

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

Nadia K. Qureshi, Shaohui Yin, Susan Boyle-Vavra*

Section of Infectious Diseases, Department of Pediatrics, University of Chicago, Chicago, Illinois, United States of America

Abstract

Vancomycin is often the preferred treatment for invasive methicillin-resistant *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 *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 β -lactam-inducible three-component regulatory system encoded on the *S. aureus* chromosome that modulates the cell-wall stress response to cell-wall acting antibiotics. Mutation in *vraTSR* has shown to increase susceptibility to β -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 (VRS1 Δ vra) by PCR. Broth MIC determinations, demonstrated that the vancomycin MIC of VRS1 Δ vra (64 μ g/ml) decreased by 16-fold compared with VRS1 (1024 μ 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 VRS1 Δ vra 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 *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 *S. aureus* strains.

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* E-mail: sboyleva@uchicago.edu

Introduction

Invasive methicillin-resistant *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 *S. aureus* (VISA) are those isolates with minimum inhibitory concentrations (MICs) between 4 μ g/mL and 8 μ g/mL, and vancomycin-resistant *S. aureus* (VRSA) are defined as those having MICs \geq 16 μ g/mL [3].

The mechanism of vancomycin resistance in VRSA 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 *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 μ 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, *vanRSHAXYZ*, whose expression is inducible by the glycopeptides, vancomycin and teicoplanin. Three of these genes (*vanHAX*) are necessary for production of the D-Ala-D-Lac containing peptidoglycan precursor

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 *yvqF*) [21]. *VraT* is necessary for methicillin resistance and for the activation of the *VraTSR*-dependent cell wall 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 *vanA* operon encodes a heterologous cell wall biosynthesis gene cluster, MRSA strains might modulate *vanA* 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 *vanA* 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 *vanA* operon, VRS1 [7]. VRS1 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 VRS1 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 reisulating the donor strain. VRS1cΔvra is a *vraTSR* operon deletion mutant complemented by all 4 genes in the *vraTSR* operon in a low copy number plasmid (pVRSR2) 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 VRS1cΔvra 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 5×10^5 CFU/ml in TSB or TSB supplemented with vancomycin at 32 µg/ml, 512 µg/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 OD₆₀₀ 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 VRS1c Δvra.

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 5×10^5 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. Oxacillin 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 *vanA* 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 *vanA* locus. In the first approach, we evaluated the steady state expression of *vanA* 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 *vanA*, 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 *vanA*. Bacteria were then harvested at midlog (OD₆₀₀ of 0.5) and stationary (OD₆₀₀ of 1.0) growth phases

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)	NARSA [8]
923 M23	A USA300 MRSA strain 923 with the <i>vraTSR</i> operon deletion (Clonal cluster 8)	[24]
VRS1Δvra	<i>vraTSR</i> operon deletion strain derived from strain 923	This study
VRS1cΔvra	VRS1Δvra deletion mutant complemented with a <i>vra</i> operon expressed on a low copy number plasmid pVRASR2 harboring the <i>vraTSR</i> operon; selectable by 10 μg/ml tetracycline	This study
Plasmids		
pVRASR2	Entire <i>vraTSR</i> operon cloned into pAW8	[22]

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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 into 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 OD₆₀₀ 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 lysostaphin (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 A₂₆₀/A₂₈₀ 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® FQ at the 3’ end. (Table 2) The concentration of each primer in qRT-PCR reactions was 500 nM whereas the probe concentration was 250 nM. Differences in gene expression were calculated by relative quantification(RQ) with the comparative ΔΔCt method [26] using the indicated reference strain as the comparator.

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

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

Name	Sequence
For qRT-PCR^a	
vanR	Forward: 5’-GTGGAGTAAAGGAGCAGAACG-3’ Probe: 5’ 6-FAM/TTAATGACAAGGCCGGAGTGGACG-3’ Reverse: 5’-GTTTTACAGAGGATTCGACG-3’
vanA	Forward: 5’-TTATAACCGTTCCCGCAGAC-3’ Probe: 5’ 6-FAM/TTTGCCGTTTCTGTATCCGTCCTC-3’ Reverse- AACATATCCACACGGGCTAG-3’
vanX	Forward: 5’-ATCGCATGTAGGGACATACG-3’ Probe: 5’ 6-FAM/AGTTGGCTGAATCGCTTTTGAAAGGC-3’ Reverse: 5’-AAGCAATCCGTACCTTGG-3’
gyrB	Forward: 5’-AACGGACGTGGTATCCCAGTTGAT-3’ Probe: 5’ Cy5/AAATGGGACGTCCAGCTGTGCAAGTT-3’ Reverse: 5’-CCGCCAAATTTACCACCAGCATGT-3’
16S rRNA	Forward 5’-CAA TGG ACA ATA CAA AGG GCA G-3’ Probe 5’ Cy5/CGC GAG GTC AAG CAA ATC CCA TAA AG 3’ Reverse 5’-TGC AGA CTA CAA TCC GAA CTG-3’
For PCR	
vraS	Forward: 5’-ATGAACCACTACAATAG-3’ Reverse: 5’-TTTAATCGTCATACGAATC-3’
vraR	Forward: 5’-ATGACGATTAAGTATTG-3’ Reverse: 5’-TTCGATACGAACTATTGA-3’
vanA	Forward: 5’-GGGAAACGACAATTGC-3’ Reverse: 5’-GTACAATGCGGGCGTTA -3’

^aprobes have 3’ Iowa Black Quencher and an internal second quencher ZEN (IDT).

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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

growth curves of VRS1 and VRS1Δ*vra* 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 VRS1Δ*vra* 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 VRS1Δ*vra* 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 VRS1Δ*vra* did not grow. Growth of VRS1Δ*vra* 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 VRS1*c*Δ*vra*). 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 VRS1Δ*vra* 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 VRS1Δ*vra* 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 VRS1Δ*vra* 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 *vraR* 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 VRS1Δ*vra*, *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 VRS1Δ*vra* compared with the wildtype strain (8.4 fold, p<0.0001).

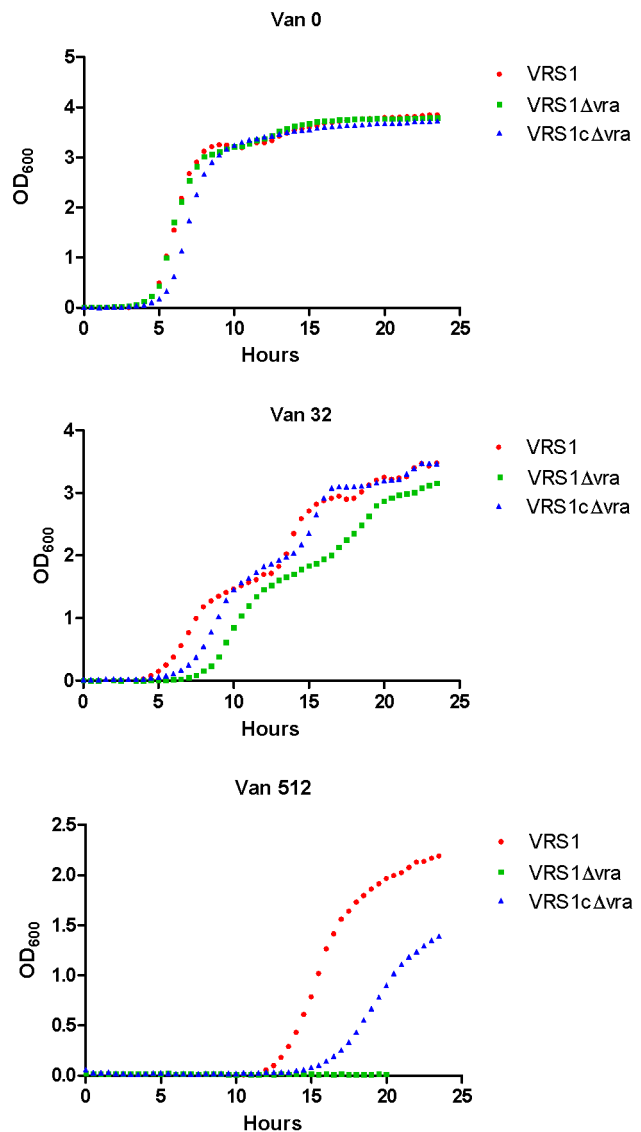


Figure 1. Comparison of VRS1, VRS1Δ*vra* and VRS1 *c*Δ*vra* growth curves in absence (Van0) and presence of vancomycin at concentrations of 32 μg/ml and 512 μg/ml (Van 32 and Van 512). Colonies were picked from TSA, diluted to a density equivalent to a 0.5 McFarland standard in 0.9% saline. This inoculum was diluted to 5×10⁵ CFU/ml and dispensed in a volume of 0.6 ml in each well. doi:10.1371/journal.pone.0085873.g001

***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 VRS1Δ*vra*, *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 VRS1Δ*vra* 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 VRS1Δ*vra*, *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 VRS1Δ*vra* compared with VRS1 (2.6 fold, p 0.0004). In contrast, the expression of *vanA* or *vanX* was

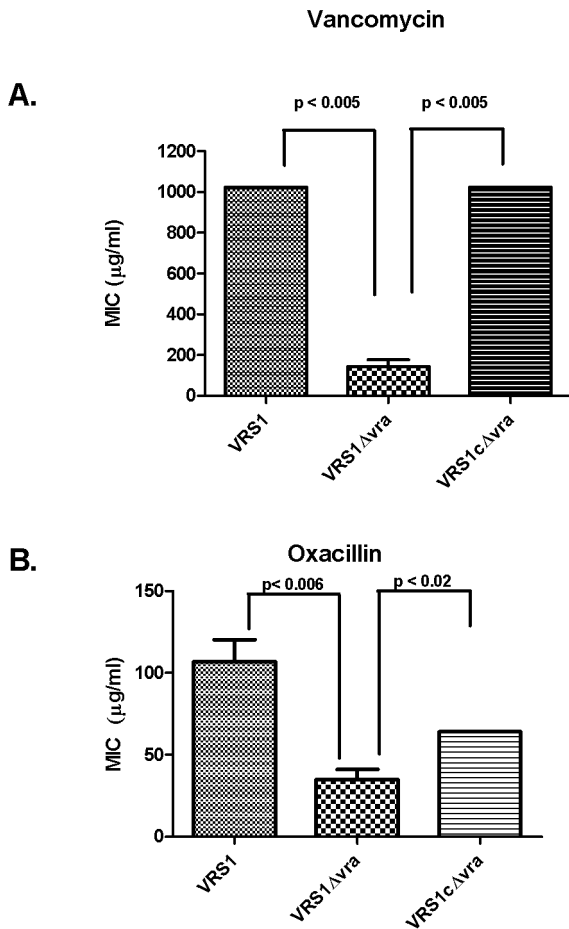


Figure 2. Comparison of MICs of the wild type VRS1, isogenic *vraTSR* deletion strain VRS1Δvra and complemented strain, VRS1cΔvra. (A) MIC for Vancomycin (B) MIC for Oxacillin. The bars represent the SD of the mean MIC from at least 4 independent determinations. The lack of error bars indicates a SD of 0. Vancomycin was tested in BHI at 0, 8, 16, 32, 64, 128, 256, and 512 μg/ml. Oxacillin was tested in TSB containing 2% NaCl at 0, 8, 16, 32, 64 and 128 μg/ml; Tetracycline (5 μg/ml) was used to test strain VRS1cΔvra to maintain the complementation plasmid pVRASR2. doi:10.1371/journal.pone.0085873.g002

not significantly different at midlog phase between the wildtype and the mutant. Nevertheless, the significantly lower abundance of the *vanA* transcript in the *vraTSR* mutant compared with the VRS1 wildtype strain by the time cells reached stationary phase, is consistent with the findings for *vanA* and *vanX* and suggests that overall, expression of the *van* operon is lower in the *vraTSR* operon deletion strain.

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 *van* 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*

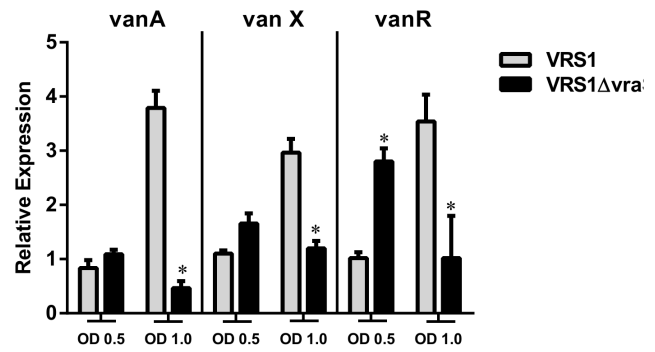


Figure 3. Effect of deletion of the *vraTSR* operon on steady state expression of the *vanA* operon genes by qRT-PCR. Subinhibitory concentration of vancomycin (2 μg/ml) was present in the growth media in two successive passages to maintain constitutive expression of *vanA*. The cultures were harvested at midlog (OD₆₀₀ 0.5) and stationary (OD₆₀₀ 1.0) growth phases as described in Materials and Methods. Expression was quantified by relative quantification (RQ) using the comparative ΔΔCt method and the wild type strain VRS1 at midlog growth phase (OD₆₀₀ 0.5) as the reference (after correction of each probe with an endogenous control). The *gyrB* gene probe labeled with the fluorophore Cy5 was used as the endogenous housekeeping gene and FAM-labeled probes for target genes *vanA*, *vanX* and *vanR* were used. The reactions were performed with three biological replicates with three technical replicates. * indicates p<0.05 compared with strain VRS1 at same time point. Error bars reflect propagated error calculated starting with the SD from the average C_T in each replicate. doi:10.1371/journal.pone.0085873.g003

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 in the *vraTSR*-complemented mutant, VRS1cΔvra, demonstrating that the loss of *vanA* induction in the *vraTSR* mutant involved *VraTSR*.

Comparing expression of *vanR* in the presence of vancomycin, there was a 3.6 fold decrease in *vanR* expression in strain VRS1Δvra compared with strain VRS1. *vanR* expression was restored to 88% of that of the wildtype in the presence of vancomycin in the complemented mutant VRS1cΔvra. *vanR* 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, *vanR* 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.

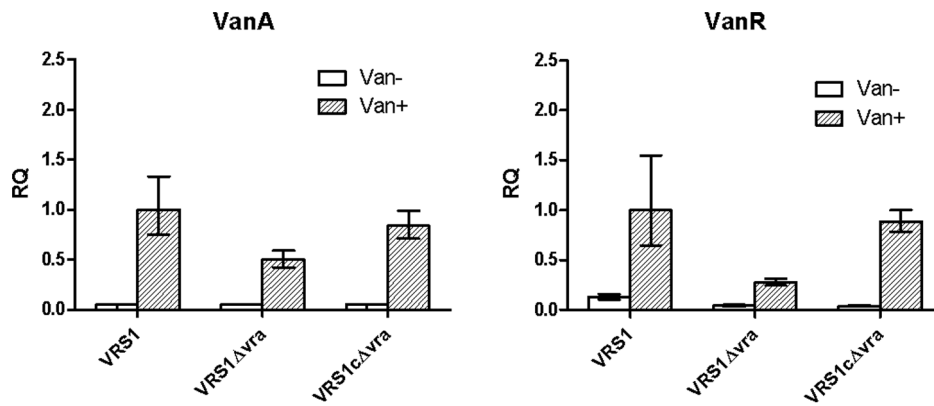


Figure 4. Effect of deletion of *vraTSR* on *vanA* and *vanR* gene induction by vancomycin. Expression of *vanA* operon genes in wildtype VRSA clinical strain (VRS1), the *vraTSR* deletion mutant (VRS1 Δ vra) and the *vraTSR*-complemented mutant (VRS1c Δ vra), as measured by qRT-PCR. Vancomycin was added to early log cultures (when cultures reached an OD₆₀₀ of 0.2) to induce *vanA* expression and RNA was isolated 1 hour later. Expression of each gene target was evaluated using relative quantification (RQ) with the comparative $\Delta\Delta$ Ct method using strain VRS1 treated with vancomycin as the reference (after each probe was normalized to an endogenous control). Error bars reflect the range of RQ values from 3 experimental triplicates. The *vanA* gene probe was labeled with FAM and used in duplex reactions with the *16S* rRNA gene probe labeled with fluorophore Cy5 as the endogenous control. A FAM-labeled *vanR* probe was used in a duplex reaction with a *gyrB* probe labeled with fluorophore Cy5 as the endogenous control. The choice of the endogenous controls were based on compatibility with the target in the duplex reaction. doi:10.1371/journal.pone.0085873.g004

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 *ppp2* [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 COLVA₂₀₀ Δ vraS by introducing the plasmid from strain VRS1 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 VRS1 strain which is the source of the plasmid used to construct COLVA₂₀₀ Δ vraS. 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 COLVA₂₀₀ Δ vraS 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 VRS1, 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 VRS1 Δ vra and VRS1 expressed similar levels of *vanA* and *vanX*, *vanR* expression was drastically higher in VRS1 Δ vra compared with VRS1. Nevertheless, at stationary phase *vanR* gene expression was drastically lower in 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 of VRSA and VISA infections. This proof of principle 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 *vanA* 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 SBV. Performed 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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