitivity to daptomycin and the emergence of hVISA are related (37, 71). In light of these findings, it is conceivable that disruption of VraRS TCS signaling could also predictably attenuate the emergence of glycopeptide cross-resistance evoked by a nonglycopeptide drug encounter.

Derivatives of <i>S. aureus</i> strain 8325, such as ISP794 used in our study, are known to have an 11-bp deletion in <i>rsbU</i>, and consequently such strains display reduced availability of free α<sup>Bo</sup>, an alternative stress sigma factor that has been shown to regulate more than 200 genes in response to a variety of stress inducers (6, 23, 39). Free α<sup>Bo</sup> is sequestered by the anti-sigma factor RsbW and is thought to be released in times of stress by the anti-sigma factor RsbV, provided it is dephosphorylated by the RsbV-specific phosphatase, RsbU (65). The absence of a functional RsbU in 8325 derived strains thus explains the constitutive sequestration of α<sup>Bo</sup> by RsbW. Previous studies (5, 7) have hinted that α<sup>Bo</sup> controls gene(s) required for glycopeptide resistance in <i>S. aureus</i> and that teicoplanin exposure stress can select for enhanced σ<sup>Bo</sup> activity. One recently identified α<sup>Bo</sup>-regulated gene contributing to reduced glycopeptide sensitivity is <i>spoVG</i>, although its mechanism of action in <i>S. aureus</i> remains to be clarified (64).

In our study, we examined the role of σ<sup>Bo</sup> specifically at the level of the emergence of first step teicoplanin resistance mutants and found that strains derived from ISP794 lacking sigB (encoding σ<sup>Bo</sup>) or the entire <i>rsbUVW-sigB</i> operon were still able to generate teicoplanin-resistant mutants, thus formally demonstrating that a functional σ<sup>Bo</sup> was not strictly necessary for the emergence of glycopeptide resistance in this strain background. In contrast, the restoration of wild-type <i>rsbU</i> in ISP794, and thus the concomitant restoration of fully functional σ<sup>Bo</sup> stress response, led to markedly enhanced emergence of teicoplanin resistance. Our inability to isolate first-step teicoplanin mutants in an ISP794 <i>rsbU</i> strain carrying vraS<sup>(H156A)</sup> indicates that disrupting VraRS signaling can apparently override any contribution to the emergence of low-level teicoplanin resistance dependent upon σ<sup>Bo</sup> in this strain background.

Using quantitative transcript analysis, we have shown that the <i>vraS</i><sup>(H156A)</sup> mutation abolishes most, but not all transcription induction of the <i>vraSR</i> operon after brief exposure to a subinhibitory amount of oxacillin to provoke cell wall stress. Only a single major transcription start site upstream of SA1703, the first open reading frame of the four-gene operon, has been detected by primer extension (77) and confirmed independently by our laboratory using 5′ rapid amplification of cDNA ends (5′RACE; A. Renzoni, unpublished results). The <i>vraRS</i> operon is not known to be transcriptionally regulated by σ<sup>Bo</sup> under any condition tested (6). We therefore believe that additional regulatory pathways exist for this promoter that are not governed by VraS-VraR phosphotransfer. Experiments to uncover these accessory regulatory factors are in progress.

The consequences of genetic disruption of <i>vraS</i> by insertional inactivation or by deletion of the entire <i>vraSR</i> coding region have been studied independently by several laboratories (9, 16, 22, 40, 51, 56, 77). These reports link the loss of function of VraRS to oxacillin resistance in C-MRSA, altered drug sensitivity profiles in a range of clinical MSSA and MRSA isolates, and disruption of VraR-dependent target gene activation such as <i>pbp2</i>. In addition, a growing inventory of clinical hVISA and GISA isolates that have been partially sequenced reveals numerous examples of missense mutations mapping in <i>vraS</i>, <i>vraR</i>, or SA1702 (16, 32–34, 52). How missense mutations such as VraS-ISN modify VraRS-dependent signaling and gene expression is unknown. A reasonable hypothesis is that such mutations result in enhanced activation of the VraRS signaling pathway, even in the absence of an external inducing signal such as drug stress. With the VraSR pathway set in an ON, or semi-ON state, the cells realign their cell wall biosynthetic machinery to survive in the presence of glycopeptide.

Our study clearly predicts that pharmacologically blocking VraS signaling by interfering with its sensor histidine kinase function will significantly block the emergence of glycopeptide resistance or restore fully, or partially, sensitivity to glycopeptide antibiotics in strains showing preexisting reduced sensitivity to these drugs. The idea of using kinase inhibitors to target bacterial TCS systems has been suggested for many years, especially in light of the fact that TCS systems are not found in humans (2, 25, 58, 62). Our genetic study reported here therefore provides a firm foundation for this strategy and further predicts that the efficacy of existing antistaphylococcal cell wall-active antibiotics might be prolonged if such kinase inhibitor compounds were to be ultimately identified and coadministered.

Evidence presented to date underscores the importance of the <i>vraRS</i> operon at the crossroads mediating <i>S. aureus</i> response to cell wall stress and cell wall-active antibiotics. The ensemble of downstream events that are regulated by VraR-dependent transcription may be quite extensive judging by the number of genes suspected to be regulated by this system. Additional response pathways mediated by other TCS systems (AgrAB, GraRS, and WallKR) and global regulators such as σ<sup>Bo</sup> would appear to contribute to the establishment of endogenous glycopeptide resistance. By targeting the initial molecular features of the VraRS signaling response pathway at the onset of this multifactorial regulatory cascade, we strongly believe that emergence of glycopeptide resistance could be contained.

ACKNOWLEDGMENTS

This study was supported by Swiss National Science Foundation grant 31000A-120428 (to W.L.K.), a Novartis Consumer Health Foundation postdoctoral fellowship grant (to A.R.), and an F. Hoffmann-La Roche M.D.-Ph.D. training grant (to D.O.A.). Portions of this study were financed by a student exchange fellowship from the Italian Ministry of Education and Research (E.G.). We thank Markus Bischoff, Brigitte Berger-Bachi, Reinhold Bruckner, and Ambrose Cheung for generously providing strains and plasmids and members of the laboratory for their encouraging support and helpful comments on the manuscript.

REFERENCES


histidine kinase: new chemical leads to combat Staphylococcus epidermidis infections. BMC Microbiol. 6:96.


