Molecular Insight Into the Mutation Within Critical Zinc²⁺-Binding Site in the PAS Domain of Walk in Vancomycin-Intermediate Resistant *Staphylococcus Aureus*

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Abstract

Vancomycin-intermediate resistant *Staphylococcus aureus* (VISA), one of the common causes of nosocomial infection, is developed by mutations, including in *walkR*, with unclear molecular mechanisms. Although studies have verified some of these mutations, there are a few studies to pay attention to the importance of molecular modeling of mutations. Here, the Sanger sequencing for comparing gene sequences of *WALKR* between a VISA and its parental strain revealed mutation Walk-H364R. Structural protein mapping showed that H364R was located in a functional zinc ion coordinating residue within the cytoplasmic Per-Arnt-Sim (PAS) domain. The structural and functional effects of this mutation were analyzed using molecular computational approaches based on the recently determined crystal structures of the PAS domain of *S. aureus*. Walk-H364R was predicted to destabilize protein and decrease Walk interactions with proteins and nucleic acids. The qRT-PCR method showed downregulation of *walkR* and *WalkR*-regulated autolysins, which verified the molecular computational results.

Overall, Walk-H364R within a critical metal-coordinating site is linked to VISA development through *the walkR* gene expression changes as well as the destructive effects on protein.

Therefore, molecular modeling can be provided detailed insight into the molecular mechanism of VISA development, in particular, where complementation experiments are not readily available.

Introduction

Vancomycin-intermediate resistant *Staphylococcus aureus* (VISA) that causes a wide range of life-threatening infections has been recovered worldwide ¹. Nevertheless, the molecular mechanism of VISA development is incompletely understood. Although the molecular studies on clinical or laboratory VISA isolates have confirmed that VISA phenotype is caused by the diverse non-synonymous single nucleotide polymorphisms (nsSNPs), only a few of these mutations have been verified by allele swapping or complementation experiments. Most of these nsSNPs have been reported in *walkR*, *graSR*, and *vraTSR* two-component systems (TCSs) ².

The WalkR system, specific TCS to low G + C Gram-positive bacteria, is the only essential TCS in *S. aureus* viability ^{3,4}. This locus plays role in cell wall biosynthesis, virulence, and antibiotic resistance due to possessing the extensive protein-protein interaction network such as interaction with GraSR, VraSR, Rpo, and autolysins (AtlA, Sle1, and LytM) ^{3,5,6}. The identical features among VISA isolates, such as reduced autolytic activity and reduced virulence, can arise from the destructive regulation of these genes due to missense mutations ⁷.

We previously emphasized the importance of mutation sites in a VISA strain. For instance, a deleterious mutation was detected in the ATP-lid, which is a conserved and essential motif in Walk ⁸. Due to the

importance of ATP-lid, the studies have suggested that it can be an appropriate site for novel antimicrobial compounds ⁹.

In *S. aureus*, the cytoplasmic Per-Arnt Sim (PAS) domain (amino acids: from 261 to 375) is conserved among members of *Staphylococcus* species and contains four critical and highly conserved residues (His 271, Asp 274, His 364, Glu 368) as metal-binding sites, which directly bind to a Zinc ion (Zn^{2+}) ¹⁰.

The critical role of the cytoplasmic PAS (PAS^{CYTO}) domain of *Bacillus subtilis* in the localization of Walk to the division septum is confirmed ¹¹. Recently, structural and functional analyses of Zn^{2+} -binding residue 271 within the PAS domain of *S. aureus* Walk showed that the Zn^{2+} -binding site regulates *S. aureus* WalkR ¹⁰. On the other hand, a substitution mutation in H271 was recently reported in a VISA strain ¹². In *B. Subtilis*, residue D274 was confirmed to be related to the regulatory pathways of peptidoglycan synthesis ¹³.

Missense mutations can disrupt drug-bacterial interactions and protein function through protein stability and interactions with biological molecules such as proteins and nucleic acid. The previously deposited crystal structure of the desired bacterial species in the mutation site is utilized for the analysis of the effect of nsSNP on the protein structure and interactions of protein using molecular computational approaches. These methods for predictions of these impacts usually are used to understand the mechanism of human genetic disease and drug resistance in infections such as *Mycobacteria* infections ^{14–17}. Despite the role of WalkR in VISA development, the molecular modeling of mutations in VISA strains is still unreviewed.

Therefore, besides the gene expression and complementation experiments to understand the VISA molecular mechanisms, the importance of knowledge about protein structure mapping of mutations and predicting of the impact of the mutation on the disruption of protein function using molecular computational approaches can provide new insight into the mechanisms underlying VISA development and help to design novel therapeutic strategies ¹⁸.

In the present study, we detected mutation Walk-H364R, which locates in the Zn^{2+} -binding residue of the PAS^{CYTO} domain, in a VISA laboratory strain compared to its wild-type strain. This mutation was also previously reported in *S. aureus* strain after nisin selection ¹⁹. Besides phenotypical and transcriptional methods, we predicted the impact of H364R nsSNP occurred at a critical site of Walk on protein function, protein stability, and the interaction of the protein with proteins and nucleic acid using computational approaches. Previously published crystal structure of PAS domain from *S. aureus* Walk was used as a wild-type template in computational approaches.

Material And Methods

Bacteria parental strain and growth conditions

A clinical MRSA (Methicillin-resistant *S. aureus*) strain was called VAN-S and served as the parental vancomycin susceptible strain (MIC of vancomycin 1 µg/ml). This strain harbored type III SCC*mec* and *agr* type III.

S. aureus was cultured within the brain-heart infusion (BHI) broth and BHI agar (Ibresco, Italy) at 35 ±2 °C with shaking at 200 rpm unless otherwise stated.

***In vitro* selection of VISA strain**

Selection of VISA mutants from *S. aureus* clinical strains has been done to the comparison of VISA/VSSA isogenic pairs with the purpose of the study of the mechanism of VISA development ².

Here, vancomycin 500 mg vial (Dana Co, Iran) was used for *in vitro* selection of VISA strain (VAN-I). It was reconstituted in sterile distilled water to achieve the desired concentrations.

Overnight culture of VAN-S strain was adjusted to 5×10⁵ CFU/ml concentration into 3 ml of BHI broth containing 1/4× MIC of vancomycin. After 24 h, the culture was serially passaged into BHI broth containing 1/2× MIC of vancomycin and then 1× MIC of vancomycin. After culturing into 2× MIC of vancomycin, the process was repeated from 1/4× MIC of vancomycin. The cultures in each step were spread on BHI agar plates to maintain vancomycin tolerance colonies.

This process was repeated for 50 days. The MIC of vancomycin was recorded every 48 h.

Stability of VISA phenotype and isogenicity between parental and mutant strains

After the selection of VAN-I mutant, it was cultured on vancomycin-free BHI agar plates over five passages to evaluate the stability of VAN-I.

To confirm the isogenicity of VAN-S and VAN-I, pulsed-field gel electrophoresis (PFGE) was performed using SmaI endonuclease (Takara, Japan), as previously described ²⁰. 1% agarose gel containing digested DNA samples were run on the CHEF-Mapper (Bio-Rad Laboratories, CA) for 19 h at 6 V/cm and a field angle of 120°, with switch times of 5 and 40 seconds at a temperature of 14°C.

Determination of minimum inhibitory concentration of vancomycin

The broth macrodilution method was used to determine of MIC of vancomycin according to 2018 CLSI guidelines ²¹. The cation-adjusted Mueller Hinton broth media (Merk, Germany) was used in the MIC assay.

Doubling time calculation

The doubling time was calculated using an obtained graph of the growth kinetic curve, as previously described with modifications ²². Briefly, The *S. aureus* culture was adjusted to 0.01 at an optical density at 660 nm (OD₆₆₀) in 20 ml BHI broth and incubated for 15 h. The OD₆₆₀ of these cultures were recorded

every 1 hour using a spectrophotometer (Biochrom WPA Biowave II, UK). The formula for calculating the doubling time (DT) was $[(t_2 - t_1) \times \log 2] / [\log \text{OD}_{660} \text{ at } t_2 - \log \text{OD}_{660} \text{ at } t_1]$. The abbreviations t_1 and t_2 are the times at the start and the end of the exponential phase, respectively ²². The experiment was performed in triplicates.

Triton X-100 induced autolysis kinetic assay

20 ml of the *S. aureus* culture was collected by centrifugation (6800 g for 10 min), washed twice with 20 ml of cold distilled water, and adjusted to an OD₆₀₀ of 1 in 10 ml cold distilled water supplemented with 0.1% Triton X-100. Autolysin activity was measured (using a spectrophotometer) as the decline in the absorbance at 600 nm within 0, 1, 2, 3, 4, and 16 hours of incubation ²³. The experiments were done on three independent occasions.

Hemolysis assay

The α -hemolysin activity was examined as previously described ²⁴, with minor modifications. The human RBCs were separated from plasma using centrifugation (900 g for 2 min), washed three times with 1X PBS solution, and diluted (300 μ l of RBCs in 10 ml PBS). 200 μ l of *S. aureus* cells (OD₆₀₀ = 0.3) added into diluted RBCs, incubated for 1 hour (with shaking at 250 rpm), and centrifuged at 6000 g for 10 min. The absorbance of the supernatant was measured (using the spectrophotometer) at OD₅₄₃ nm and analyzed as hemolytic activity in three independent experiments.

Triton X-100 and diluted RBCs in PBS without bacteria were used as positive and negative controls, respectively. The percent of hemolysis activity was calculated from the average of three experiments by the following formula: (A₅₄₃ of the sample $\div$ A₅₄₃ of negative control) / (A₅₄₃ of positive control $\div$ A₅₄₃ of negative control) $\times$ 100.

RNA extraction and quantitative real-time PCR

The RNA was extracted from VAN-S and VAN-I cells in the mid-logarithmic phase using the GeneAll Hybrid-R™ RNA isolation kit (Geneall Biotechnology, Korea) with DNase I treatment (Thermo Fisher Scientific, USA) of 1 μ g of RNA template (final concentration: 0.1 μ g/ μ l).

After cDNA synthesis (Yekta Tajhiz Azma, Iran), qRT-PCR was run on a Rotor-Gene Q (Qiagen, Hilden, Germany) in the One-Step analysis using RealQ Plus 2x Master Mix SYBR Green (Ampliqon, Denmark), primers (Pishgam Biotech, Iran) (See Supplementary Table S1 online) to amplify of *walR*, *vraR*, *graR*, *atlA*, *lytM*, *sle1*, and *sceD*, and *gyrA* as the internal control. Each sample was run at least in triplicate assays.

DNA extraction, gene sequencing, and protein mapping of the mutation

Genomic DNA from overnight cultures of VAN-S and VAN-I was extracted using the Gene Transfer kit (Pioneers, Iran).

PCR was performed to amplify *walK* and *walR* genes using the primers (See Supplementary Table S1 online) within a T100™ Thermal cycler (BioRad, USA).

After confirmation of PCR products by electrophoresis on a 1% agarose gel, those were purified and sequenced (by Microsynth AG Co, Switzerland) using the Sanger sequencing method (with forward and reverse reads).

The results were checked using Chromas software (V2.6.6; <https://technelysium.com.au/chromas.htm>) and aligned using allele ID software (V6.00; Premier Biosoft, USA) and also against the BLAST database.

After detection of nonsynonymous substitution mutation in WalK protein, we determined the protein domain mapping¹⁰ of mutation detected in this study as well as some nsSNPs in previously reported WalK mutations in vancomycin nonsusceptible *S. aureus* strains, including T492R²⁵; R555C⁸; A243Y, S9T, V15L, H271D, A342R, G358S¹²; L7Q^{12,26}; A582E^{12,27}; Q371del^{12,28,29}; A117V, A468T, R222K³⁰, Y225N²⁶, S221P³¹, R86C, I287T²⁷, I544M³², Q369R, M220I³³; V380I, V383I, S9P, A243T, R282C, T217, G223S, H576R, S437F³⁴; R263C, S273N²⁸; G223D, V268F⁵.

Furthermore, the conservation of the mutation site among *staphylococcal* species and other closed genera was examined using the alignment of WalK sequences.

Protein stability analysis

The sequence alignment of WalK protein VAN-S using the protein data bank (PDB) database was performed to obtain the crystal structure of *S. aureus* WalK protein. Finally, 4mn5 and 4mn6 PDB IDs were selected based on the coverage of the mutation site in VAN-S (residue 364) with 96/03% and 100% identifies, respectively, as wild-type templates for future protein analyses. 4mn5 and 4mn6 display the crystal structure of the PAS^{CYT0} domain from *S. aureus* WalK based on the X-ray diffraction method at 2 Å and 2.1 Å resolutions, respectively.

The DUET web server (<http://biosig.unimelb.edu.au/duet/>) was applied to predicting the effects of mutation H364R on the WalK protein stability.

DUET predicts the changes in protein stability upon the introduction of nsSNP using consolidating mCSM (mutation Cutoff Scanning Matrix) and SDM (Site Directed Mutator) computational approaches³⁵.

Protein-Protein interactions analysis

Predicting the impact of mutation H364R on the protein-protein affinity of WalK was carried out by the mSCM-PPI2 web server (http://biosig.unimelb.edu.au/mcsm_ppi2/). The mSCM-PPI2 applied an optimized graph-based signature approach to better evaluate the molecular mechanism of the nsSNP using modeling the effects of variations in the inter-residue non-covalent interaction network³⁶.

Protein-nucleic acid interactions analysis

Protein-DNA interaction changes upon mutation Walk-H364R was determined using mCSM-NAv2 web server (http://biosig.unimelb.edu.au/mcsm_na/), which predicts the effects of nsSNP in protein-coding regions on nucleic acid binding affinities ³⁷.

Prediction of the impact of nsSNP mutation on protein function

SIFT web server was used to the prediction changes in the protein function. SIFT performs PSI-BLAST search and predicts changes based on sequence homology and the physical properties of amino acid residues.

Statistical analysis

SPSS24 statistical software (SPSS inc., Chicago, IL) was used for statistical analyses of data. A P value ≤ 0.05 was considered as a significant difference between the isogenic pair of VAN-S/VAN-I. The results were reported as mean $\pm$ standard deviation (SD).

Parametric paired-sample Student's *t*-test was used to compare the autolysis activity of the isogenic pair of VAN-S/VAN-I. Data of hemolysis assay and doubling time were analyzed using the nonparametric two-tailed Wilcoxon Signed Ranks Test.

The results of qRT-PCR were analyzed by $\Delta\Delta C_t$ method using the Relative Expression Software Tool (REST) 2009 (v2.0.13; Qiagen, Valencia, CA, USA).

SIFT predictions are based on the scores (Ranges: 0-1) and median sequence conservation (Ranges: 0-4.32; ideally: 2.75-3.5). The amino acid substitution was predicted deleterious if the score was ≤ 0.05 and tolerated if the score was ≥ 0.05 . Median sequence conservation shows the diversity of the sequences used for prediction. If it was greater than 3.25, there was a warning.

Predicted results of the DUET, mSCM-PPI2, and mCSM-NAv2 methods include the variation in Gibbs Free Energy ($\Delta\Delta G$ in kcal/mol).

Results

***In vitro* development, stability, and isogenicity of VAN-I mutant**

VAN-I strain was selected as a VISA strain from a clinical vancomycin susceptible *S. aureus* strain (VAN-S) after a serial adaptation process. In this process, the MIC of vancomycin was increased to 2, 4, and 8 $\mu\text{g/ml}$ after 6, 10, and 18 days, respectively.

The constant MIC of vancomycin in VISA mutants on vancomycin-free media showed stability of VAN-I mutant over five passages.

The same PFGE profile confirmed the isogenicity of VAN-S and VAN-I.

Decreased doubling time, autolytic kinetic, and hemolytic activity in VAN-I

In this study, VAN-I mutant had longer doubling times (DT) than that in VAN-S ($DT^{VAN-S} = 27.28$ min versus $DT^{VAN-I} = 45.47$ min).

Furthermore, autolytic activity at the tested time points in VAN-I significantly was decreased ($P \leq 0.05$) compared to that in VAN-S (Fig. 1a).

Furthermore, the hemolytic activity of VAN-I was decreased compared to that in VAN-S (23.43% versus 81.03%) (Fig. 1b).

Transcriptional changes in VAN-I mutant

The analysis of transcriptional profiles of *walkR*, *vraTSR*, and *graSR* systems indicated that the expression of *walkR* (0.14-fold; 7.1 times) was significantly ($P \leq 0.05$) downregulated in VAN-I mutant. Furthermore, the comprising of *walkR*-dependent peptidoglycan hydrolase genes (*lytM*, *atlA*, *sle1*, and *sceD*) showed the downregulation ($P \leq 0.05$) of *lytM* (0.14-fold; 7.14 times), *atlA* (0.04-fold; 23.25 times), and *sle1* (0.45-fold; 2.18 times) genes in VAN-I mutant.

Identification of nsSNP in *walk* VAN-I mutant and mapping of the mutation

After aligning the *walk* in parental (VAN-S) and mutant (VAN-I) strains, nsSNP was detected in VAN-I mutant (*walk* nucleotide 1091, base change: A to G). In this mutation, Histidine 364 was replaced by Arginine (Walk- H364R) in Walk protein. Mapping of mutation to Walk protein revealed that Walk-H364R was located in the Zn^{2+} -binding site within the PAS^{CYTO} domain ¹⁰. The Zn^{2+} -binding sites of Walk protein is displayed in Fig. 2.

Furthermore, the protein structural mapping of 38 nsSNPs reported in previous studies in Walk of vancomycin non-susceptible *S. aureus* strains is shown in Fig. 3. The results displayed that most nsSNPs were located in the PAS^{CYTO} domain (12 nsSNPs) and HAMP domain (9 nsSNPs).

We also observed that one nsSNP (H271D) within the Zn^{2+} -binding site (residue 271) was detected in a VISA strain ¹².

Conservation of Zn^{2+} -binding sites in Walk protein

After alignment of Walk sequences in the selected region from staphylococci, enterococci, and bacilli, the potential conservation of Zn^{2+} -binding residues (including H364) among Walk of *staphylococcus* species and *enterococci* were confirmed (Fig. 4).

Protein stability change upon mutation H364R in Walk

We analyzed the impact of H364R in a zinc²⁺-binding residue of Walk protein, which was observed in the VISA strain VAN-I, using DUET method. DUET method predicted that the nsSNPs can directly influence protein stability via changes in $\Delta\Delta G$ (kcal/mol) value.

Overall, the presence of mutation H364R was estimated as a destabilizing effect on Walk protein stability. The results are displayed in Table1 and include the combined DUET prediction and the mCSM and SDM individually predicted changes.

Protein-protein complex affinity change upon mutation H364R in Walk

The mSCM-PPI2 predicted that the protein-protein affinity of Walk destabilized (Reduced affinity) upon mutation H364R. The $\Delta\Delta G^{\text{affinity}}$ against 4mn5 was -0.045 kcal/mol in chain A and -0.084 in chain B. The $\Delta\Delta G^{\text{affinity}}$ against 4mn6 was -0.069 kcal/mol in chain A and -0.106 kcal/mol in chain B. The inter-residue non-covalent interaction network of VAN-I mutant and wild-type (PDB ID 4mn6) are displayed in Fig. 5.

Protein-nucleic acid binding affinity change upon mutation H364R in Walk

The mCSM-NAv2 displayed that protein-nucleic acid binding affinity was reduced. Therefore, Walk-H364R had a destabilizing effect on protein-nucleic acid interaction. $\Delta\Delta G^{\text{affinity}}$ was 0 kcal/mol against both PDB structures in chain A and chain B. $\Delta\Delta G^{\text{stability effect}}$ against 4mn5 and 4mn6 was -1.4 kcal/mol and -1.364 in chain A and -1.478 kcal/mol and -1.53 in chain B, respectively.

Prediction of the impact of mutation H364R in Walk on protein function

SIFT estimated that substitution at position 364 from H to R is predicted to be tolerated with a score of 1.00 and median sequence conservation: 3.02.

Due to the importance of the mutation site, the result of SIFT prediction was surprising.

The SIFT alignment results in FASTA format showed that Walk sequences are homologous to *S. aureus* SsrB and ResE in *B. subtilis* in some residues (fig. 4). The alignment of Walk showed that an R364 residue (similar to the amino acid substituted in H364R) in protein sensor kinases SsrB in *S. aureus* and ResE in *B. subtilis* was matched with residue 364 (Arginine) of VAN-I mutant.

(Fig. 4). Therefore, this could be the cause of tolerating the prediction of H364R in the SIFT method. We searched the binding sites in SsrB protein using PLIP (Protein-Ligand Interaction Profiler) web service (<https://projects.biotec.tu-dresden.de/plip-web/plip/index>) (input PDB ID: 6PAJ; data not shown). No Zn²⁺-binding site was detected in SsrB. Furthermore, residue 348 in SsrB showed no evidence of a ligand-binding site. Therefore, this residue has a different function in SsrB compared to it in Walk. This result showed a low accuracy of the SIFT method in classifying nsSNPs within critical sites of *S. aureus* Walk.

Discussion

Although most reported nsSNPs in VISA strains occur in regulatory TCSs (e.g. *walkR*), the molecular mechanisms of the development of vancomycin resistance in VISA remain still unclear². In this study, we have reported a VISA strain with an nsSNP in a functional residue within the PAS^{CYT0} domain of Walk protein, which binds to a specific ligand.

The complementation experiments have shown that in some strains, only one nsSNP in *walkR* system can lead to a decrease in vancomycin susceptibility. For instance, Walk-G223D or Walk-Q371del can convert VSSA to hetero-resistant VISA (hVISA)^{5,29}. Walk-Q371del is located in the PAS^{CYT0} domain.

Recently, the X-ray crystal structure of the PAS^{CYT0} domain of *S. aureus* Walk has revealed the presence of four Zn²⁺-binding sites, including His 271, Asp 274, His 364, Glu 368 within the PAS^{CYT0} domain. Zn²⁺ is tetrahedrally-coordinated by four Zn²⁺-binding sites¹⁰. In the present study, Walk-H364R occurred in residue His 364 in VISA strain VAN-I. This nsSNP was also previously reported to involve in nisin resistance in an *S. aureus* strain after nisin selection¹⁹. Nisin disrupts the cell membrane as a primary target. Furthermore, it inhibits the peptidoglycan biosynthesis in Gram-positive bacteria through specific interaction with lipid II³⁸. Interestingly, lipid II is also a target for vancomycin³⁹. Coates-Brown et al. suggested that Walk-H364R could limit pore formation and nisin interaction with lipid II through the increased cell wall thickness, which is a common feature in VISA strain¹⁹. This hypothesis also provides new insight into the importance of Walk-H364R in the development of vancomycin resistance in VISA. High conservation of Zn²⁺-binding sites among staphylococci Walk and the importance of residue H364 in binding to Zn²⁺ ligand and regulating *walkR* can support this hypothesis (Fig. 4).

Molecular mapping and computational approaches have been used for predicting the effects of nsSNP on the development of drug resistance, including isoniazid and rifampicin resistance in *Mycobacterium tuberculosis*¹⁵, rifampin resistance in *Mycobacterium leprae*¹⁶, and carbapenem resistance in *Acinetobacter baumannii*¹⁷. However, studies on VISA development are limited to allele swapping and complementation experiments². Although these studies are needed to confirm the effect of nsSNPs in the development of VISA, the knowledge on molecular modeling of nsSNPs helps to a better insight into the molecular mechanism of VISA development. These approaches are easy and fast and the only necessity is the published crystal structure of the desired bacterial species in the mutation site.

In the present study, an overview of the protein structure mapping of several previously reported nsSNPs in VISA strains indicated that Walk nsSNPs frequently occurred in the PAS^{CYT0} domain^{5,12,27–29,33}. However, we observed that nsSNP could occur in every Walk protein domain. These results explain the diversity of nsSNPs in VISA strains. This diversity may occur due to the differences in the genetic backgrounds of isolates³⁴, which needs future analyses.

Here, the molecular computational analyses showed that substitution H364R in the Zn²⁺-binding site of the PAS^{CYT0} domain decreased the stability, flexibility, and interactions (with proteins and nucleic acids) Walk. The destabilizing effect of mutations on protein leads to significant disruption of protein function

or its regulation ⁴⁰. Here, decreasing the affinity of Walk interactions (with proteins and nucleic acids) in VAN-I mutant was confirmed by the downregulation of *walkR* and *walkR*-regulated autolysins (*sle1*, *lytM*, and *atlA*). Thus, H364 can be a destructing mutation in the Walk and involve in VISA development. These results showed the importance of nsSNP in the Zn²⁺-binding site of the PAS^{CYT0} domain of Walk.

The molecular models have suggested that the activation of Walk histidine kinase in *S. aureus* induces through the specific interaction of the PAS^{EC} domain with D-Ala-D-Ala residue, which leads to phosphorylation of the WalR ⁴. Recently, Monk et al. have suggested that Zn²⁺-binding sites within the PAS^{CYT0} domain have a regulatory role within *S. aureus* Walk. They showed that substitution in Zn²⁺-binding residue 271 (Walk-H271Y) led to inhibition of Zn²⁺ binding, activation of *walkR* regulon, increased hemolysis, increased *atl* expression, and increased vancomycin sensitivity ¹⁰. These features were in contrast to our VAN-I mutant, which had a substitution in the Zn²⁺ + binding site. However, H271Y mutant, similar to VISA strains and VAN-I mutant, displayed reduced size and pigmentation of colonies, reduced growth rate, and increased doubling time ¹⁰. Recently, similar to our study, the genome sequencing of a VSSA/VISA pair revealed a mutation in the Zn²⁺-binding site (residue 271: Walk-H271D) was associated with increased resistance to vancomycin in the VISA strain¹². Indeed, in contrast to Walk-H271Y substitution, Walk-H271D substitution in Zn²⁺-binding led to increased vancomycin resistance.

The complementation experiments have revealed that molecular events associated with the development of VISA result in typical phenotypic features observed in VISA isolates. For instance, reduced autolysis and hemolysis can be related to dysfunctional *walkR* ^{2,41}. Here, *walkR* and *walkR*-related autolysins, including *sle1*, *lytM*, and *atlA*, were downregulated in VAN-I compared to those in VAN-S. Collectively, these events could be related to H364R, which might negatively regulate the *walkR* regulon. Subsequently, it led to increased resistance to vancomycin. Thus, the nsSNPs in Zn²⁺-binding sites likely induce different effects on the regulation of *walkR* regulon.

Conclusion

Walk gene sequencing of a VSSA/VISA pair reveals a mutation H364R in a critical Zn²⁺ coordinating site of the PAS^{CYT0} domain, which might play a role in VISA development. Molecular computational approaches show that this mutation can induce destabilizing effects on Walk protein function in VISA strain through decreasing stability and interactions of protein-protein and protein-nucleic acid. The *walkR* and *walkR*-related genes that downregulated in VISA strain have verified these results. Collectively, molecular mapping and computational approaches can be useful in understanding the mechanism of VISA development and designing of novel antimicrobial drugs.

Declarations

Data Availability

The gene sequences from this study are available in the GenBank database (<https://www.ncbi.nlm.nih.gov/>) under the accession numbers of MN503664, MN503665, MN503668, and MN503669. Primers used in this study are described in the Supplementary Information file online (Table S1).

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Author Contributions

N.B. and Sh.N.P. designed the study. N.B. performed the experiments and analyzed the data. Sh.N.P. gave scientific suggestions and controlled experiments. Sh.N.P. and B.B. supervised the study. N.B. wrote the manuscript. All authors reviewed the manuscript.

Competing interests

We have no conflicts of interest.

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Tables

Table 1

Predicted stability changes upon mutation H364R using DUET prediction web server. The negative $\Delta\Delta G$ s (kcal/mol) indicate the destabilizing effect of mutation H364R.

Method	mCSM Predicted Stability Change ($\Delta\Delta G$: kcal/mol)	SDM Predicted Stability Change ($\Delta\Delta G$: kcal/mol)	DUET Predicted Stability Change ($\Delta\Delta G$: kcal:mol)
Wild-type			
4mn5-chain A	-1.4	-1.0	-1.371
4mn5-chain B	-1.478	-1.19	-1.484
4mn6-chain A	-1.364	-1.58	-1.423
4mn6-chain B	-1.478	-1.19	-1.484

Figures

Figure 1

Comparative phenotypic characteristics of an isogenic pair of VAN-S and VAN-I (with missense mutation H364R). The error bars indicate the standard deviation ($\pm$ SD) of values obtained by spectrophotometer in three independent tests. A P value ≤ 0.05 was considered as a significant difference between VAN-S and VAN-I. (a) A kinetic data set of autolysis of the *S. aureus* culture (in 20 ml BHI broth) washed (two times) and adjusted to an OD600 of 1 in 10 ml cold distilled water supplemented with 0.1% Triton X-100 was measured at OD600 in the tested time points. There was a statistically significant difference ($P < 0.01$) in the autolysis kinetic activity of VAN-I versus VAN-S (paired-sample Student's t-test) in each tested point. (b) The percent of hemolysis of 300 μ l of human RBCs diluted in 10 ml PBS in exposure to *S. aureus* culture (adjusted to an OD600 of 0.3 in BHI broth) was measured at OD 543 using the following formula: $(A_{543} \text{ of the sample} - A_{543} \text{ of negative control}) / (A_{543} \text{ of positive control} - A_{543} \text{ of negative control}) \times 100$. Negative control: diluted RBC in PBS without bacteria; Positive control: Triton X-100; The symbol (***) indicates a significant difference ($P < 0.001$) using the two-tailed Wilcoxon Signed Ranks Test.

Figure 2

The Zn²⁺-binding sites of Walk protein. Blue rods show the Zn²⁺-binding residues (HIS 271, ASP 274, HIS 364, and GLU 368). The coordinating bonds are shown with purple lines and numbers (1, 2, 3, 4, and 5). The image is generated by PLIP (Protein-Ligand Interaction Profiler) web service (<https://projects.biotec.tu-dresden.de/plip-web/plip/index>). PDB ID 4mn6 was used as a wild-type template.

Figure 3

Schematic map of Walk protein domains and locations of non-synonymous single nucleotide polymorphism (nsSNP) in sensor protein kinase Walk of *S. aureus*. H364R detected in the present study is indicated in red color. Other nsSNPs were reported previously in vancomycin nonsusceptible *S. aureus* strains. The nsSNP occurring in the same domain are shown with the same color. The nsSNP with the red symbol (*) indicates the presence in a Zn²⁺-binding site. PASEC: extracellular PAS domain; HAMP: present in Histidine kinases, Adenyl cyclases, Methyl-accepting proteins, and Phosphatases domain; PASCYTO: cytoplasmic PAS domain, DHp: dimerization and histidine phosphorylation domain; CAT: catalytic and HATPase (ATP-binding) domain.

Figure 4

Alignment of residues 271-370 of Walk protein in *S. aureus* with other staphylococci, bacilli, and enterococci. Zn²⁺-binding residues are shown in red color. The mutated residue in VAN-I is highlighted in yellow. The nonconserved residues are highlighted in blue.

Figure 5

Three dimensional (3D) view of the differences of interatomic interactions of HIS364 (wild-type) and ARG364 (mutant) residues in chain A and chain B of WalK in the mutant (VAN-I) and wild-type (PDB ID as a template: 4mn6). Binding interactions are reduced in the mutant than those in the wild-type. (a) Wild-type, chain A. (b) Wild-type, chain B. (c) Mutant, chain A. (d) Mutant, chain B. The color of interactions: clash: purple; VDM: pale blue; hydrogen bond: red; ionic: yellow; aromatic: pale green; hydrophobic: green; carbonyl: blue; polar: orange. The image is generated by the mCSM-PPI2 web server (http://biosig.unimelb.edu.au/mcsm_ppi2/).

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