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VraR Binding to the Promoter Region of *agr* Inhibits Its Function in Vancomycin-Intermediate *Staphylococcus aureus* (VISA) and Heterogeneous VISA

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ABSTRACT Acquisition of vancomycin resistance in *Staphylococcus aureus* is often accompanied by a reduction in virulence, but the mechanisms underlying this change remain unclear. The present study was undertaken to investigate this process in a clinical heterogeneous vancomycin-intermediate *S. aureus* (hVISA) strain, 10827; an hVISA reference strain, Mu3; and a VISA reference strain, Mu50, along with their respective series of vancomycin-induced resistant strains. In these strains, increasing MICs of vancomycin were associated with increased expression of the vancomycin resistance-associated regulator gene (*vraR*) and decreased expression of virulence genes (*hla*, *hlb*, and *coa*) and virulence-regulated genes (RNAIII, *agrA*, and *saeR*). These results suggested that VraR might have a direct or indirect effect on virulence in *S. aureus*. In electrophoretic mobility shift assays, VraR did not bind to promoter sequences of *hla*, *hlb*, and *coa* genes, but it did bind to the *agr* promoter region. In DNase I footprinting assays, VraR protected a 15-nucleotide (nt) sequence in the intergenic region between the *agr* P2 and P3 promoters. These results indicated that when *S. aureus* is subject to induction by vancomycin, expression of *vraR* is upregulated, and VraR binding inhibits the function of the Agr quorum-sensing system, causing reductions in the virulence of VISA/hVISA strains. Our results suggested that VraR in *S. aureus* is involved not only in the regulation of vancomycin resistance but also in the regulation of virulence.

KEYWORDS *Staphylococcus aureus*, global regulatory networks, two-component regulatory systems, vancomycin resistance, virulence regulation

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Staphylococcus aureus is an important pathogen that can be acquired in both community and hospital environments and is responsible for illnesses ranging from superficial skin infections to deep-seated and life-threatening diseases (1, 2). Infections caused by methicillin-resistant *S. aureus* (MRSA) are associated with mortality and morbidity, an aggressive course, multidrug resistance, and hospital outbreaks (3). Vancomycin is the first-choice drug for treatment of MRSA infection, but its increasing clinical use has led to the emergence of *S. aureus* strains with reduced vancomycin susceptibility, including vancomycin-resistant *S. aureus* (VRSA), vancomycin-intermediate *S. aureus* (VISA), and heterogeneous VISA (hVISA) (4–6). According to the Clinical and Laboratory Standards Institute (CLSI) (7), VRSA is defined by a vancomycin MIC of ≥16 μg/ml, whereas VISA isolates have MICs between 4 and 8 μg/ml. hVISA strains appear to be susceptible to vancomycin (MICs of ≤2 μg/ml) but contain a subpopulation of vancomycin-resistant cells.

Most VRSA isolates carry plasmid-borne copies of transposon Tn*1546*, which was acquired from vancomycin-resistant *Enterococcus* species (8). Tn*1546*-based vancomycin resistance involves alteration of the D-alanyl-D-alanine dipeptide residue in the *S.*

aureus cell wall precursor lipid II to D-alanyl-D-lactate, which has substantially lower affinity for vancomycin (9). Unlike in VRSA, the molecular mechanisms of resistance in VISA/hVISA strains are not well understood. Comparative genomics shows diverse genetic mutations in VISA relative to vancomycin-susceptible *S. aureus* (VSSA), and only a few of these mutations (such as *vraSR*, *graSR*, *walKR*, *stk1/stp1*, *rpoB*, *clpP*, *sigB*, and *trfAB* genes) have been experimentally verified (10–17). Transcriptomic studies have revealed changes in expression levels in two-component regulatory systems (TCRSs), in particular, *vraSR*, *walKR*, and *graSR* (10, 18–20). These TCRSs seem to be involved in cell wall synthesis and thickening, which restricts the access of vancomycin to its target sites. A particularly interesting member of this group is *vraSR*, which encodes a histidine kinase (VraS) and a response regulator (VraR) that can rapidly sense and transduce cell wall stress (20). The VraSR system is highly expressed in VISA/hVISA strains (18, 21–23). After induction by an inhibitor of cell wall synthesis, VraS and VraR autoactivate the expression of the *vra* operon and about 46 other unlinked genes known collectively as the cell wall stimulon, which positively regulate synthesis of the cell wall, leading to thickening and subsequent resistance to vancomycin (21–24).

Acquisition of vancomycin resistance in VISA/hVISA strains is often accompanied with a decrease in virulence (25–27). Our previous report has also demonstrated that VISA/hVISA strains have reduced coagulase activity and reduced or no hemolysis (28). Hattangady et al. performed complete genome comparison, along with transcriptomic and metabolomic studies, of two laboratory-selected VISA strains and found that expression of surface-associated virulence determinants was decreased in VISA isolates (29). Majcherczyk et al. and Peleg et al. used a rat model and a Galleria mellonella model, respectively, to show that virulence and infectivity of VISA/hVISA strains attenuate as the vancomycin MIC for the strains increases (26, 27). However, the specific mechanism that underlies the attenuation of virulence in VISA/hVISA strains was still not clear.

In our study, a clinical hVISA strain, 10827; an hVISA reference strain, Mu3; and a VISA reference strain, Mu50, were exposed to increased concentrations of vancomycin and produced their series of vancomycin-induced resistant strains. Quantitative real-time PCR (qRT-PCR) was performed to investigate the expression of *vraR* (encoding a vancomycin resistance-associated regulator); virulence genes *hla* (encoding alpha-toxin), *hlb* (encoding β-toxin), and *coa* (encoding coagulase); and virulence-regulated genes (RNAIII, *agrA*, and *saeR*) in all strains. Electrophoretic mobility shift assays (EMSAs) were conducted to identify the potential of VraR to affect virulence through transcriptional regulation, and the precise location of VraR binding to promoter sequences was determined by DNase I footprinting.

RESULTS

Expression of *vraR* and virulence-associated genes is altered in VISA strains. Expression of *vraR*, *hla*, *hlb*, *coa*, RNAIII, *agrA*, and *saeR* was analyzed by qRT-PCR. As shown in Fig. 1A and Table 1, transcription of *vraR* was upregulated in the vancomycin-resistant strains 10827-V32 (3.11-fold), Mu3-V32 (2.92-fold), and Mu50-V32 (2.33-fold) compared to their parental isolates ($P < 0.001$). Transcription of *hla*, *hlb*, and *coa* was downregulated in the vancomycin-resistant strains 10827-V32 (0.43-fold, 0.30-fold, and 0.29-fold, respectively), Mu3-V32 (0.33-fold, 0.31-fold, and 0.28-fold, respectively), and Mu50-V32 (0.46-fold, 0.44-fold, and 0.58-fold, respectively) compared to their parental strains ($P < 0.001$) (Fig. 1B). Transcription of *agrA* and *saeR* was downregulated in the vancomycin-resistant strains 10827-V32 (0.26-fold and 0.59-fold, respectively), Mu3-V32 (0.26-fold and 0.57-fold, respectively), and Mu50-V32 (0.41-fold and 0.83-fold, respectively) compared to their parental strains ($P < 0.05$) (Fig. 1C). Moreover, we also observed a trend toward reduced transcription of RNAIII with higher vancomycin MIC, although this was not statistically significant.

Oligomeric state of VraR in solution. The analysis of purified VraR protein by gel filtration chromatography revealed a single peak with an elution volume of 17.06 ml. On the assumption that the shape and partial specific volume of VraR are similar to

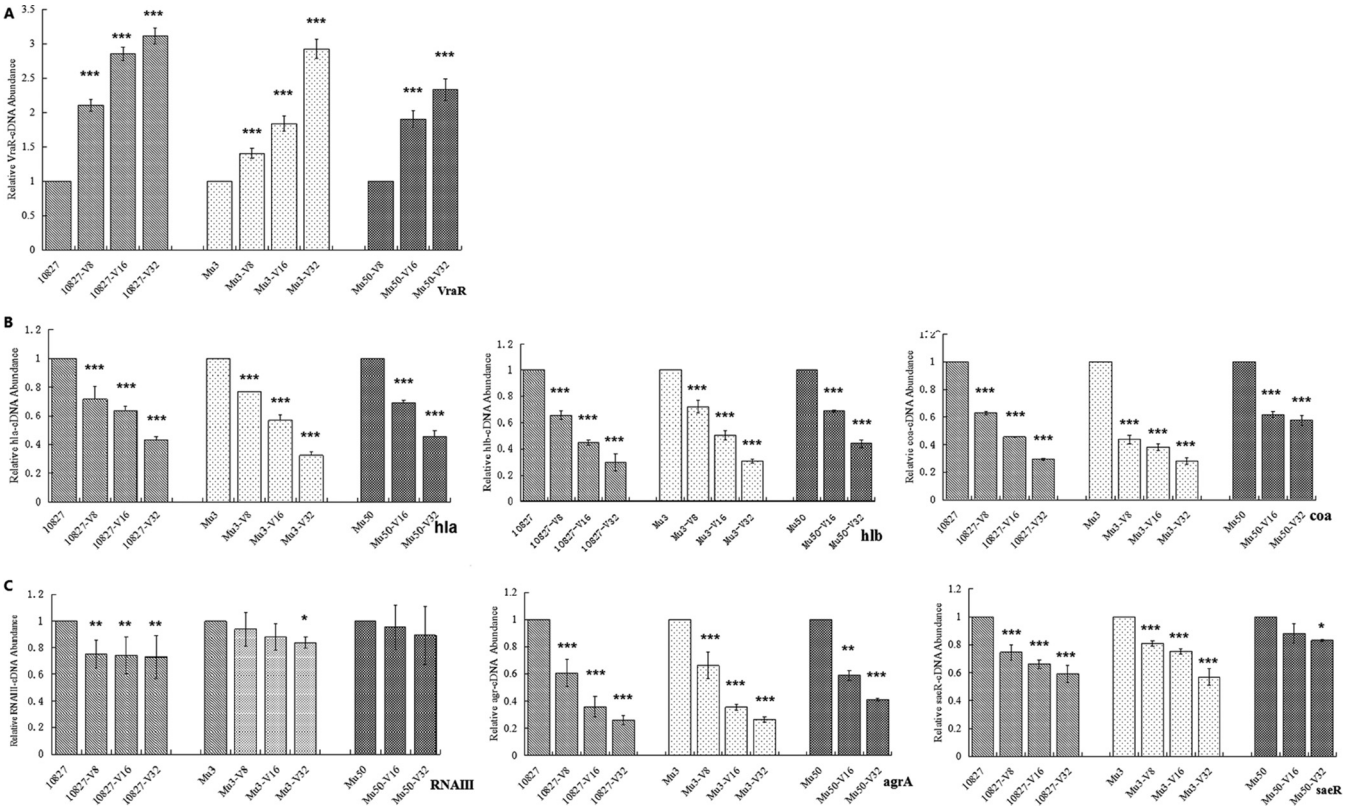

FIG 1 Quantitative real-time PCR analysis of the expression of resistance-associated and virulence-associated genes in *S. aureus* strains 10827, Mu3, and Mu50 and their series of vancomycin-resistant induced strains. Results are presented relative to their respective parental strain 10827, Mu3, or Mu50, the value for which has been normalized to 1. (A) Expression of *vraR*. (B) Expression of *hla*, *hlb*, and *coa*. (C) Expression of RNAIII, *agrA*, and *saeR*. Bars represent mean values from three or more independent experiments. Error bars indicate standard deviations. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

those of the standard proteins, the native molecular mass of VraR was estimated to be 24,058 Da, which was calculated from a standard linear regression equation, lgMw = 5.9466 − 2.7119 × K_{av} (Fig. 2). The native molecular mass is approximately the same as the molecular mass of a VraR monomer (~23.5 kDa). Native PAGE analysis also indicated that purified VraR protein was present in a single oligomeric state in solution. Thus, we conclude that VraR in solution is a stable monomer.

VraR binds specifically to the *agr* promoter region. The *vraR* gene was cloned, and His-tagged VraR protein was expressed and purified to perform EMSA with DNA probes containing the putative promoter sequences of the *coa*, *hla*, *hlb*, and *agr* target genes. With an increasing concentration of VraR in the assays, the amount of free *agr*

TABLE 1 Quantitative real-time PCR analysis of gene expression in *S. aureus* strains 10827, Mu3, and Mu50 and their series of vancomycin-resistant induced strains

Strain	Vancomycin MIC (μg/ml)	Relative cDNA abundance						
		vraR	*hla*	*hlb*	*coa*	RNAIII	*agr*	*sae*
10827	1	1	1	1	1	1	1	1
10827-V8	8	2.10 ± 0.09	0.71 ± 0.09	0.66 ± 0.03	0.63 ± 0.01	0.75 ± 0.10	0.61 ± 0.10	0.75 ± 0.06
10827-V16	16	2.85 ± 0.10	0.64 ± 0.03	0.45 ± 0.02	0.46 ± 0.00	0.74 ± 0.14	0.36 ± 0.08	0.66 ± 0.03
10827-V32	32	3.11 ± 0.12	0.43 ± 0.02	0.30 ± 0.06	0.2939 ± 0.0081	0.73 ± 0.16	0.26 ± 0.03	0.59 ± 0.06
Mu3	2	1	1	1	1	1	1	1
Mu3-V8	8	1.40 ± 0.07	0.77 ± 0	0.72 ± 0.05	0.44 ± 0.03	0.94 ± 0.13	0.66 ± 0.10	0.81 ± 0.02
Mu3-V16	16	1.84 ± 0.11	0.57 ± 0.04	0.51 ± 0.04	0.38 ± 0.02	0.88 ± 0.10	0.3555 ± 0.0224	0.75 ± 0.02
Mu3-V32	32	2.92 ± 0.14	0.33 ± 0.02	0.31 ± 0.02	0.28 ± 0.02	0.84 ± 0.04	0.26 ± 0.02	0.57 ± 0.60
Mu50	8	1	1	1	1	1	1	1
Mu50-V16	16	1.90 ± 0.12	0.70 ± 0.01	0.69 ± 0.01	0.62 ± 0.02	0.95 ± 0.17	0.59 ± 0.04	0.88 ± 0.07
Mu50-V32	32	2.33 ± 0.16	0.46 ± 0.04	0.44 ± 0.03	0.58 ± 0.04	0.89 ± 0.22	0.49 ± 0.01	0.83 ± 0.01

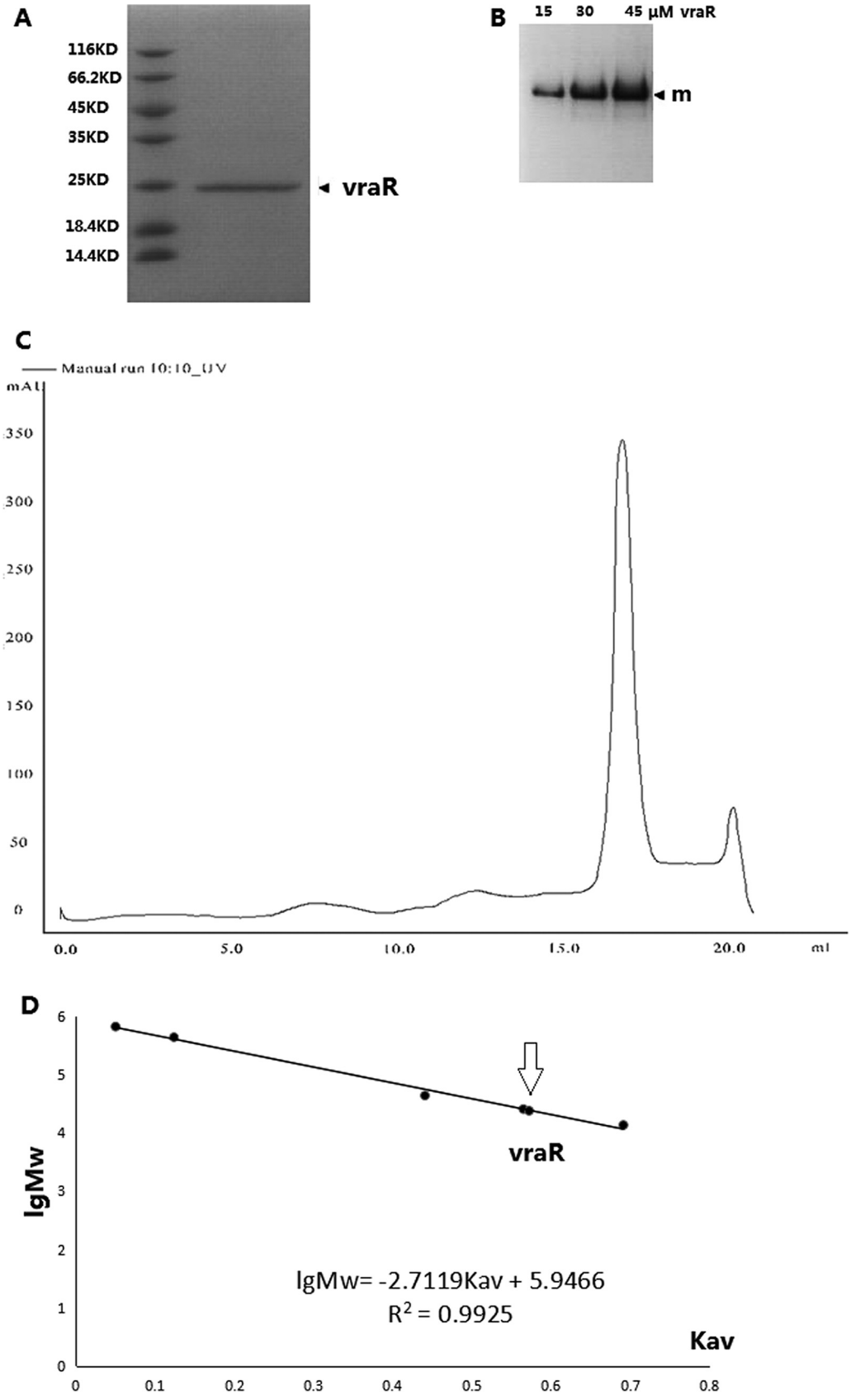

FIG 2 Determination of VraR purity and oligomeric state. (A) SDS-PAGE analysis of VraR. (B) Native PAGE analysis of VraR ("m" denotes the monomer). (C) Gel filtration chromatographic analysis of VraR. (D) Molecular size calibration for standard proteins. Molecular size estimated from the K_{av} value for VraR is indicated by an arrow.

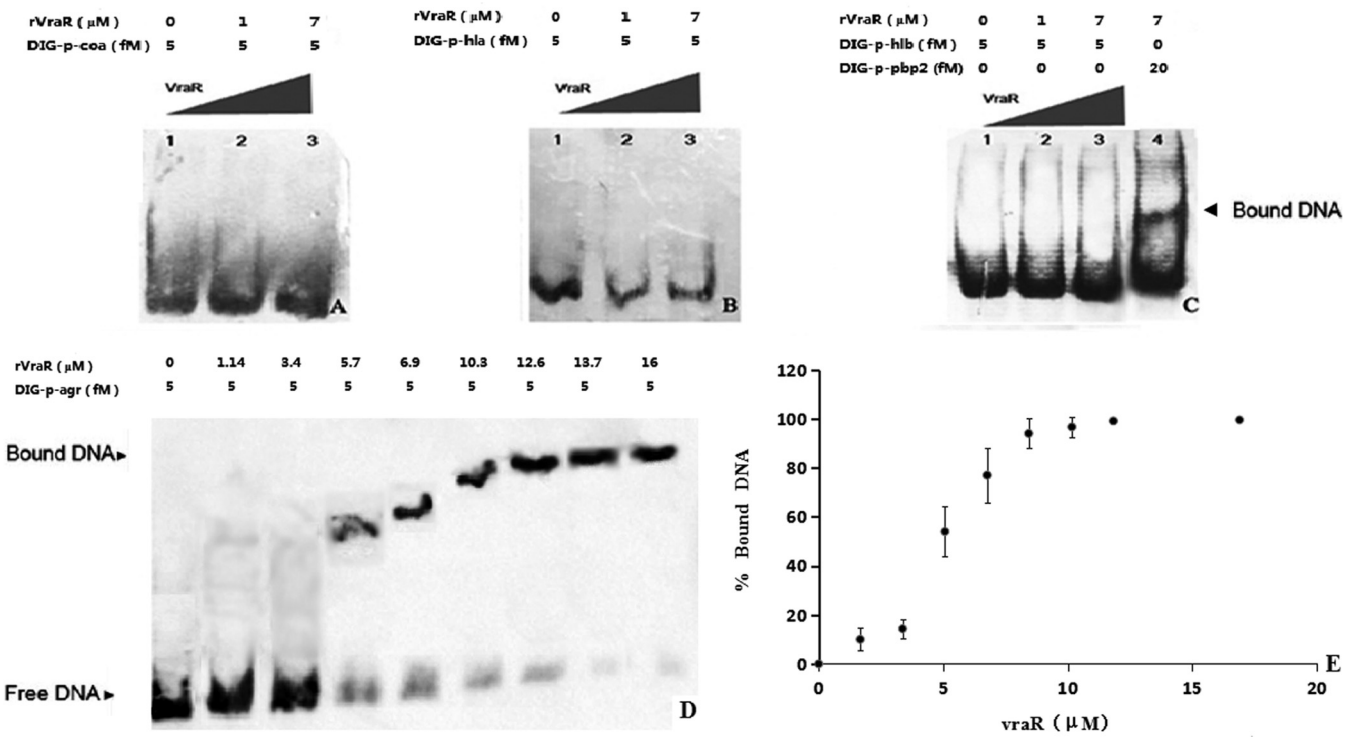

FIG 3 Electrophoretic mobility shift assay (EMSA) for VraR. (A) EMSA with the *coa* promoter. (B) EMSA with the *hla* promoter. (C) EMSA with the *hlb* promoter. (D) Analysis of the DNA-binding affinity of VraR. (E) Plot of the level of bound *agr* promoter in the EMSA against the VraR concentration in the assay.

promoter DNA substrate decreased, and the intensity of the shifted band increased (Fig. 3). The VraR concentration that resulted in shifting of half of the *agr*-promoter probe gave a computed dissociation constant (K_d) of 5.06 ± 0.90 μM. In these assay, VraR did not bind to the promoter regions of *coa*, *hla*, and *hlb*.

Characterization of the VraR binding site in the *agr* promoter. To further investigate the mechanism of regulation of the *agr* locus by VraR, the location of potential VraR binding sites in the promoter region of the *agr* locus was investigated by DNase I footprinting analysis. As shown in Fig. 4A, VraR protected a 15-nucleotide (nt) region of the *agr* promoter (5′-ATTTAACAGTTAAGT-3′ on the *agrA* coding strand) against DNase I digestion. The protected sequence was located in the intergenic region (IR) between the P2 and P3 promoters (Fig. 4B).

DISCUSSION

It has been found that the natural parental strains of VSSA were alterable to hVISA and VISA by serial passage with stepwise increases of vancomycin concentrations (30, 31). A link has also been implied between the acquisition of vancomycin resistance in *S. aureus* and attenuated virulence (10, 26, 27, 29, 32). In our study, vancomycin resistance was induced in three strains of *S. aureus*, including Mu3 and Mu50, which both carry the mutation in *vraS*(*I5N*) and result in replacement of isoleucine at residue 5 of the protein with asparagine (31, 33). The third strain (10827) was a clinical hVISA isolate, and its genome has not been fully sequenced. We found that the vancomycin MICs of the three strains were increased to 32 μg/ml by incrementally increasing induction concentrations, and the MICs were positively correlated with the expression of *vraR* and negatively correlated with the expression of virulence-associated genes. These results correspond well with those of previous studies. Whether similar trends also exist in other clinical strains remains to be determined.

The virulence of *S. aureus* is largely determined by surface-associated proteins, secreted toxins, and enzymes, expression of which is tightly regulated by regulatory loci, such as *agr*, *saeR*, *sarA*, and *sigB*, which form a complex and delicate regulatory

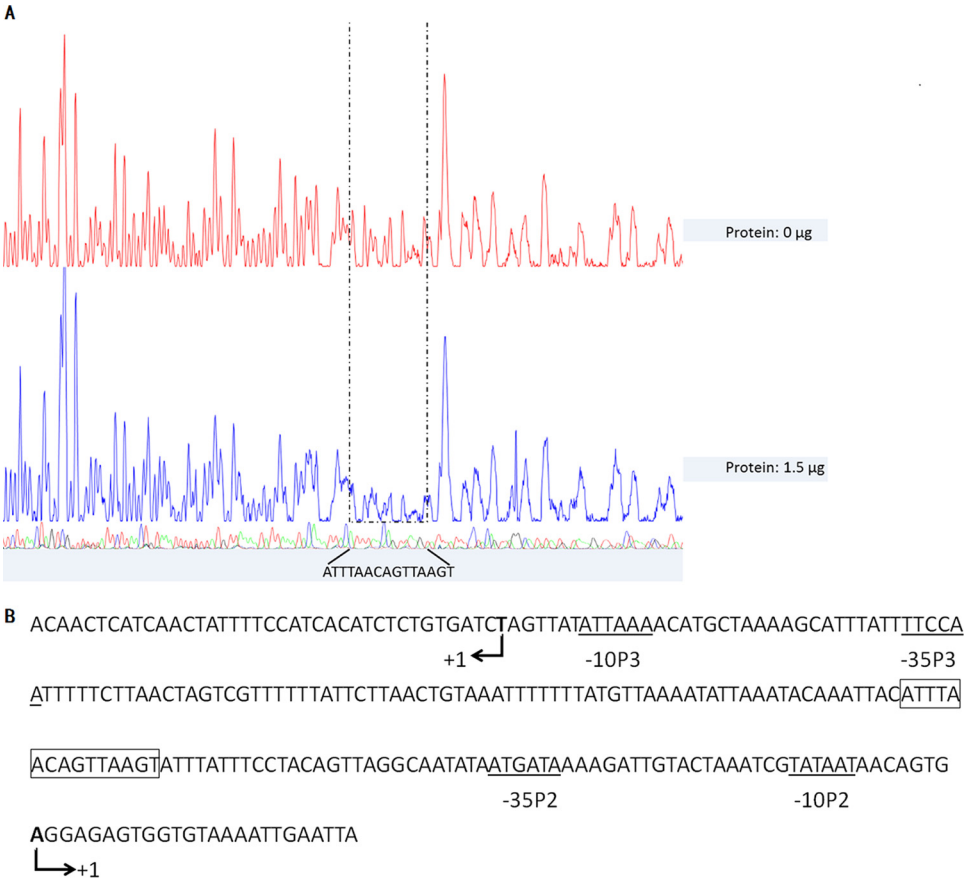

FIG 4 Identification of VraR binding sequences. (A) DNase I footprinting analysis of the *agr* promoter with VraR. (B) *agr* promoter sequence with a summary of the DNase I footprinting assay results. The −10 and −35 promoter regions are indicated by solid lines below the sequence. VraR-protected regions are in solid boxes. The translational start site is indicated by an angled arrow, and the corresponding nucleotide is in boldface.

network that acts to coordinate temporal expression of virulence genes (34). The Agr quorum-sensing system is a global regulatory system which downregulates the expression of many genes encoding surface-associated proteins (such as *coa* and *fnb*) and upregulates expression of genes encoding secreted toxins (such as *hla* and *hlb*) (35, 36). The SaeSR system regulates expression of many virulence genes, including those encoding surface proteins and toxins, primarily at the transcriptional level (37). Giraudo et al. showed by Northern blot analysis that the SaeSR system can regulate transcription of *hla*, *hlb*, and *coa* independently of the Agr system (37). Another regulatory locus, *sarA*, encodes the 14.5-kDa DNA-binding proteins that also regulate virulence factor expression. Unlike *agr*, SarA activates the synthesis of both surface-associated proteins and exoproteins in *S. aureus* (35, 38). Moreover, the alternative transcription factor sigma B (encoded by *sigB*) affects the expression of several genes that encode virulence factors and stress response proteins and seems to counterbalance the influence of the *agr* system on the expression of virulence factors. In our study, when *S. aureus* was under the induction of vancomycin, the expression of *vraR* was increased, and expression of *hla*, *hlb*, *coa*, RNAIII, *agrA*, and *saeR* was decreased. The results of EMSAs showed that VraR did not bind to the promoter regions of *hla*, *hlb*, and *coa* but it did bind to the *agr* promoter. This result indicated that VraR was indirectly involved in the regulation of virulence through binding to the *agr* promoter region. Previous studies have shown that *agr* activates the transcription of *hla* and *hlb* and represses the transcription of *coa* (35, 36). Therefore, we speculated that VraR inhibits other virulence regulation loci such as *saeRS* by an indirect path (Fig. 5).

The *agr* locus is composed of two divergent transcriptional units, the transcription

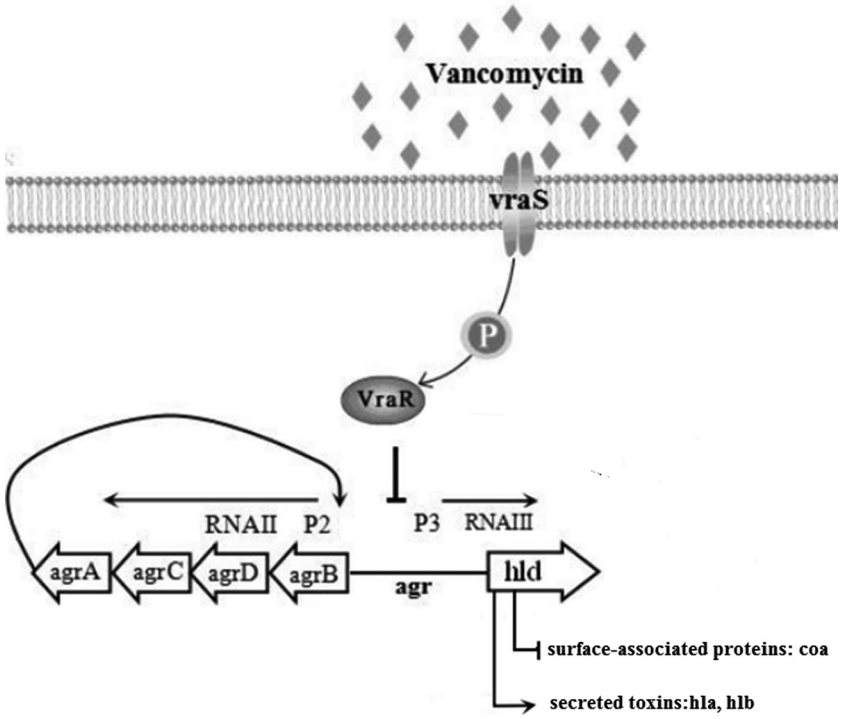

FIG 5 Proposed model of the molecular events in VraR-mediated gene regulation. Direct repression and activation of genes are shown by the solid bars and arrows, respectively. Based on experimental data from this study and others, we propose that *vraS* responds to vancomycin-elicited cell wall stress and results in transphosphorylation of VraR, which is followed by directly targeting and binding the *agr* promoter region to inhibit the expression of virulence factors.

of which is activated from divergent promoters, P2 and P3 (39). The P2 promoter drives the transcription of the *agrBDCA* operon (40). The P3 promoter drives the synthesis of the RNAIII molecule (41). Previous research has shown that the intracellular signaling molecules that bind the *agr* promoter are AgrA, SarA, and SarR (42, 43). AgrA can bind as a dimer to each of two 9-bp direct repeats in the IR between the P2 and P3 promoters to modulate *agr* transcription (44). The SarA binding site on the *agr* promoter covered a 29-bp region between the P2 and P3 promoters devoid of any direct repeats (45). SarR is a dimer that recognizes and binds to an overlapping site between two AgrA binding direct repeats (45, 46). Reyes et al. reported that AgrA activates *agr* P2 and P3 promoters, whereas SarA activates and SarR represses P2 transcription (47). In our study, DNase I footprinting showed that VraR protects a 15-nt sequence in the P2 and P3 interpromoter region of *agr* and that this sequence contains overlapping AgrA, AarA, and SarR binding sites.

The *agr* locus has been shown to be polymorphic and can be divided into four distinct genetic groups (48, 49). Previous studies have shown that induction of the VISA phenotype is more likely in *agr* group II strains than in other groups (50). However, Sirichoat et al. reported that the *S. aureus* strains from *agr* groups I to IV all developed to become VISA strains, and when hVISA or VISA developed from VSSA, a reduction in levels of *agr* expression occurred in the resistant isolates (51). Sakoulas et al. examined Agr function in VISA/hVISA and found that all VISA strains were defective in Agr function (52). Harigaya et al. and Sirichoat et al. have also confirmed that VISA/hVISA strains have been detected with reduced or absent Agr activity (51, 53). As yet, the molecular mechanisms of *agr* dysfunction in VISA/hVISA strains have not been elucidated. In this study, we found that with the increase of *vraR* expression, the *agr* expression gradually decreased and VraR did bind to the *agr* promoter. This observation may partly explain the finding that reduced *agr* function in VISA/hVISA strains was frequently detected. However, there have been reports that loss of Agr function directly

contributes to the development of vancomycin resistance in *S. aureus*. Tsuji et al. used an *in vitro* pharmacodynamic model to evaluate the role of Agr in wild-type and knockout *S. aureus* strains and found that strains with a disruption in the *agr* locus were more likely to develop intermediate resistance to vancomycin (54). Therefore, whether a direct or indirect feedback-regulation mechanism exists would be determined by further study.

Conclusion. It has been reported that VISA/hVISA strains with attenuated virulence may represent a "stealth" strategy to evade host immune surveillance and promote clinical persistence and chronic infections (10). The VraSR system is known as vancomycin resistance associated. Our results suggest that the VraSR system, as the central regulation factor, is not only involved in the regulation of vancomycin resistance but is also involved in the regulation of virulence. This finding indicates that the resistance and virulence regulatory network of *S. aureus* is more complex than we previously recognized, which is worth further attention and exploration.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. aureus* strains 10827 (vancomycin MIC, 1 μg/ml), Mu3 (vancomycin MIC, 2 μg/ml), and Mu50 (vancomycin MIC, 8 μg/ml) were induced by continuous passage through medium containing increasing concentrations of vancomycin. Briefly, overnight cultures of the strains were diluted to an optical density at 600 nm (OD_{600}) of 0.5 in fresh brain heart infusion (BHI) broth, and then 10 μl of each suspension was streaked onto vancomycin-supplemented BHI plates (starting concentration of 2 μg/ml), which were incubated overnight at 35°C. Individual colonies were picked and grown in BHI broth containing the same concentration of vancomycin. This procedure was repeated with increasing concentrations of vancomycin (up to 32 μg/ml) until the isolate grew stably in the presence of vancomycin. These homogenous series of vancomycin-induced resistant strains were named according to the MIC values: 10827-V8, 10827-V16, and 10827-V32; Mu3-V8, Mu3-V16, and Mu3-V32; and Mu50-V16 and Mu50-V32.

RNA preparation and qRT-PCR assays. RNA preparations were made as described previously (55). Briefly, the parental strains 10827 and Mu3 was grown to exponential phase in BHI broth at 35°C, and the viability of bacteria was determined via spectrophotometry (OD_{600} of 0.7). A homogenous series of vancomycin-induced resistant strains was harvested until they reached an OD_{600} of 0.7 in BHI broth, and then the cells were collected by centrifugation for 10 min at 5,000 $\times$ *g* and used for total RNA extraction using TRIzol (Invitrogen). cDNA was synthesized and labeled according to the manufacturer's recommendations for *S. aureus* antisense genome arrays (Affymetrix Inc.). Transcript levels of *vraR*, *hla*, *hlb*, *coa*, RNAIII, *agrA*, and *saeR* were quantified by quantitative real-time PCR (qRT-PCR) using a Kapa SYBR qPCR kit (Kapa Biosystems) in a LightCycler (LC-32; Roche, USA). The 16S rRNA gene was used as an internal control as described previously (56). All qRT-PCR assays were repeated three times. The primer sets for expression analysis of all genes are described in Table 2.

Cloning, expression, and purification of VraR. Cloning and expression of VraR proteins were performed as described previously (20). Briefly, the *vraR* gene was amplified using the primers 5′-GA*GATCC*ATGACGATTAAAGTATTGTTTG-3′ (forward) and 5′-GCCTCGAGCTATTGAATTAAATTATGTTGG-3′ (reverse), containing BamHI (italicized) and XhoI (underlined) sites, respectively. The amplified product was cloned into pGEM-T vector (Promega) using the TA cloning procedure and transformed into *Escherichia coli* XL1-Blue cells (Invitrogen). *vraR* was subcloned into the expression vector pET28a (Invitrogen), and the recombinant plasmid was transformed into *E. coli* BL21(DE3). The transformants were checked for the insert by using colony PCR and DNA sequencing. The clones were incubated in Luria-Bertani (LB) broth with shaking at 35°C. The overnight cultures were diluted 1:50 in LB broth and incubated with shaking again at 35°C until the OD_{600} reached 0.3. The cultures were then induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated at 25°C for 12 h. Cells were harvested by centrifugation and resuspended in 50 ml of lysis buffer (20 mM Tris-HCl, pH 8.0, and 0.5 M NaCl). Then, cells were lysed by sonication and centrifuged at 12,000 $\times$ *g* for 30 min at 4°C. The supernatant was loaded onto a nickel affinity column as recommended by the manufacturer (His-select nickel affinity gel; Sigma), and the protein was eluted using an elution buffer composed of 50 mM Tris (pH 8.0), 300 mM NaCl, and 250 mM imidazole. The purity of the eluted protein was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (SDS-PAGE). The protein concentration was measured using a Bradford assay, with bovine serum albumin as a standard.

Determination of the oligomerization state of His-tagged VraR protein. The oligomerization state of His-VraR protein was analyzed by gel filtration chromatography and native polyacrylamide gel electrophoresis (native PAGE; Tris-glycine system). The methods were performed as described previously (20). Briefly, gel filtration chromatography was carried out by the Äkta fast protein liquid chromatography (FPLC) system (Amersham Biosciences). In brief, a purified VraR sample (1 mg/ml) in 500 mM Tris buffer, pH 7.0, 0.5 mM $MgCl_2$, was applied to a Superdex 200 HR 10/300 column (GE Healthcare) equilibrated with the same buffer. The column was operated at a flow rate of 0.36 ml/min, and the proteins were detected at 280 nm. The column was calibrated with proteins of known molecular mass: thyroglobulin (669 kDa), ferritin (440 kDa), ovalbumin (44 kDa), chymotrypsinogen A (25.7 kDa), and RNase A (13.7 kDa). The K_{av} values for the standard proteins and VraR were calculated from the equation $K_{av} = (V_e - V_0)/$

TABLE 2 Primers used in this study

Name	Primer sequence (5′–3′)
16sRNA-1	CGTGCTACAATGGACAATACAAA
16sRNA-2	ATCTACGATTACTAGCGATTCCA
vraR-1	AAGACTAAACACCAACAAAACAGAG
vraR-2	GAAAAGTTACTTACGCCAATCACA
coa-1	GAGATACAGACAATCCACATAA
coa-2	CTACCTTCAAGACCTTCTAAAA
hla-1	GTAAGTCGTATTAGAACTAAAGCGG
hla-2	GCACGCAAGAATCTTGTAGTTC
hlb-1	AATCAATTTTGCATCTATTTTGTTG
hlb-2	CAAAACGGTCGATAACATATAAACG
RNAIII-1	TTCACTGTGTCGATAATCCA
RNAIII-2	GGAAGGAGTGATTTCAATGG
agrA-1	ATGGTATCGAGAATCTTAAAGTACG
agrA-2	TACTTACTTCATCGGGTATTTCG
saeR-1	CGCCTTAACTTTAGGTGCAGATGAC
saeR-2	ACGCATAGGGACTTCGTGACCATT
coa-promoter-1	GTGTTGTCATGCTTTGTTACTCC
coa-promoter-2	GCGCCTAGCGAAATTATTTGC
hla-promoter-1	TTTTCATCATCCTTCTATTT
hla-promoter-2	CTAACCCTCGAAATTGAAAT
hlb-promoter-1	TACTCAAAAAACATTTACTTAAAAATATAAATTCGAT
hlb-promoter-2	TTTTATATAGCTTACAACAAAATAGATGCAAAATTG
agr-promoter-1	ATCAACTATTTTCCATCACATCT
agr-promoter-2	TTACACCACTCTCCTCACT

($V_c - V_0$), where V_0 is the column void volume, V_e is the elution volume, and V_c is the geometric column volume. The retention time of VraR was interpolated to obtain an approximate mass of VraR protein. The purified His-VraR protein also was examined by native PAGE to verify the major oligomeric forms. Concentrations of VraR sample analyzed by native PAGE were 15, 30, and 45 μM.

EMSA. DNA fragments containing the *coa*, *hla*, *hlb*, and *agr* promoters were amplified from the *S. aureus* Mu50 chromosome. The primers are described in Table 2. The resulting PCR products were labeled using a digoxigenin gel shift kit (Roche) according to the manufacturer's instructions. The labeled fragments were then incubated at 25°C for 15 min with various amounts of purified VraR protein (1 to 16 μM) in 10 μl of incubation buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 3 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol). Following incubation, the mixtures were separated by electrophoresis in a 4.5% native polyacrylamide gel in 0.5× Tris-borate-EDTA buffer. Band shifts were detected and analyzed by a Universal Hood 2 electrophoresis imager (Bio-Rad). The quantitative analysis of the bands was carried out using NIH ImageJ (version 1.3). Determination of the dissociation constants was based on the results obtained from three independent experiments.

DNase I footprinting assay. For preparation of fluorescent 6-carboxyfluorescein (FAM)-labeled probes, the promoter region of *agr* was amplified from plasmid pEASY-Blunt Simple-*agr* using Dpx DNA polymerase (Tolo Biotech) and primers *Agr-p*-1 (5′-ATCAACTATTTTCCATCACATCT-3′; FAM labeled) and *Agr-p*-2 (5′-TTACACCACTCTCCTCACT-3′). The amplified 235-bp probes consist of the full lengths of the P2 and P3 promoters. The FAM-labeled probes were purified using the Wizard SV gel and PCR cleanup system (Promega) and quantified using a NanoDrop 2000C spectrophotometer (Thermo). DNase I footprinting assays were performed according to the method of Wang et al. (57). Briefly, 400 ng of probe was incubated with different amounts of protein (0, 0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 μg) in a total volume of 40 μl, and 1.5 μg VraR protein was chosen to carry out the experiment. Following incubation for 30 min at 25°C, 10 μl of solution containing ~0.015 U of DNase I (Promega) and 100 nmol of freshly prepared $CaCl_2$ was added to each mixture and then further incubated for 1 min at 25°C. The reaction was stopped by the addition of 140 μl of DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA, and 0.15% SDS). Samples were first extracted with phenol-chloroform and then precipitated with ethanol, and the resulting pellets were dissolved in 30 μl of Milli-Q ultrapure water (Millipore). Then, we added 200 ng/μl poly(dI-dC) in binding buffer to exclude the possibility of nonspecific binding. Preparation of the DNA ladder, electrophoresis of the reaction products, and data analysis were performed as described previously (57), except for use of the GeneScan-LIZ500 size standard (Applied Biosystems).

Statistical analysis. All statistical analyses were performed using SPSS Statistics 16.0 (SPSS Inc., Chicago, IL). Statistical differences between each group were determined by one-way analysis of variance (ANOVA). A P value of <0.05 was considered statistically significant.

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We declare that we have no conflicts of interest.

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