VraCP regulates cell wall metabolism and antibiotic resistance in vancomycin-intermediate Staphylococcus aureus strain Mu50

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Received 8 December 2020; accepted 13 March 2021

Objectives: Vancomycin-intermediate Staphylococcus aureus (VISA) is increasingly being reported. Previous studies have shown that vraC and vraP may be involved in vancomycin resistance, although the molecular mechanism remains elusive.

Methods: The vraC (SAV0577), vraP (SAV0578) and vraCP mutants were constructed in Mu50 by allelic replacement. Some common VISA phenotypes were assessed in mutants, such as, susceptibility to the cell wall-associated antibiotics, cell wall thickness, autolysis activity and growth rate. RT-qPCR was performed to reveal the differential genes associated with these phenotypes. The binding abilities of VraC and VraCP to the promoters of target genes were determined by electrophoretic mobility shift assay (EMSA).

Results: VraP forms a stable complex with VraC to preserve their own stability. The vraC, vraP and vraCP mutants exhibited increased susceptibility to the cell wall-associated antibiotics and thinner cell walls compared with the WT strain. Consistent with these phenotypes, RT-qPCR revealed downregulated transcription of glyS, sgtB, ddl and alr2, which are involved in cell wall biosynthesis. Moreover, the transcription of cell wall hydrolysis genes, including sceD, lytM and isaA, was significantly downregulated, supporting the finding that mutants exhibited reduced autolysis rates. EMSA confirmed that both VraC and VraCP can directly bind to the sceD, lytM and isaA promoter regions containing the consensus sequence (5’-TTGTAAN2AN3TGTAA-3’), which is crucial for the binding of VraCP with target genes. GFP-reporter assays further revealed VraC and VraCP can enhance promoter activity of sceD to positively regulate its expression.

Conclusions: vraCP plays a significant role in cell wall metabolism and antibiotic resistance in Mu50.

Introduction: Staphylococcus aureus is an important human pathogen that can cause a variety of infections ranging from mild skin and soft-tissue infections to life-threatening infections, such as endocarditis, bacteraemia, pneumonia and chronic osteomyelitis. The advent and use of antibiotics initially proved effective against S. aureus. However, the rise and widespread prevalence of MRSA poses a serious threat to human health. Vancomycin has been introduced to treat severe MRSA infections. However, S. aureus strains with reduced susceptibility to vancomycin have been increasingly reported worldwide.

Based on levels of vancomycin resistance, vancomycin-non-susceptible S. aureus has been classified into vancomycin-intermediate S. aureus (VISA, MIC = 4–8 mg/L) and vancomycin-resistant S. aureus (VRSA, MIC ≥16 mg/L). Because of high prevalence of VISA, VISA is a much greater problem in the clinic than VRSA. The cell wall is crucial to bacterial survival. Any compromise of the cell wall plays an integral part in antibiotic resistance, because it is targeted by many antibiotics, including β-lactams (oxacillin), glycopeptides (vancomycin and teicoplanin) and other cell wall-associated antibiotics (daptomycin). Vancomycin interferes with late-stage peptidoglycan synthesis by forming non-covalent hydrogen bonds with the penultimate d-Ala-d-Ala residues of newly synthesized UDP-MurNac-pentapeptides, thereby disrupting downstream peptidoglycan assembly, cell wall synthesis is ultimately inhibited. VISA strains share some common characteristics, including thickened cell wall, decreased autolytic activity, reduced cross-linking of peptidoglycan, slower growth rate and attenuated virulence. Since VISA strain was first reported in 1997, multiple approaches had been used to investigate the molecular genetic basis of the VISA phenotype. These studies revealed several genes and/or mutations in genes that contribute to the...
development of VISA, such as graRS, vrrS, walkKR, sigB, rpoB, vtm, sceD, and mpr. So far, however, the molecular mechanisms underlying vancomycin resistance in VISA have been incompletely defined. It is clear that resistance to vancomycin is complex and involves more genes than those that have been identified to date.

Previously, Kuroda et al. reported that the transcription of vraC (measured by cDNA differential hybridization) was remarkably up-regulated in Mu3 and Mu50 as compared with VSSA strain Mu50a. Many differently expressed genes observed in VISA/VSSA pairs could be induced by treating the corresponding parental strain with vancomycin. The transcription profiles of N315 strain exposed to vancomycin revealed that 139 genes are induced, including SA0536, whose sequence is identical to that of SAV0577. These findings indicated that vraC and vraP may collaborate and contribute to vancomycin resistance. In this study, we investigated the mechanism behind increased susceptibility to the cell wall-associated antibiotics, reduced cell wall thickness and decreased autolysis in vraC, vraP and vraCP mutants with the Mu50 strain background.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1 (available as Supplementary data at JAC Online). Unless otherwise indicated, S. aureus strains were grown with shaking (220 rpm) in tryptic soy broth (TSB; Difco) or on tryptic soy agar (TSA) at 37°C. Escherichia coli strains were grown in lysogeny broth (LB; Oxoid) medium or on lysogeny broth agar (LA) at 37°C. For plasmid maintenance, media were supplemented with ampicillin (150 mg/L) or kanamycin (50 mg/L) for E. coli and chloramphenicol (15 mg/L) for S. aureus, as needed.

Construction of mutant strains

To construct the vraC, vraP and vraCP mutants, the temperature-sensitive shuttle vector pBTs was used as previously described. Briefly, the upstream and downstream fragments of individual genes were amplified and ligated by overlap to form up-down fragments. The product fragments were digested with KpnI/Sall and then ligated into the plasmid pBts with T4 ligase. The resulting plasmids were first transformed into S. aureus RN4220 and then transformed into S. aureus Mu50. Allelic replacement mutants were screened using a previously described method and were further confirmed by PCR and sequencing.

Growth curves

Overnight cultures of S. aureus were diluted into fresh TSB and grown at 37°C with shaking. The optical density was measured at 600 nm each hour using a microplate reader (Elx800; Bio-Tek). To analyse the growth defect of the mutants in vancomycin-containing medium, Mueller-Hinton broth supplemented with vancomycin at concentrations of 1, 2 and 3 mg/L was used.

Antibiotic susceptibility assay

Antibiotic susceptibility testing was performed by the broth microdilution method, as recommended by the CLSI. Population analysis was performed as described previously, which is established by plating appropriate dilutions of direct colony suspension on TSA containing increasing concentrations of antibiotics. The numbers of cfu were determined after incubation at 37°C.

Triton X-100-induced autolysis assay

Overnight cultures were diluted to an OD600 of 0.05 in TSB. Cells were harvested at an OD600 of 0.5, washed twice with PBS, resuspended in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.2% (vol/vol) Triton X-100, incubated at 37°C with shaking, and tested for lysis by measuring the absorbance (OD600) each hour using a microplate reader (Elx800; Bio-Tek). The experiment was repeated at least three times.

Expression and purification of proteins

E. coli BL21 containing protein expression plasmid was cultivated in LB at 37°C to an OD600 of 0.5 and induced with 0.5 mM IPTG at 16°C for 20 h. Cells were harvested, resuspended in protein buffer (50 mM Tris-HCl, 300 mM NaCl, pH 8.0) and lysed by sonication on ice. Proteins with C-terminal His tag were purified using a nickel-nitrilotriacetic acid agarose solution (Novagen) according to the manufacturer’s instructions. The bound protein was eluted with elution buffer (50 mM Tris-HCl, 300 mM NaCl, 200 mM imidazole, pH 8.0). The imidazole in the eluent was removed with the protein buffer. Proteins were stored at −80°C until use.

RNA extraction, reverse transcription and quantitative reverse transcription-PCR

Overnight cultures of S. aureus were diluted to an OD600 of 0.05 in TSB, grown to the indicated cell density and collected by centrifugation. The pellet was treated with RNase plus (Takara) and lysed with 0.1 mm diameter silica beads in the FastPrep-24 system (MP Biomedicals). The total RNA was extracted. cDNA was synthesized using a PrimeScript first-strand cDNA synthesis kit (Takara), RT-qPCR was performed with SYBR Ex Taq premix (Takara) using the StepOne real-time PCR system (Applied Biosystems) to quantify relative gene expression. pta served as an internal reference gene to normalize the gene expression abundance. All RT-qPCR assays were repeated at least three times. The primers used in this study are listed in Table S2.

Transmission electron microscopy

Strains were cultured until exponential growth phase and harvested. Samples were prepared as described previously, and sent to the Core Facility Center for Life Science (USTC, China). Specimens were examined with a transmission electron microscope (TEM) operated at an accelerating voltage of 120 kV. Fifteen cells of each strain were measured by Image J.

Electrophoretic mobility shift assay (EMSA)

The biotin-labelled DNA fragments containing promoter regions of target genes were amplified from Mu50 genomic DNA. Various amounts of proteins were incubated with the biotin-labelled probes at 25°C for 30 min. Samples were mixed with gel loading buffer and electrophoresed in a native polyacrylamide gel in 1× Tris-borate-EDTA (TBE) buffer, and then transferred to a nylon membrane in 0.5× TBE buffer. The band shifts were detected according to the manufacturer’s instructions. The images were obtained using ImageQuant LAS 4000 mini (GE, Piscataway, NJ). The unlabelled fragment of each promoter was added as specific competitors. The unlabelled DNA fragment of hu ORF was added as a non-specific competitor.

Fluorescence-based promoter activity assay

Bacteria were cultivated, harvested and washed twice with PBS buffer. Promoter activities were analysed by measuring OD600 and GFP.
fluorescence (excitation, 488 ± 9 nm; emission, 518 ± 20 nm) using CLARIOstar plate reader (BGM labtech).

**Zymographic analysis**

Zymographic analyses were performed as described previously. Cells were grown until exponential growth phase and harvested. Substrate cells were autoclaved for 15 min at 121°C, lyophilized overnight in a speed-vac, then resuspended thoroughly in water. Proteins were loaded onto 15% SDS-PAGE gels containing 2 mg/L substrate from *S. aureus* NCTC8325-4. The gels were washed and incubated in renaturation buffer (0.1% Triton X-100, 10 mM CaCl$_2$, 10 mM MgCl$_2$, 50 mM Tris-HCl, pH 7.5) at 37°C with gentle agitation.

**Statistical analysis**

All experiments were performed in biological triplicates. Graphing and analysis were performed using Origin 2019. Statistically significant differences calculated by the unpaired two-tailed Student’s t test are indicated: NS, not significant (P>0.05); *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

**Results**

**vraC and vraP are co-transcribed and induced by vancomycin treatment**

vraCP encodes two proteins of uncharacterized function since no gene homologue was found in extant genes. vraP (SAV0578) is located 2 nt downstream of vraC (SAV0577) (Figure 1a). BioCyc software predicted that vraC and vraP are co-transcribed. To verify this, PCR analysis was performed with primer pairs spanning ORFs of vraC and vraP. Genomic DNA, reverse-transcription cDNA and the same amount of total RNA from Mu50 were used as templates. This result suggested that vraC and vraP could be co-transcribed (Figure 1b).

![Figure 1](https://academic.oup.com/jac/article/76/7/1712/6264898/29661712/1714.png)

**Figure 1.** vraC and vraP are co-transcribed and induced by vancomycin treatment. (a) Schematic of the vraCP locus in *S. aureus* Mu50 and the positions of primers used in the PCR assay. The black arrows indicate the genes we studied, and the grey arrows indicate neighbouring genes. (b) Gel electrophoresis analysis of PCR products amplified with primers spanning fragments of vraP and vraCP. Genomic DNA, reverse-transcription cDNA and the same amount of total RNA from strain Mu50 were used as templates. G, Genomic DNA; C, reverse-transcription cDNA; R, RNA. (c) Transcription of vraC and vraP is growth-phase dependent. (d) Transcription of vraC and vraP was induced by vancomycin treatment. (e) Protein electrophoresis of VraC, VraA and VraCP by SDS-PAGE.
Besides, the transcription profiles of vraC and vraP in Mu50 were determined by RT-qPCR. As shown in Figure 1c, the transcriptional levels of vraC and vraP are similar in the same growth phase, and their expression is growth-phase dependent. Meanwhile, vancomycin treatment induced similar transcriptional levels of vraC and vraP (Figure 1d), which further confirmed that vraC and vraP are co-transcribed.

To study the function of these genes, the expression plasmids pE\textit{vraC}, pE\textit{vraP} and pE\textit{vraCP} were constructed. pE\textit{vraC} and pE\textit{vraP} contained a C-terminal His\textsubscript{6} fusion tag, respectively, pE\textit{vraCP} only contained a C-terminal His\textsubscript{6} fusion tag at vraP gene. We found that VraC and VraP are unstable during the protein purification. However, the expression product of pE\textit{vraCP} displayed two clear bands corresponding to VraC and VraP (Figure 1e), which indicated that VraC and VraP could form a stable complex by protein–protein interaction. Thus, we named the SAV0578 protein as a partner of VraC (VraP).

### Table 1. MICs for different cell wall-targeting antimicrobials in the vraC, vraP and vraCP mutants and Mu50 WT strain

| Strain           | Vancomycin MIC (mg/L) | Daptomycin MIC (mg/L) | Teicoplanin MIC (mg/L) | Oxacillin MIC (mg/L) |
|------------------|------------------------|-----------------------|------------------------|----------------------|
| WT               | 8                      | 4                     | 2                      | 512                  |
| Δ\textit{vraCP}  | 2                      | 3                     | 1                      | 256                  |
| Δ\textit{vraC}   | 2                      | 4                     | 1                      | 256                  |
| Δ\textit{vraP}   | 2                      | 4                     | 2                      | 256                  |
| Δ\textit{vraCP}-compl. | 8                      | 8                     | 4                      | 512                  |

The \textit{vraC}, \textit{vraP} and \textit{vraCP} mutants exhibit increased susceptibility to cell wall-associated antibiotics

To understand the effects of \textit{vraC}, \textit{vraP} and \textit{vraCP} on antibiotic resistance in VISA, we constructed the \textit{vraC}, \textit{vraP} and \textit{vraCP} mutants in hospital-associated VISA strain Mu50. Antibiotic susceptibilities of these mutants were evaluated by measuring the MICs of antibiotics according to CLSI.\textsuperscript{41} Significant decreases in the vancomycin, teicoplanin, oxacillin and daptomycin MICs were detected in the \textit{vraC}, \textit{vraP} and \textit{vraCP} mutants compared with that of WT strain, however, the susceptibilities to other classes of antibiotics exhibited no obvious change (Table S3). Specifically, the MICs of all three mutants decreased from a vancomycin intermediate resistant level of 8 mg/L to a vancomycin susceptible level of 2 mg/L, and the \textit{vraCP} chromosomal complemented strain exhibited increased MIC, phenocopying the parent strain, which confirmed that the MIC decrease was due to deletion of the \textit{vraCP} (Table 1).

One of the characteristics of VISA is a significant slow-down in growth rates relative to their susceptible counterparts.\textsuperscript{13,46} Thus,

![Figure 2](https://academic.oup.com/jac/article-fig/76/7/1712/F2){#fa} Growth curves of \textit{S. aureus} Mu50 and its derivative strains in different concentrations of vancomycin. Growth of the WT strain (a), the \textit{vraCP} mutant (b), the \textit{vraC} mutant (c), the \textit{vraP} mutant (d) and the \textit{vraCP} complemented strain (e) in MH broth at 37°C containing 0, 1, 2 and 3 mg/L of vancomycin.
we measured the growth rates of the mutants and WT strain in TSB, and found that the mutants exhibited faster growth rates compared with the WT strain (Figure S1). In addition, we also tested cell growth in MH broth without antibiotic or exposed to different vancomycin concentrations (1, 2 and 3 mg/L). This result showed that the WT and complemented strains displayed slow and steady growth rates at all three concentrations of vancomycin, whereas the three mutants did not display any growth at 2 mg/L or higher vancomycin concentrations (Figure 2). These data confirmed the increased susceptibility of the \textit{vraC}, \textit{vraP} and \textit{vraCP} mutants to vancomycin.

To further characterize the population of the mutants with regard to the cell wall-associated antibiotics resistance, population analysis was performed as described previously \cite{15,47}, and the results showed that resistance to the cell wall-associated antibiotics (vancomycin, teicoplanin, daptomycin and oxacillin) in these mutants was significantly reduced (Figure 3), which is consistent with the MIC assay results. The increased susceptibilities of the mutants to cell wall-associated antibiotics suggested that the cell walls of the mutants were compromised.

\textbf{Deletion of the vraC, vraP and vraCP decreases cell wall thickness}

To confirm whether cell wall defects exist in the \textit{vraC}, \textit{vraP} and \textit{vraCP} mutants, cell wall morphology was evaluated using TEM. These data revealed that the mutants showed significant reduction (approximately 46\%) in cell wall thickness compared with the WT strain (Figure 4a–f and Table 2). These results suggested that \textit{vraC}, \textit{vraP} and \textit{vraCP} play important roles in cell wall synthesis and maintenance in Mu50.

\textbf{Deletion of the vraC, vraP and vraCP influences the expression of cell wall biosynthesis genes}

The above data clearly indicated that \textit{vraC}, \textit{vraP} and \textit{vraCP} participate in the cell wall-associated antibiotic resistance and cell wall synthesis. Many genes involved in cell wall synthesis and antibiotic resistance have been reported. To further explore the mechanism behind these phenotypes, RT-qPCR was performed and revealed that the expression of \textit{glyS} (glycine tRNA synthetase), \textit{sgtB} (monofunctional peptidoglycan glycosyltransferase), \textit{ddl} (D-alanine-D-alanine ligase) and \textit{alr2} (alanine racemase) significantly
decreased in the vraC, vraP and vraCP mutants (Figure 4g), which is consistent with a thinner cell wall and decreased cell wall-associated antibiotic resistance. These data supported this opinion that vraC, vraP and vraCP play an integral part in cell wall synthesis and maintenance in Mu50.

The vraC, vraP and vraCP mutants exhibit decreased autolysis rate and downregulated transcription of cell wall hydrolysis genes

Beyond the thickened cell wall, reduced antibiotic susceptibility and slower growth rate, the change in autolytic activity is also a common feature of VISA. To determine whether vraC, vraP and vraCP also confer this phenotype, we examined Triton X-100-induced autolytic activity of Mu50 strain as well as its derivatives.

These mutants showed reduced autolytic activity compared with the WT strain, And the phenotype was restored in vraCP complemented strain (Figure 5a). Meanwhile, we observed significantly downregulated transcription of sceD, lytM and isaA (Figure 5b). Zymographic assays confirmed cell wall hydrolytic activity of SceD, LytM and IsaA proteins (Figure 5c,d). We therefore hypothesized that vraC, vraP and vraCP influenced cell wall hydrolysis by regulating the expression of sceD, lytM and isaA.

VraC and VraCP can enhance promoter activity of sceD

To further verify the regulatory role of vraCP in the expression of sceD, we constructed the sceD promoter-GFP fusion reporter plasmid to detect the promoter activity in Mu50 as well as its derivatives by measuring GFP fluorescence intensity. As predicted, the sceD promoter activity was dramatically weaker in the vraC, vraP and vraCP mutants than WT strain (Figure 5e). These data suggested that VraCP can enhance the promoter activity of sceD, and then promote its expression.

Both VraC and VraCP can directly bind to the promoter regions of sceD, lytM and isaA

To investigate the mechanism underlying altered expression of sceD, lytM and isaA, EMSA was performed with biotin-labelled putative promoter regions and the recombinant VraC and VraCP.
results showed that VraCP can retard the mobility of the \textit{sceD}, \textit{lytM} and \textit{isaA} promoters in a dose-dependent manner (Figure 6a–c). These shifted bands disappeared in the presence of an approximately 100-fold excess of individual unlabelled promoter regions, but not in the presence of a 100-fold excess of an unlabelled coding sequence DNA of \textit{hu}. As for VraC, similar band shift patterns were observed (Figure 6d–f). These data suggested that both VraC and VraCP can directly bind to \textit{sceD}, \textit{lytM} and \textit{isaA} promoter regions.

\textbf{The consensus sequence (5'-TTGTAAN2AN3TGTAA-3') plays the important role in the binding of VraCP with target genes}

Sequence alignment revealed that the potential consensus sequence (5'-TTGTAAN2AN3TGTAA-3') is present in the promoters of \textit{sceD}, \textit{lytM} and \textit{isaA}, this sequence contains two pentanucleotide direct repeats (TGTAAT) (Figure 7a).\textsuperscript{48} To confirm the role of the consensus sequence in the binding of VraCP, truncated probes with different lengths were designed to perform EMSA (Figure 7b–d). The binding ability of VraCP to PsceD was abolished when the probe length was truncated from 168 bp to 118 bp (Figure 7e). The binding ability of VraCP to PlytM was abolished when the probe length was truncated from 202 bp to 144 bp (Figure 7f). The binding ability of VraCP to PsiSa was abolished when the probe length was truncated from 162 bp to 142 bp (Figure 7g). These data demonstrated that the consensus sequence (5'-TTGTAAN2AN3TGTAA-3') is crucial for the binding of VraCP with target genes.

\textbf{Discussion}

VISA has attracted considerable attention because of widespread prevalence and treatment failure.\textsuperscript{9,11} Comparative genome, transcriptome and proteome have been employed to explore the mechanism behind vancomycin resistance.\textsuperscript{23,25,26,38} Hundreds of...
mutations were discovered by comparing the genomes of VSSA/VISA pairs. Allelic exchange experiments replacing the normal allele in VSSA with the mutated allele from VISA were performed to evaluate whether allele swapping is responsible for vancomycin resistance. However, reverse-direction studies are rare, because genetic manipulation is challenging in clinical VISA strains, such as Mu50. Meanwhile, several differential expression genes were also detected by transcriptome and proteome analysis. It is obvious that the mechanism of the generation of VISA is complex and varied.

Herein, we explored the mechanism through which vraCP contributes to antibiotic resistance and cell wall metabolism in VISA strain Mu50. The vraC, vraP and vraCP mutants exhibited increased susceptibility to the cell wall-associated antibiotics and reduced cell wall thickness, which are consistent with the VSSA phenotypes. Previous studies revealed that the cell wall thickness and antibiotic resistance are strongly correlated. VISA strains display thickened cell wall and increased binding of vancomycin to ‘false targets’, thereby contributing to reduced vancomycin susceptibility. Daptomycin needs to penetrate through the cell wall before reaching lethal targets. Therefore, one possible reason leading to increased vancomycin and daptomycin susceptibility in mutants is the thinner cell-wall. RT-qPCR was performed to examine the expression of genes involved in cell wall biosynthesis (Figure 4g, Figure S2a), and revealed that the transcriptional levels of glyS, sgtB, ddi and alr2 were decreased in mutants, which may result in thinner cell wall. Ddl and Alr are responsible for terminal stem peptide D-Ala-D-Ala. The decreased transcription of ddi and alr2 results in reduced free D-Ala-D-Ala residues, which are ‘false targets’ for vancomycin. The reduced cell wall thickness and the decreased proportion of ‘false targets’ accelerate penetration of vancomycin molecules to lethal targets, which may be the reason for increased susceptibility to vancomycin and teicoplanin in mutants.

In addition, the mutants exhibited reduced autolytic activity, which seems contradictory to their VSSA-like phenotype. Previous studies revealed that reduced autolytic activity of VISA resulted...
from decreased expression of cell wall hydrolytic genes, such as atlA, sle1, lytM and lytN. However, in this study, the transcriptional levels of atlA, sle1 and lytN showed no difference between mutants and WT strain (Figure S2b). In contrast, the transcriptional levels of sceD, lytM and isaA were significantly downregulated, which may play the leading roles in reduced autolytic activity of mutants. Among the differentially regulated genes, the change of sceD was the most pronounced. Studies have reported that high expression of sceD is related to vancomycin resistance in VISA, which is consistent with our study. Both VraC and VraCP can directly bind to sceD, lytM and isaA promoter regions, while VraP is too unstable to be employed for EMSA. The consensus sequence (5'-TTGTAAN2AN3TGTAA-3') in sceD, lytM and isaA promoter regions is crucial for the binding of VraCP to target genes, which is similar to the WalR binding site (5'-TGTWAH-N5-TGTWAH-3'). However, whether an association between VraCP and WalKR exists in the Staphylococcus regulatory network needs further exploration. Meanwhile, GFP-reporter assays revealed that VraC, VraP and VraCP can enhance the sceD promoter activity to promote its expression. However, GFP fluorescence intensity has not been detected in all strains carrying the pPlytM-GFP plasmid. This might be due to the lytM promoter region containing 15 ATG codons, which affects the expression of GFP. Besides, only 4-fold difference of GFP fluorescence intensity in mutants carrying the pPsced-GFP plasmid was detected compared with the WT strain, but a 20-fold difference was revealed by RT-qPCR. Thus, it is possible that the change of isaA promoter activity is not sufficient to be detected.

Moreover, vraCP was induced by vancomycin and regulated by GraSR and VraSR (Figure S3a, b). Meanwhile, vraCP was expressed at slightly higher levels in Mu50 than its isogenic strain Mu3, overexpression of vraCP in Mu3 could increase vancomycin resistance (Figure S3c, d, Table S4), indicating that the expression of vraCP plays important roles in vancomycin resistance with the Mu50 strain background. However, we constructed vraCP knockdown strains in clinical VISA strain XN108 by CRISPRi, and no vancomycin resistance change was observed in knockdown strains (Figure S4, Table S5), which is due to the difference in vancomycin resistance mechanism between Mu50 and XN108.

Figure 7. The consensus sequence (5'-TTGTAAN2AN3TGTAA-3') plays an important role in the binding of VraCP with target genes. (a) The consensus sequence (5'-TTGTAAN2AN3TGTAA-3') in promoter regions of sceD, lytM and isaA was identified by sequences alignment. The locations of Psced, PlytM and PisaA truncated probes with different lengths are marked by black arrows. The partial relative dispositions of sequence are illustrated in the lower panel. The consensus sequences (5'-TTGTAAN2AN3TGTAA-3') are marked in red, the two pentanucleotide direct repeats (TGTAAT) are highlighted in yellow (b–d). EMSA analysis of VraCP with Psced, PlytM and PisaA truncated probes (e–g).
VraCP regulates cell wall metabolism and AMR in VISA strain Mu50

**Supplementary data**
Figures S1 to S4 and Tables S1 to S5 are available as Supplementary data at JAC Online.

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