The Role of the Staphylococcal VraTSR Regulatory System on Vancomycin Resistance and vanA Operon Expression in Vancomycin-Resistant \textit{Staphylococcus aureus}

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Abstract

Vancomycin is often the preferred treatment for invasive methicillin-resistant \textit{Staphylococcus aureus} (MRSA) infection. With the increase in incidence of MRSA infections, the use of vancomycin has increased and, as feared, isolates of vancomycin-resistant \textit{Staphylococcus aureus} (VRSA) have emerged. VRSA isolates have acquired the enterococcal vanA operon contained on transposon (Tn) 1546 residing on a conjugal plasmid. VraTSR is a vancomycin and \beta-lactam-inducible three-component regulatory system encoded on the \textit{S. aureus} chromosome that modulates the cell-wall stress response to cell-wall acting antibiotics. Mutation in vraTSR has shown to increase susceptibility to \beta-lactams and vancomycin in clinical VISA strains and in recombinant strain COLVA-200 which expresses a plasmid borne vanA operon. To date, the role of VraTSR in vanA operon expression in VRSA has not been demonstrated. In this study, the vraTSR operon was deleted from the first clinical VRSA strain (VRS1) by transduction with phage harvested from a USA300 vraTSR operon deletion strain. The absence of the vraTSR operon and presence of the vanA operon were confirmed in the transductant (VRS1Δvra) by PCR. Broth MIC determinations, demonstrated that the vancomycin MIC of VRS1Δvra (64 \mu g/mL) decreased by 16-fold compared with VRS1 (1024 \mu g/mL). The effect of the vraTSR operon deletion on expression of the van gene cluster (vanA, vanX and vanR) was examined by quantitative RT-PCR using relative quantification. A 2–5-fold decreased expression of the vanA operon genes occurred in strain VRS1Δvra at stationary growth phase compared with the parent strain, VRS1. Both vancomycin resistance and vancomycin-induced expression of vanA and vanR were restored by complementation with a plasmid harboring the vraTSR operon. These findings demonstrate that expression in \textit{S. aureus} of the horizontally acquired enterococcal vanA gene cluster is enhanced by the staphylococcal three-component cell wall stress regulatory system VraTSR, that is present in all \textit{S. aureus} strains.

Introduction

Invasive methicillin-resistant \textit{Staphylococcus aureus} (MRSA) has emerged as a major public health problem, implicated in 18,000 deaths annually with an estimated 94, 360 invasive MRSA infections in 2005 [1]. Vancomycin is a glycopeptide antimicrobial agent that has been one of the most frequently used antibiotics for invasive MRSA infections. With the increased usage of vancomycin, there has been an increase in the number of MRSA isolates with reduced vancomycin susceptibility in the last decade [2]. According to the Clinical and Laboratory Standards Institute (CLSI), vancomycin-intermediate \textit{S. aureus} (VISA) are those isolates with minimum inhibitory concentration (MIC) between 4 \mu g/mL and 8 \mu g/mL, and vancomycin-resistant \textit{S. aureus} (VRSA) are defined as those having MIC≥16 \mu g/mL [3].

The mechanism of vancomycin resistance in VISA strains is entirely different from that of VISA strains. Whereas vancomycin intermediate resistance involves chromosomal point mutations and a thicker cell wall [4–6], VRSA isolates to date have acquired the vanA operon contained on transposon (Tn)1546 residing on a conjugal plasmid [7,8]. VanA mediated resistance has been well studied in enterococci since the first \textit{Enterococcus faecium} isolate with transmissible vancomycin resistance was reported in France in 1988 [9]. The vanA locus typically confers high-level vancomycin resistance (MICs 512–1024 \mu g/mL) to enterococcal species [10] by encoding the genes necessary for producing an altered peptidoglycan precursor in which the final dipeptide, D-alanyl-D-alanine (D-Ala-D-Ala) is replaced by depsipeptide, D-alanyl-D-lactate (D-Ala-D-Lac). Vancomycin binds with decreased affinity to this D-Ala-D-Lac terminus, thus rendering the bacteria resistant to the drug [11]. The vanA locus consists of seven genes, \textit{vanRSHLXYZ}, whose expression is inducible by the glycopeptides, vancomycin and teicoplanin. Three of these genes (\textit{vanHLY}) are necessary for production of the D-Ala-D-Lac containing peptidoglycan precur-
sors [11]. VanR and VanS, encoded immediately upstream of vanHAX, comprise a two-component regulatory system responsible for the glycopeptide inducibility of vanHAX expression and the vanSR gene clusters. VanS is a membrane localized histidine kinase with an extracellular loop that has been proposed to be involved in sensing vancomycin leading to autophosphorylation of a conserved histidine residue [12]. This phosphoryl group is transferred to an aspartate in the cognate transcriptional activator, VanR, similar to other two-component systems [13]. It has been shown in enterococci that upon induction with vancomycin, the VanRS two-component system activates its own promoter and that of vanHAX leading to altered peptidoglycan precursors that confer resistance. VanY is a carboxypeptidase that is not necessary for resistance, but contributes to the resistance level [14]. VanZ confers resistance to teicoplanin by an unknown mechanism.

Of the 12 cases of VRSA reported in the USA, each has independently acquired the vancomycin-resistance transposon, Tn1546, independently from enterococcal donors [15]. Interestingly, vanA-containing S. aureus isolates exhibit a wide range of vancomycin MICs (vancomycin MIC 32 to 1024 μg/ml). We hypothesized that differential expression of native housekeeping genes amongst different S. aureus isolates could affect the phenotypic expression of acquired VanA-mediated vancomycin resistance.

Staphylococci have the ability to adapt quickly to antibiotic selection pressures resulting in development of resistant strains [16]. Exposure of antibiotics targeting the cell wall of S. aureus, activates the transcription of numerous genes encoding for cell-wall biosynthesis and metabolic pathways, known as the ‘cell wall stress stimulon’ [17–20]. VraTSR is a vancomycin- and β-lactam-inducible three-component regulatory system that modulates a large proportion of genes comprising this cell-wall stress response of S. aureus [19–21]. The VraTSR regulatory system includes VraS, a sensor histidine kinase, and VraR a response regulator [22]. VraS and VraR are encoded together on a transcript downstream of two other genes, which we recently named vraU and vraT (previously called yqfP) [21]. VraT is necessary for methicillin resistance and for the activation of the VraTSR-dependent cell wall stress stimulon whereas vraU is not required for either of these activities [17,21]. Thus, although vraU is encoded in the operon, we refer to the vraTSR operon since the role for vraU has not been found to date. Other studies have also shown that mutations in vraTSR genes can increase or decrease susceptibility to β-lactams and/or vancomycin in clinical MRSA and VISA strains [18,19,21,22]. We reasoned that since the acquired enterococcal vra and operon encodes a heterologous cell wall biosynthesis gene cluster, MRSA strains might modulate vraA mediated vancomycin resistance by using VraTSR. To test this hypothesis, we deleted the vraTSR operon from the first reported clinical VRSA strain and determined the effect on both vancomycin resistance and vraA operon expression.

**Materials and Methods**

All experiments were conducted under Biosafety level 2 conditions with the approval of the Institutional Biosafety Committee at the University of Chicago.

**Bacterial strains and plasmids**

The bacterial strains and plasmids used in this study are listed in Table 1. The VRSA parent strain used for deletion of the vraTSR operon was the first described clinical VRSA isolate carrying the Tn1546-borne vraA operon, VRSL1 [7]. VRSL1 was provided by the Network for Antimicrobial Resistance in S. aureus (NARSA) repository.

**Transduction**

Propagation of phage and transduction of S. aureus was carried out according to standard procedures as described [23]. To produce a phage lysate, bacteriophage Φ 11, was propagated in the vraTSR deletion strain, 923-M23 which has all 4 genes of the vraTSR operon (vraU, vraT, vraS, and vraR) replaced with a cat gene as described [24]. The lysate was used to infect VRSA1 at a multiplicity of infection of 1:1 (phage-to-recipient). Transductants carrying a vraTSR operon deletion were selected on tryptic soy agar (TSA) supplemented with chloramphenicol at 10 μg/ml. As a control, the phage lysate was streaked alone to evaluate sterility and the possibility of reisolating the donor strain. VRSL1Δvra is a vraTSR operon deletion mutant complemented by all 4 genes in the vraTSR operon in a low copy number plasmid (pVRASTR2) selectable by 5 or 10 μg/ml tetracycline as described [22].

**Effect of vraTSR deletion on growth**

The growth of wild type strain VRS1, VRS1Δvra and complemented strain VRS1Δvra were monitored using an incubated multi-mode plate reader (FLUOstar OPTIMA, BMG LABTECH) using conditions similar to an MIC assay. Briefly, the bacterial strains were grown overnight in tryptic soy agar (TSA) at 37°C. A colony from the overnight culture was inoculated in 0.9% saline and diluted to a cell concentration of 5 × 10^7 CFU/ml and 1 ml each was transferred to wells of a 48-well culture dish (Corning, Inc., Corning, NY) in quadruplicate and incubated at 37°C and the OD_600 was measured every 20 min for 24 hrs. The plates were agitated by orbital shaking prior to each reading. Tetracycline was included in the wells containing VRSL1Δvra.

**Broth Minimum Inhibitory Concentrations (MIC) determinations**

MICs were determined using the broth dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) [3] with exception of the use of BHI medium to optimize the vancomycin resistance phenotype as described previously [25]. An inoculum of 5 × 10^6 CFU was applied to each well of a 24-well culture dish (Corning, Inc., Corning, NY) containing duplicates of two fold increasing concentrations of vancomycin from 0–1024 μg/ml. Oxaclin was tested at 0, 2, 4, 6, 12, 16, 24, 32, 48, 64, 96, 128 μg/ml. The dishes were incubated at 37°C and MICs were recorded at 24 hrs. Each MIC experiment was repeated at least 4 times.

**Growth conditions for evaluating the effect of vraTSR deletion on van gene expression**

Since vraA and vraTSR expression are both inducible by vancomycin, two approaches were used to grow strains to evaluate the effect of the vraTSR deletion on expression of the vraA locus. In the first approach, we evaluated the steady state expression of vraA under continuous vancomycin inducing conditions. Strains were revived from frozen stocks stored in skim milk at −80°C onto TSA plates and incubated overnight at 37°C. The following day, a colony was inoculated into TSB supplemented with 2 μg/ml of vancomycin to induce vraA, followed by incubation for 16 hours at 37°C with aeration. The next day, the overnight culture was diluted 1:100 in fresh TSB, again with 2 μg/ml of vancomycin to maintain expression of vraA. Bacteria were then harvested at midlog (OD_600 of 0.5) and stationary (OD_600 of 1.0) growth phases.
as assessed by spectrophotometer. (Bausch & Lomb; Spectronic 21)

The second approach evaluated the effect of a vraTSR deletion on induction of vanA expression shortly after exposure to vancomycin. To this end, a colony was inoculated in TSB and incubated for 16 hours at 37°C with aeration. The next day, the overnight culture was diluted 1:100 in fresh TSB lacking vancomycin. When the culture reached an OD_{600} of 0.2, 2 μg/mL vancomycin was added to the medium. The RNA was harvested from the culture one hour later.

RNA isolation and purification
At the desired times during growth, bacteria were pelleted by centrifugation and frozen at −80°C. To isolate RNA, the cells were thawed on ice, resuspended in the appropriate volume of TE buffer containing recombinant lysozyme (Sigma, 1000 μg/mL) and incubated at room temperature for 10 mins to facilitate digestion of cell walls. The RNA was then extracted using the RNeasy kit (Qiagen) as directed by manufacturer’s instructions, including treatment with DNase prior to RNA precipitation. The RNA concentration was determined from the optical density at 260 nm, and the quality was determined from the A_{260}/A_{280} ratio and by analysis of rRNA using Agilent Bioanalyzer 2100.

Quantitative real-time reverse transcription PCR (qRT-PCR) assay conditions
Reverse transcription was performed using 2 μg of total RNA using the High Capacity Archive cDNA Kit (Applied Biosystems) for cDNA synthesis. The real-time PCR was carried out using ABI 7500 Fast RT-PCR instrument. Prime Time primer design software was used to design primer/probe mixes for a 5′ nuclease assay from Integrated DNA Technologies (IDT). The qRT-PCR probes were each labeled at the 5′ end with the indicated fluorophore and were double quenched with internal ZEN and a Iowa Black fluorophore and were double quenched with internal ZEN and a second internal quencher. The qRT-PCR was carried out using ABI 7500 Fast RT-PCR instrument. Prime Time primer design software was used to design primer/probe mixes for a 5′ nuclease assay from Integrated DNA Technologies (IDT). The qRT-PCR probes were each labeled at the 5′ end with the indicated fluorophore and were double quenched with internal ZEN and an internal second quencher ZEN (IDT).

Data Analysis
Relative quantitation of gene expression by qRT-PCR and MIC data were compared using Mann-Whitney test. All statistical data were analyzed by using Prism 5 program. (GraphPad Software, Inc., San Diego, CA). A p value of ≤0.05 was considered significant.

Results
Characterization of growth of the vraTSR mutant and the mutant complemented with the vraTSR operon
The comparison of growth curves of wild type VRS1 and the operon deletion strain VRS1Δvra demonstrate that deletion of vraTSR had minimal effect on fitness as shown by the similar

### Table 1. Strains and Plasmids used in this study.

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| Strains           |             |                     |
| VRS1              | First clinical VRSA isolate from Michigan (Clonal cluster 5) | NARS [8] |
| 923 M23           | A USA300 MRSA strain 923 with the vraTSR operon deletion (Clonal cluster 8) | [24] |
| VRS1Δvra          | vraTSR operon deletion strain derived from strain 923 | This study |
| VRS1Δvra          | vraTSR operon deletion mutant complemented with a vra operon expressed on a low copy number plasmid pVRASR2 harboring the vraTSR operon; selectable by 10 μg/ml tetracycline | This study |
| Plasmids          |             |                     |
| pVRASR2           | Entire vraTSR operon cloned into pAW8 | [22] |

### Table 2. Oligonucleotides and qRT-PCR probes used in this study.

| Name | Sequence |
|------|----------|
| For qRT-PCR* |                     |
| vanR Forward | 5′-GTGGAGTAAAGAGGACAGAAGC-3′ |
| Probe | 5′-6-FAM/TTAATGCAAGCCCGGTAAGCGGAC-3′ |
| Reverse | 5′-GTTTTCAAGAGGATCCGCAGGAC-3′ |
| vanA Forward | 5′-TTATAACGTTCCCACGACGACG-3′ |
| Probe | 5′-6-FAM/TGTCGGTACTCCGTACCTCATCC-3′ |
| Reverse | 5′-AAAGCAATCGTTCCATCTTGAGGAC-3′ |
| gyrB Forward | 5′-AACGGAGCTGTAGTACCAGTTGAT-3′ |
| Probe | 5′-Cy5/AAATGGGACGTCCAGCTGGAATGAG-3′ |
| Reverse | 5′-CCGCCAATTTACCACCCAGATT-3′ |
| 16S rRNA Forward | 5′-CAA TGG ACA ATA CAA AGG GCA G-3′ |
| Probe | 5′-Cy5/CGC GAG TGC AAA ATC CCA TAA AG-3′ |
| Reverse | 5′-TGC AGA CTA CAA TCC GAA CTG-3′ |
| For PCR |                     |
| vraS Forward | 5′-ATGAACCAACTACAATAAG-3′ |
| reverse | 5′-TAAATGCGTACTAAGCAGAC-3′ |
| vraR Forward | 5′-ATGACGTTATTAAGTTGAC-3′ |
| reverse | 5′-TCTGAGACGACTATTTG-3′ |
| vanA Forward | 5′-GCCGAAACAGCAATTCG-3′ |
| reverse | 5′-GTCAATTCGGCGGCTA-3′ |

*probes have 3′ Iowa Black Quencher and an internal second quencher ZEN (IDT). doi:10.1371/journal.pone.0085873.t002
growth curves of VRS1 and VRS1Δvra in absence of vancomycin (Fig. 1). At a subinhibitory vancomycin concentration (32 µg/ml), the duration of the lag phase increased by about 3.5 hrs in strain VRS1Δvra compared with VRS1; however the growth rates of the two strains were similar in the presence of this amount of vancomycin. Complementation with the vraTSR operon decreased the duration of the lag phase of VRS1Δvra by 2 hrs when grown with 32 µg/ml of vancomycin, which is intermediate between the wildtype and mutant strains. The presence of 512 µg/ml of vancomycin increased the lag phase to over 10 hrs for strain VRS1 whereas strain VRS1Δvra did not grow. Growth of VRS1Δvra in 512 µg/ml of vancomycin was partially restored to that of the wildtype strain by complementation with the vraTSR operon in trans on a plasmid (strain VRS1cΔvra). These data demonstrate that at a sub-MIC of vancomycin, the vraTSR operon deletion has an effect on the lag phase rather than the growth rate but has little effect on fitness in the absence of vancomycin.

Deletion of vraTSR decreased vancomycin resistance phenotype in vitro

As expected from a previous study [7], the MIC of vancomycin for the clinical strain VRS1 at 24 hrs was 1024 µg/ml. Deleting the vraTSR operon from strain VRS1 significantly reduced resistance to vancomycin (mode MIC, 64 µg/ml) by 16 fold (p-value 0.0003) (Fig. 2A). Complementation of strain VRS1Δvra with the vraTSR operon, restored the vancomycin resistance phenotype to that of the wildtype.

Deletion of vraTSR reduced resistance to oxacillin in vitro

The VraTSR three component regulatory system has been shown to influence the methicillin resistance phenotype [18,19,21,27]. Therefore, we determined the MIC of oxacillin of the mutant strain VRS1Δvra to assess the effect of the vraTSR deletion on methicillin resistance in a VRSA background. The MIC of oxacillin for the clinical strain VRS1 at 24 hrs was 128 µg/ml. Deleting the vraTSR operon from strain VRS1 significantly reduced resistance to oxacillin compared with the wildtype strain (MIC of 32 µg/ml, p-value <0.006) (Fig. 2B). The complementation of strain VRS1Δvra with the vraTSR operon expressed in trans increased the oxacillin MIC to 64 µg/ml (p-value <0.03).

Effect of deletion of vraTSR on steady state vanA, vanX and vanR expression (Figure 3)

Both the vanA and vraTSR operons are inducible by vancomycin [12,22]. Thus we evaluated the effect of the vraTSR deletion on vanA, vanX and vanR expression under conditions that would ensure expression of vanA during growth. We first evaluated the effect of a vraTSR deletion on vanA operon expression during midlog and stationary growth phases. To maintain continuous steady state induction of vanA, subinhibitory vancomycin was present in the growth medium during an overnight passage and during the experiment. Expression of vanA, vanX and vanR were evaluated by qRT-PCR at midlog (OD600 of 0.5) and stationary (OD600 of 1.0) growth phases. (Fig. 3)

vanA. In strain VRS1, steady state expression of vanA increased from mid-log to stationary growth phase (4.7 fold, p 0.0004). In contrast, in VRS1Δvra, vanA expression decreased 2.1 fold from mid-log to stationary growth phase. Moreover, the difference of vanA expression was significantly lower at the stationary growth phase in VRS1Δvra compared with the wildtype strain (3.4 fold, p <0.0001).

vanX. Similar to vanA, steady state vanX expression in strain VRS1 increased from mid-log to stationary growth phase (3.1 fold, p 0.01). In contrast, in VRS1Δvra, vanX gene expression was similar in mid-log and stationary growth phases (1.2 fold, p 0.48). Moreover, vanX expression at stationary growth phase was significantly lower in VRS1Δvra compared with VRS1 (2.2 fold, p 0.002).

vanR. The steady state expression of vanR in strain VRS1 increased from mid-log to stationary growth phase (3.5 fold, p 0.004) as it did for vanA and vanX. In contrast, in VRS1Δvra, vanR expression precipitously dropped in stationary phase compared with the midlog phase by 8.8 fold (p<0.0001). At mid-log growth phase, a paradoxical effect was observed. The expression of vanR was higher in the deletion strain VRS1Δvra compared with VRS1 (2.6 fold, p 0.0004). In contrast, the expression of vanA or vanX was

![Figure 1. Comparison of VRS1, VRS1Δvra and VRS1 CΔvra growth curves in absence (Van0) and presence of vancomycin at concentrations of 32 µg/ml and 512 µg/ml (Van 32 and Van 512). Colonies were picked from TSA, diluted to a density equivalent to a 0.5 McFarland standard in 0.9% saline. This inoculum was diluted to 5×10^8 CFU/ml and dispensed in a volume of 0.6 ml in each well. doi:10.1371/journal.pone.0085873.g001](image)
Deletion of vraTSR attenuates vanA and vanR gene induction by vancomycin (Fig. 4)

We also examined the effect of vraTSR deletion on the induction of the vanA operon and whether the decreased expression of vanA in the vraTSR mutant could be complemented by overexpression of vraTSR in trans. To this end expression of vanA gene expression was evaluated 1 hr after addition of vancomycin to the medium in strains VRS1, VRS1Δvra and the vraTSR complemented mutant strain, VRS1cΔvra. As shown in Figure 4, expression of vanA and vanR were induced by vancomycin in all three strains but induction was attenuated in the vraTSR mutant. With restoration of vraTSR operon expression in the complemented strain, VRS1cΔvra, the expression of both vanA and vanR genes increased compared with the mutant VRS1Δvra and was comparable to the wild type, VRS1 (Fig. 4).

In cultures grown in the presence of vancomycin, the expression of vanA decreased two-fold in strain VRS1Δvra compared with the wildtype strain VRS1. Moreover, expression of vanA was restored to 84% of the wildtype control strain VRS1. vanA expression also decreased in the absence of vancomycin 2.9-fold in mutant strain VRS1Δvra compared with the wildtype strain. However, in the absence of vancomycin, vra expression was expressed at the same level in both VRS1Δvra and VRS1cΔvra (Fig. 4). Thus, there was no complementation of vanR expression in the absence of vancomycin as there was in the presence of vancomycin. This pattern was similar to that observed with vanA expression. This demonstrates that VraTSR has a greater influence on vancomycin-dependent induction of vanA and vanR than it does on constitutive expression.

Although the deletion of vraTSR decreased expression of vanR and vanA in the presence of vancomycin, the fact that vanR and vanA expression did not diminish to that of the uninduced condition lacking vancomycin suggests that another factor besides VraTSR, such as VraR itself, is also involved in inducing vanR and vanA expression.
Vancomycin was added to early log cultures (when cultures reached an OD₆₀₀ of 0.2) to induce the endogenous control. The choice of the endogenous controls were based on compatibility with the target in the duplex reaction.

**Discussion**

Vancomycin and other glycopeptides interfere with the terminal stages of peptidoglycan synthesis by forming a complex with the terminal D-Ala-D-Ala di-peptide of peptidoglycan precursors thereby preventing their incorporation with the growing peptidoglycan polymer. Horizontal acquisition of the vanA gene cluster results in vancomycin resistance by producing an alternative peptidoglycan precursor to replace the wildtype precursors [28]. But the van genes remain relatively silent unless the bacteria are exposed to a glycopeptide. Similarly, the native vraTSR regulatory system is designed to have increased expression in response to cell wall biosynthesis stress that is elicited by antibiotics such as vancomycin [19,22,29], oxacillin [18,21,27] or daptomycin [30]. Likewise vraTSR expression has been shown to increase in response to decreased expression of the native cell wall synthesis enzyme pbp2 [18]. The result of upregulating the VraTSR system is a coordinated increase in expression of a regulon consisting of cell wall and metabolic genes that coordinately facilitate survival. The significance of this response is demonstrated by the fact that deletion or insertional mutagenesis of the vraTSR three-component regulatory system has been shown to increase the susceptibility of staphylococci to vancomycin, daptomycin and oxacillin in varying genetic backgrounds [18,19,21,27,29,30].

This study provides further evidence for the important role of VraTSR in the adaptation to vancomycin, and shows for the first time that VraTSR plays a role in vancomycin resistance in clinical VRSA strains through regulation of vanA gene expression. This was demonstrated by an increased lag phase of growth at sub MIC of vancomycin in the vraTSR mutant and by the requirement of an intact vraTSR operon for maximal induction of vanA and vanR by vancomycin. Furthermore, we show that the increase in vanA operon gene expression from midlog to stationary growth phase is dependent upon an intact VraTSR cell wall stress sensing system. This suggests that vanA expression is induced as cells sense that growth is slowing. This phenomenon could be linked to a signal generated by increased autolysis and slowing of peptidoglycan precursor incorporation into the cell wall. It is worth noting that although prior studies have examined the inducibility of growth and D-Ala-D-Lac peptidoglycan precursor production in VRSA strains [31], this is the first study to examine vanA gene expression in **S. aureus**.

Previously, vraTSR has only been shown to influence expression of native staphylococcal genes. This study now shows that despite being a native gene encoded on the staphylococcal chromosome, vraTSR can be utilized by *S. aureus* to control the expression of heterologous cell wall biosynthesis operon that is acquired horizontally with the advantage of conferring antibiotic resistance. This represents a particularly clever strategy since both van operon expression and vraTSR are induced by vancomycin.

This study confirms and extends a prior study in which the effect of vraTSR on vancomycin resistance had been tested in a VRSA strain containing the vanA operon. Gardete et al, produced a strain COLVA₃₀₀ΔvraS by introducing the plasmid from strain VRSA₁ into strain COL (a strain isolated in 1961) and deleting vraTSR [18]. In contrast, the approach taken in this study was to delete the vraTSR operon from the native clinical VRSA strain which is the source of the plasmid used to construct COLVA₃₀₀ΔvraS. Moreover, the strain used in our study belongs to the same clonal cluster as all other clinical VRSA isolates reported, clonal cluster 5 [32]. In contrast COLVA₃₀₀ΔvraS belongs to ST250 from clonal cluster 8. It was interesting that the vraTSR deletion in the clinical VRSA isolate decreased the vancomycin MIC to a greater extent (16-fold) than seen in the lab derived-strain (4-fold). Since both strains harbor the same vanA containing plasmid from VRSA₁, this provides evidence that factors in addition to vraTSR can account for differences in the level of vanA mediated vancomycin resistance among naturally occurring clinical isolates. This is consistent with historical data for oxacillin resistance. Although vraTSR affects resistance to oxacillin, strain specific factors other than vraTSR also influence the level of oxacillin resistance [33].

We observed a slight paradox on the effect of vraTSR during steady state vanA induction in midlog phase expression of vanA and vanX genes relative to vanR expression. Whereas midlog phase cultures of VRSAΔvra and VRS1 expressed similar levels of vanA and vanX, vanR expression was drastically higher in VRS1Δvra compared with VRS1. Nevertheless, at stationary phase vanR gene expression was drastically lower in the the vraTSR mutant compared with the wildtype, as it was for vanA and vanX.
Although vancomycin resistance decreases by 16 fold with a vraTSR operon deletion and is statistically significant, it may not be clinically important, as the vancomycin MIC still remains in the resistant range. It is possible however, that chemical inhibitors of VraTSR might be able to synergize with vancomycin to improve therapy against VRSA and VISA infections. This question remains to be tested in animal models of vancomycin therapy of vraTSR mutants, as we have done for oxacillin therapy of vra mutants of MRSA [21,24].

The molecular mechanism by which VraTSR affects vra operon expression remains to be determined. It is possible that there is cross talk between the two regulatory systems, VraTSR and VanRS. Indeed, it has been shown in Enterococcus faecalis that VanR is activated in the absence of VanS by another histidine kinase [12]. It is also possible that one of the 40 genes that are activated by VraTSR in response to vancomycin is responsible for the activation of vanA by VraTSR. These possibilities will be explored in future studies.

**Author Contributions**
Conceived and designed the experiments: NKQ SY. Analyzed the data: NKQ SBV. Contributed reagents/materials/analysis tools: NKQ SBV. Wrote the paper: NKQ SBV.

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