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A novel mutation of *walK* confers vancomycin-intermediate resistance in methicillin-susceptible *Staphylococcus aureus*

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ABSTRACT

With the treatment failure by vancomycin and poor clinical outcomes, the emergence and spread of vancomycin intermediate-resistant *Staphylococcus aureus* (VISA) has raised more concerns in recent years. While most VISA strains are isolated from methicillin-resistant *S. aureus* (MRSA), the mechanism underlying the generation of VISA from methicillin-susceptible *S. aureus* (MSSA) is still largely unknown. Here, we identified a total of 10 mutations in 9 genes through comparative genome analysis from laboratory-derived VISA strain. We verified the role of a novel mutation of WalK (I237T) and our results further indicated that the introduction of WalK (I237T) by allelic replacement can confer vancomycin resistance in MSSA with common VISA characteristics, including thickened cell walls, reduced autolysis, and attenuated virulence. Consistent with these phenotypes, real-time quantitative reverse transcription-PCR revealed the altered expression of several genes associated with cell wall metabolism and virulence control. In addition, electrophoretic mobility shift assay indicated that WalR can directly bind to the promoter regions of *oatA*, *sle1,* and *mgt*, fluorescence-based promoter activity and β-galactosidase assays revealed WalK (I237T) can alter promoter activities of *oatA*, *mgt, and sle1*, thus regulating genes expression. These findings broaden our understanding of the regulatory network by WalKR system and decipher the molecular mechanisms of developmental VISA resistance in MSSA with point mutations.

1. Introduction

Staphylococcus aureus is an important human pathogen that can lead to various infections, ranging from mild skin and soft tissue infections to life-threatening bacteremia, endocarditis, sepsis, and toxic shock syndrome (Dayan et al., 2016; Lowy, 1998). Antibiotics such as penicillin and methicillin were introduced to effectively treat penicillin-susceptible *S. aureus* (PSSA) and penicillin-resistant *S. aureus* (PRSA) infections in the mid-20th century. However, the MRSA strains were quickly generated and then spread globally, making vancomycin as the last line in the clinical treatment of serious staphylococcal infections (Levine, 2006). Unfortunately, the prevalence and spreading of VISA and heterogenous VISA (hVISA) strains poses serious challenges, which were usually accompanied by vancomycin treatment failure and poor clinical outcomes (Burnham et al., 2016; Holmes et al., 2012).

Since the first report of VISA in 1997, many studies have been carried out to investigate the molecular mechanisms involved in the generation of VISA (Hiramatsu et al., 1997). Generally, VISA strains exhibit some common characteristics, such as thickened cell walls (Cui et al., 2003), reduced autolysis (Sun et al., 2013), altered surface protein profile, and attenuated virulence (Howden et al., 2014), while diverse mutations have been identified in different isolates by comparative genomics profile analyses of VSSA/VISA pairs or series (Howden et al., 2011). Notably, mutations observed are often located on two-component systems (TCSs) in *S. aureus* such as *vraSR* (Berscheid et al., 2014; Katayama et al., 2009), *graSR* (Howden et al., 2008b; Neoh et al., 2008), and *walKR* (Howden et al., 2011; Shoji et al., 2011), other genes, including *sle1* and *msrR* associated with cell wall synthesis or hydrolysis (Katayama et al., 2016), *cmk* and *fdh2* associated with metabolism (Katayama et al., 2016; Matsuo et al., 2013), *stp1* and *clpP* associated with post-translational modification (Cameron et al., 2012; Shoji et al., 2011), and *rpoB* associated with transcription regulation (Matsuo et al., 2011) are identified and further verified by experiments, suggesting complicated mechanisms underlying VISA resistance.

WalKR (also known as YycFG) is the conserved TCS among low-G + C gram-positive bacteria, which is originally identified in *Bacillus subtilis* (Fabret and Hoch, 1998; Martin et al., 1999). TCSs are important signal transduction regulatory pathways for bacteria to adapt to a variety of

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external stimuli. In *S. aureus*, the WalKR system is the only essential TCS within 16 TCSs identified, which is mainly responsible for cell wall metabolism (Dubrac et al., 2007). WalK (histidine kinase) is a sensor and WalR (response regulator) is reported to regulate the expression of genes, including *ssaA*, *isaA*, and *lytM*, by directly binding to promoter regions of these genes (Dubrac and Msadek, 2004). In addition to regulating cell wall metabolism, the WalKR system is also involved in biofilm formation and exhibits significant roles in virulence regulation and host inflammatory response by controlling autolytic activity (Delaune et al., 2012).

Mutations in *walKR* have been identified during the generation of VISA strains, where positions are distributed across the spectrum of the domains that contribute to two-component regulatory function (Howden et al., 2011). Among these mutations, only four mutated forms of WalKR, including WalK (G223D) in JKD6009 or MW2 (Howden et al., 2011; Hu et al., 2015), WalR (K208R) in JKD6004 or JKD6005 (Howden et al., 2011), WalK (ΔQ371) in LR5P1 (Shoji et al., 2011), and WalK (S221P) in XN108 (Peng et al., 2017) are verified by allelic replacement to confirm their roles in VISA resistance. In our previous work, one MW2-derived VISA isolate, designated SV-1, was generated by serial passage with progressively increasing concentrations of vancomycin (Hu et al., 2015). The study reveals that the WalK (G223D) mutation displays a decreased autophosphorylation, thereby reducing the phosphorylation of the WalR and decreasing the binding capacity of WalR to the *altA* promoter. While most VISA isolates evolved from MRSA, sporadic cases were reported to elucidate the generation of VISA from MSSA and mechanisms behind it (Pillai et al., 2009; Wang et al., 2017). In this study, we selected MSSA strain Newman to incubate with vancomycin and isolated a laboratory-derived VISA strain SVNM. Ten mutations in nine genes were identified by comparative genome analysis. Our results reveal that a novel mutation of WalK (I237T) confers vancomycin resistance in Newman and influences the promoter activities of *oatA*, *mgt*, and *sle1* to regulate the expression of these genes in VISA strain.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 4. Newman is a clinical MSSA which is susceptible to the vancomycin. SVNM is a derivative of *S. aureus* strain Newman, generated by serial passage of the parent strain with stepwise increasing vancomycin treatment as described previously (Shoji et al., 2011). *S. aureus* and *E. coli* strains were stored in glycerol broth at −80 °C and subcultured before being used for any experiment. *S. aureus* strains were grown with shaking (220 rpm) in tryptic soy broth (TSB; Difco) or on tryptic soy agar (TSA; Difco) plates at 37 °C. *Escherichia coli* strains were grown with shaking (220 rpm) in lysogeny broth (LB; Oxoid) medium or on lysogeny broth agar (LA) at 37 °C. When required, different antibiotics were added, 100 μg/mL ampicillin sodium salt or 50 μg/mL kanamycin sulfate for *E. coli* and 15 μg/mL chloramphenicol for *S. aureus* strains were supplemented in media.

2.2. Whole-genome sequence comparison of Newman and SVNM

Cells of *S. aureus* strains Newman and SVNM were collected and stored at −80 °C, and sent to Personal Biotechnology Company (Shanghai, China) to perform the whole-genome sequence comparison. Further, details of method are provided in Supplementary File S1.

2.3. Construction of the walK mutant strain NMK1

To create the *walK* mutant strain without extra genes introduced, the plasmid pBTs was used as previously described (Cao et al., 2016). By using the primer pair of *walK*-F-KpnI/*walK*-R-SalI and the genome of SVNM as PCR template, DNA fragment was amplified with point mutation of WalK (I237T). The fragment was digested with KpnI and SalI and cloned into the plasmid pBTs with T4 ligase. The resulting plasmid, pBTsWalK (I237T), was first introduced into *S. aureus* strain RN4220 for modification and subsequently transformed into *S. aureus* strain Newman. An allelic replacement mutant was selected using a previously described method (Bae and Schneewind, 2006) and was further confirmed by PCR and sequencing.

2.4. Construction of protein expression and reporter plasmids

To construct the protein expression plasmids pETWalK, pETWalR, and pETWalK (I237T), the partial open reading frame (ORFs) of WalK (isolated cytoplasmic histidine kinase domain, residues 220–608) and full-length WalR ORF were amplified from strain Newman genomic DNA using primer pairs of WalK-exp-F-NcoI/WalK-exp-R-XhoI and WalR-exp-F-NcoI/WalR-exp-R-XhoI (Table 5), and the partial ORF containing the point mutation of *walK* (I237T) was amplified from strain NMK1 genomic DNA using primer pair of WalK-exp-F-NcoI/WalK-exp-R-XhoI. The three DNA fragments were digested with NcoI and XhoI and then cloned into the expression vector pET28a (+) (Novagen). The recombinant expression plasmids were transformed into *E. coli* BL21 (DE3).

To construct the reporter plasmids pGFP*oatA*, pGFP*mgt*, pGFP*sle1*, pOS*oatA*, and pOS*mgt* for detection of *oatA*, *mgt*, and *sle1* expression, the fragments containing the promoter of *oatA, mgt* or *sle1* were amplified from strain Newman genomic DNA with primer pairs as Table 5 indicated. The plasmid pGFP(Ma et al., 2017) containing the reporter gene *gfp* and pOS1(Zhu et al., 2019) containing the reporter gene *lacZ* were used. These recombinant reporter plasmids were first transformed into strain RN4220 and then transformed into strain Newman and NMK1.The recombinant plasmids were sequenced to confirm the DNA sequence identity of inserted fragments to the reference genome.

2.5. Antibiotic susceptibility assay

The antibiotic susceptibility of all strains was performed by the broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute (Institute, 2006). Population analysis was performed as described previously (Wootton et al., 2001), which is established by plating appropriate dilutions of direct colony suspension on Mueller-Hinton agar with 2 % NaCl containing increasing concentrations of antibiotics. The numbers of colony-forming unit (CFU) were determined after 24 h of incubation at 37 °C.

2.6. Growth curves

The overnight cultures of *S. aureus* were diluted into an optical density at 600 nm (OD_{600}) of 0.05 in 50-mL flasks contained 10 mL of fresh TSB. The mixtures were then incubated with constant shaking (220 rpm) at 37 °C, and the OD_{600} was read hourly using a spectrophotometer (DU 800; Beckman Coulter). These assays were repeated at least three times, with similar results.

2.7. Transmission electron microscopy

To detect morphological changes during the generation of VISA, strains were cultivated in TSB for 24 h with constant shaking (220 rpm) at 37 °C. Samples were prepared as previously described (Yuan et al., 2013) and sent to the Core Facility Center for Life Science (USTC, China). Specimens were examined with a TEM which is operated at an accelerating voltage of 120 kV.

2.8. Total RNA extraction and real-time quantitative reverse transcription-PCR

S. aureus strains were cultured overnight, diluted into an optical density at OD_{600} of 0.05 in 50-mL flasks contained 10 mL of fresh TSB

and cultivated to early exponential (OD_{600} = 0.5), mid-logarithmic (OD_{600} = 2.5), and early stationary (OD_{600} = 6) phases. The cells were collected by centrifugation at 5000 g for 5 min and then immediately treated with 1 mL of RNAiso plus (TaKaRa) and lysed with 0.1-mm-diameter-silica beads in a FastPrep-24 automated system (MP Biomedicals). The residual DNA was removed with RNase-free DNase I (TaKaRa).

For reverse transcription, cDNA was synthesized by using a PrimeScript first-strand cDNA synthesis kit (TaKaRa), and qRT-PCR was performed with SYBR Ex Taq premix (TaKaRa) using the StepOne real-time PCR system (Applied Biosystems). The cDNA quantity measured by real-time PCR was normalized to the abundance of *pta* cDNA (Valihrach and Demnerova, 2012). All qRT-PCR assays were repeated at least 3 times.

2.9. RNA sequencing

Cells of *S. aureus* strains Newman and NMK1 were collected at early exponential phase (OD_{600} = 0.5) and then immediately treated with 1 mL of RNAiso plus (TaKaRa) and lysed with 0.1-mm-diameter-silica beads in a FastPrep-24 automated system. The samples in two replicates were stored at −80 °C and sent to GENEWIZ (Suzhou, China) to perform the RNA sequencing. Further, details of RNA sequencing method are provided in Supplementary File S2.

2.10. Expression and purification of WalK, WalR, and WalK (I237T)

Strains BL21 (DE3) containing the expression plasmids were used to express WalK, WalR, and WalK (I237T). The transformant was grown in LB with 50 μg/mL kanamycin at 37 °C to an OD_{600} of 0.4−0.6 and induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 37 °C for additional 3 h. The cells were harvested and lysed by sonication in a lysis buffer (50 mM Tris−HCl, pH 8.0, 300 mM NaCl). WalK, WalR, and WalK (I237T) were purified with a nickel-nitrilotriacetic acid agarose solution (Qiagen, Valencia, CA, USA) following the manufacturer's instruction. The bound protein was eluted with an elution buffer (300 mM imidazole, 50 mM Tris−HCl, pH 8.0, 300 mM NaCl). The imidazole in the eluent was removed by using a Centrifuge Biomax-5 column (Millipore, Billerica, MA, USA), and the protein was stored with storage buffer (50 mM Tris−HCl, 25 mM KCl, 100 nM DTT, 10 % [vol/vol] glycerol, 0.01 % [vol/vol] Tween 20) at −80 °C until use. The protein purity was analyzed by SDS-PAGE, and the protein concentration was determined using the bicinchoninic acid (BCA) assay with bovine serum albumin as the standard.

2.11. Electrophoretic mobility shift assay

To identify the binding ability of purified WalR, the biotin-labeled DNA fragments containing the putative promoter regions of *sle1*, *oatA*, and *mgt* were amplified from *S. aureus* strain Newman genomic DNA. The biotin-labeled probe was incubated at 25 °C for 20 min with various amounts of WalR in 10 μl of incubation buffer (50 mM Tris−HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA). After incubation, the mixtures were added with 2 μl of gel loading buffer and then electrophoresed in a 5% native polyacrylamide gel in 1 × Tris-borate-EDTA buffer, and then transferred to a nylon membrane in 0.5 × TBE buffer. The band shifts were detected and analyzed according to the manufacturer's instructions. The images were obtained using an ImageQuant LAS 4000 min. (GE, Piscataway, NJ). The unlabeled *hu* fragment was added as a nonspecific competitor.

2.12. Fluorescence-based promoter activity and β-galactosidase assays

S. aureus strains containing the reporter plasmids were used to perform the fluorescence-based promoter activity and β-galactosidase assays. These strains were cultured overnight, diluted into an optical density at OD_{600} of 0.05 in 50-mL flasks contained 10 mL of fresh TSB with 15 μg/mL chloramphenicol and cultivated to early exponential (OD_{600} = 0.5), mid-logarithmic (OD_{600} = 2.5), and early stationary (OD_{600} = 6) phases. Cells were collected during different phases and washed twice before the detection. The GFP fluorescence was monitored by CLARIOstar (BMG LABTECH, Germany) and the procedure was referred to previous study (Horn et al., 2018). The galactosidase activity detection and analysis was performed according to the method described previously (Liu and Sun, 2020).

3. Results

3.1. Comparative genome analysis revealed novel mutations in the VISA strain SVNM

To further investigate the molecular mechanisms underlying VISA resistance, the MSSA strain Newman was treated with increasing concentrations of vancomycin by following a protocol previously described (Shoji et al., 2011). Finally, a laboratory-derived VISA strain, designated SVNM, was obtained after serial passage for 3 months. The vancomycin MIC of SVNM was increased to 8 mg/liter in MH medium, compared to 2 mg/liter for parent strain Newman (Table 1). To confirm the stability of the VISA phenotype, we performed a further serial passage of SVNM in TSB without antibiotic for 14 days with consistent MIC values.

Then the deep genome sequencing of Newman and SVNM is completed, a total of 10 mutations in 9 genes were identified by comparative genome analysis (Table 2). Among these, mutations in the genes of *walK* and *vraS* are reported to develop the vancomycin resistance in MRSA strains (Howden et al., 2011; Katayama et al., 2009). WalKR is only essential TCS for the viability of *S. aureus* (Martin et al., 1999) and several mutations are studied that were involved in vancomycin resistance of VISA (Hu et al., 2015; Jiang et al., 2019; Peng et al., 2017; Shoji et al., 2011). Since a single-nucleotide polymorphism within *walK* (conferring the I237T amino acid change) is a novel mutation identified in VISA strain SVNM, we firstly focused our efforts on its possible effects on vancomycin resistance.

3.2. WalK (I237T) develops vancomycin resistance in MSSA

To study the influences of the *walK* mutation identified in SVNM, allelic replacement was performed to generate the *walK* mutant NMK1 by using plasmid pBTsWalK (I237T). We extracted genome of SVNM as the template to amplify fragment containing the point mutation by PCR, and single-nucleotide polymorphism was further confirmed by sequencing. Then we detected antibiotic susceptibilities of strain Newman and NMK1 by determining the MICs of vancomycin and daptomycin. The reconstituted NMK1 has increases of vancomycin and daptomycin MICs compared with those of the parental strain Newman (Table 1), which suggests the contribution of WalK (I237T) to vancomycin resistance. Population analysis was also performed, and the results showed the same tendency of reduced vancomycin susceptibility in NMK1 (Fig. 1A). Of note, the difference of vancomycin and daptomycin MICs between NMK1 and SVNM indicates the participation of other genes involved in the generation of VISA.

Table 1
Antibiotic susceptibilities of *Staphylococcus aureus* used in this study.

Strain	Description	MIC[a] (mg/liter)	
		Vancomycin	Daptomycin
Newman	Clinical isolate	2	2
SVNM	Derivative	8	8
NMK1	Derivative	4	4

[a] MICs were determined by broth microdilution method. The results were read after 48 h of incubation at 37 °C.

Table 2
Whole-genome sequence comparison of SVNM and Newman[a].

Mutation	Effect of mutation	Locus	Gene	Function
T to C	I237T	NWMN_0018	*walK*	Sensor protein kinase
G to T	V216F	NWMN_0306		Uncharacterized protein
A to T	D38I	NWMN_0407	*lpl4nm*	Staphylococcal tandem lipoprotein
C to A	I39K	NWMN_0407	*lpl4nm*	Staphylococcal tandem lipoprotein
T to A	L417Stop	NWMN_0538	*vraA*	Long chain fatty acid CoA ligase
GT to G	Frame shift deletion	NWMN_1213		Glutathione peroxidase
AG to GA	R57E	NWMN_1251	*lexA*	LexA repressor
C to T	A314V	NWMN_1823	*vraS*	Sensor protein VraS
A to G	E75G	NWMN_1840	*putP*	Sodium/proline symporter
G to C	A204P	NWMN_2281		Transcriptional regulator AraC family protein

[a] Vancomycin-susceptible MSSA strain used as a sequence control.

3.3. WalK (I237T) contributes to thickened cell walls and reduced autolysis

Several reports studying the mechanisms underlying VISA resistance show that VISA strains usually exhibit common characteristics, including thickened cell walls, reduced autolysis, and attenuated virulence (Boyle-Vavra et al., 2003; Cui et al., 2003; Herbert et al., 2007). To verify whether the VISA strain NMK1 share the similar characteristics, we detected cell wall thickness of Newman, NMK1, and SVNM by transmission electron microscopy (TEM). VISA strains NMK1 and SVNM represent significantly thickened cell walls compare to the parent strain Newman (Fig. 1D). Autolytic activities were measured by using 0.1 % Triton X-100 or 100 ng/mL lysostaphin to induce autolysis, the results show the reduced autolytic activities in the VISA strains NMK1 and SVNM (Fig. 1B and C).

3.4. WalK (I237T) influences expression of genes associated with autolytic activity, cell wall synthesis and modification, and virulence

WalKR, is identified as the only essential TCS in *S. aureus*, and plays important roles on cell wall biosynthesis and cell division. Genes involved in cell wall metabolism, such as *atlA*, *ssaA*, *isaA*, and *lytM*, are regulated by WalKR (Dubrac et al., 2007; Dubrac and Msadek, 2004). Considering the previous study, we selected several genes and performed RNA sequencing (File S3) to determine whether the expression of relative genes changed in NMK1. Results of qRT-PCR indicate that the expression of *atlA*, *sle1*, *lytN*, *oatA* (secretory antigen precursor SsaA homolog), and *lrgA* is significantly decreased in NMK1 (Fig. 2A), which is consistent with VISA phenotype of reduced autolysis. Besides, we also detect the genes *murD*, *ddl*, *femA*, *mgt,* and *dltA* associated with cell wall synthesis and modification. A significant increase in the transcript levels of *murD*, *femA*, *mgt,* and *dltA* was observed in the strain NMK1 compared with those in the wild-type Newman (Fig. 2B), implying the possible roles of these genes on the thickened cell wall.

Meanwhile, attenuated virulence is often accompanied by the generation of VISA strains, which is also considered as one form of fitness cost (Shang et al., 2016). Since *S. aureus* strain Newman is a human clinical isolate (Duthie and Lorenz, 1952) and displays robust virulence properties in animal models (Bae et al., 2004), we explored the change of virulence genes in the strain NMK1 and found that the transcriptional levels of *spa*, *rot*, *efb*, *clfA*, *cspC*, and *lip* were significantly decreased in NMK1 while the *capA*, *splE*, and *fnbA* were significantly increased (Fig. 2C). These data indicate that WalK (I237T) may confer phenotypes

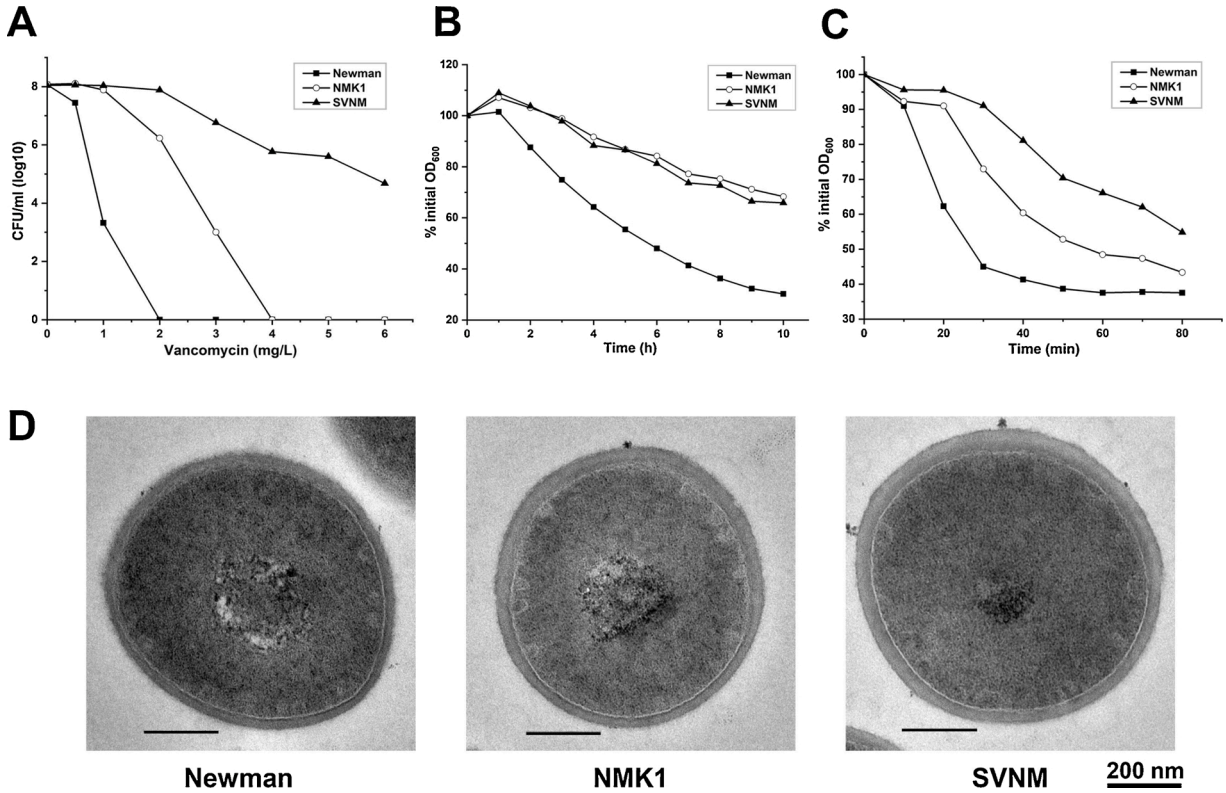

Fig. 1. Phenotypic analysis of *S. aureus* strain Newman, NMK1, and SVNM. (A) Vancomycin population analysis of Newman, NMK1, and SVNM. (B) Autolytic activity detection of Newman, NMK1, and SVNM. Cells were grown in TSB, 0.1 % Triton X-100 was added as the detergent to induce autolysis. (C) Autolytic activity detection of Newman, NMK1, and SVNM. Cells were grown in TSB, 100 ng/mL lysostaphin was added to induce autolysis. These experiments performed in triplicates were repeated three times. (D) The cell wall thickness was viewed under TEM. SVNM was a laboratory-derived vancomycin-intermediate *S. aureus* isolate evolved from the parent strain Newman. NMK1 was a point mutation strain by introducing *walK* mutation of VISA strain SVNM into the VSSA strain Newman.

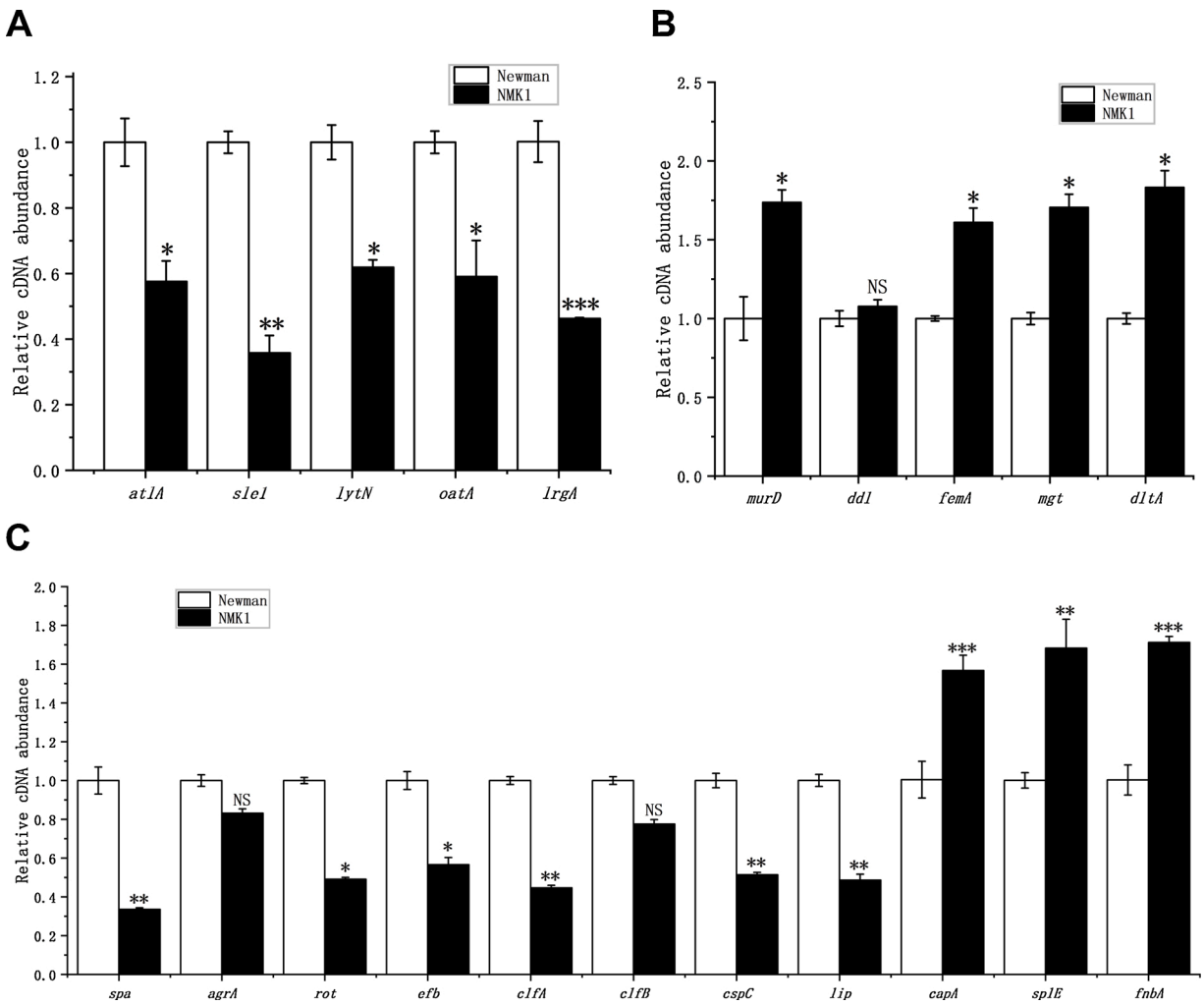

Fig. 2. Transcriptional analysis of genes associated with autolytic activity, cell wall synthesis and modification, and virulence. Cells were grown in the TSB and collected at the exponential phase (OD_{600} = 0.5). The relative transcription levels of (A) autolysis genes, including *atlA*, *sle1*, *lytN*, *oatA*, and *lrgA* (B) cell wall synthesis and modification genes, including *murD*, *ddl*, *femA*, *mgt*, *dltA*, and (C) virulence genes, including *spa*, *agrA*, *rot*, *efb*, *clfA*, *clfB*, *cspC*, *lip*, *capA*, *splE*, and *fnbA* in the Newman, NMK1, and SVNM strains are shown. Error bars represent SD (n = 3). Statistically significant differences calculated by the unpaired two-tailed Student's *t* test are indicated: NS, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

of VISA to the strain Newman by altering expression of genes involved in autolytic activity, cell wall metabolism, and virulence.

3.5. WalR can directly bind to the promoter regions of oatA, sle1, and mgt

To investigate the mechanisms underlying altered expression of genes detected, we analysed promoter regions of genes, which are significantly changed in NMK1 as WalR is reported to bind a consensus site (5′-TGTWAH-N5-TGTWAH-3′) in *S. aureus* (Dubrac and Msadek, 2004). Among these genes, *sle1* (N-acetylmuramoyl-L-alanine amidase aaa precursor), *oatA*, and *mgt* (monofunctional glycosyltransferase) were found to possess a consensus WalR binding site in their upstream regions on either the coding or the noncoding strand (Table 3). To test whether the potential target genes are under the direct control of WalKR, electrophoretic mobility shift assay (EMSA) was performed with biotin-labeled putative promoter regions and the recombinant WalR which was expressed by the plasmid pETWalR and purified by a nickel nitrilotriacetic acid agarose solution (Fig. 3A). The results showed that WalR can retard the mobility of the *oatA*, *sle1*, and *mgt* promoter in a dose-dependent manner (Fig. 3B to D). To further verify the binding ability of WalR, a 100-fold or 200-fold excess of an unlabeled DNA fragment of *hu* was added during the incubation which had no effect on the shifted band.

3.6. WalK (I237T) can alter promoter activities of oatA, mgt, and sle1

To further confirm the effect of the WalK (I237T) on genes expression, we constructed two different promoter activities reporter plasmids,

Table 3
Genes potentially related to cell wall metabolism in the strain Newman that have a consensus WalR binding site in their upstream regions.

Gene	Putative WalR binding site	Distance (bp) from translation start site	Strand location[a]
oatA (NWMN_2466)	TGTTATCATAATGTAAT	165	NCS
sle1 (NWMN_0429)	TGTAATCATAATGTCAT	103	CS
mgt (NWMN_1766)	TGTAGCCTATTTGTGAT	56	CS

[a] CS, coding strand; NCS, noncoding strand.

Table 4
Strains and plasmids used in this study.

Strain or plasmid	Description [a]	Reference or source
S. aureus strains		
Newman	Wild-type, parent VSSA	NARSA[b]
RN4220	8325−4 r^-, initial recipient for modification of plasmids that are introduced into *S. aureus* from *E. coli*	NARSA
SVNM	Laboratory-derived VISA strain	This study
NMK1	Newman strain mutant strain, WalK (I237T)	This study
E. coli strains		
Trans1-T1	Clone host strain; F^-Φ80 (*lacZ*) ΔM15 *lacX74 hsdR* ($r_K^- m_K^+$) Δ*recA*1398 *endA1 tonA*	TransGen
BL21 (DE3)	Express strain; F^-*ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (DE3)	TransGen
Plasmids		
pBTs	Shuttle vector, temp sensitive, Amp^r Chl^r	(Cao et al., 2016)
pBTsWalK (I237T)	pBTs derivative, for WalK (I237T) mutation in strain Newman, Amp^r Chl^r	This study
pET28a (+)	Expression vector with a hexahistidine tag, Kan^r	Novagen
pETWalK	pET28a (+) derivative, with ORF of *walK* from strain Newman, Kan^r	This study
pETWalR	pET28a (+) derivative, with ORF of *walR* from strain Newman, Kan^r	This study
pETWalK (I237T)	pET28a (+) derivative, with ORF of *walK* from strain NMK1, Kan^r	This study
pOS1	Shuttle vector, with *lacZ* ORF lacking first 6 amino acids, Amp^r Chl^r	(Zhu et al., 2019)
pOS*oatA*	pOS1 derivative, harboring *oatA* promoter region and 18 bp of *oatA* coding sequence from strain Newman, Amp^r Chl^r	This study
pOS*mgt*	pOS1 derivative, harboring *mgt* promoter region and 18 bp of *mgt* coding sequence from strain Newman, Amp^r Chl^r	This study
pGFP	*gfp* expression with the promoter of S10 ribosomal gene, Amp^r Chl^r	(Ma et al., 2017)
pGFP*oatA*	pGFP derivative, harboring *oatA* promoter region from strain Newman, Amp^r Chl^r	This study
pGFP*mgt*	pGFP derivative, harboring *mgt* promoter region from strain Newman, Amp^r Chl^r	This study
pGFP*sle1*	pGFP derivative, harboring *sle1* promoter region from strain Newman, Amp^r Chl^r	This study

[a] r^-, restriction system negative; Kan^r, kanamycin resistant; Amp^r, ampicillin resistant; Chl^r, chloramphenicol resistant.
[b] NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.

denominated as pOS*oatA* and pGFP*oatA*, pOS*mgt* and pGFP*mgt*, and pGFP*sle1* that carried the original sequence of the *oatA*, *mgt,* and *sle1* promoter region. Cells containing the reporter plasmid were cultured and collected at early exponential (OD_{600} = 0.5), mid-logarithmic (OD_{600} = 2.5), and early stationary (OD_{600} = 6) phases, respectively. The results showed that the promoter activity of *oatA* and *sle1* is decreased while the promoter activity of *mgt* is increased at different phases in NMK1 (Figure 4), which exhibited same tendency of the genes expression, suggesting WalK (I237T) can influence the consistent promoter activities of target genes, and then alter the expression of genes regulated by WalKR.

4. Discussion

By horizontal gene transfer of mobile genetic elements and mutations in chromosomal genes, *S. aureus* is notorious for its extraordinary ability to develop resistance after the introduction of new antibiotics for the treatment of staphylococcal infections (Gardete and Tomasz, 2014; Jensen and Lyon, 2009). Besides the rapid occurrence of PRSA and MRSA, *S. aureus* can acquire complete vancomycin resistance, known as vancomycin-resistant *S. aureus* (VRSA), through the acquisition of

Table 5
Primers used in this study.

Primer	Oligonucleotide (5′-3′)[a]	Application
walK-F-KpnI	CGGggtaccATGAAGTGGCTAAAACAACT	*walk* point mutation
walK-R-SalI	ACGCgtcgacTTATTCATCCCAATCACCGT	*walk* point mutation
WalK-exp-F-NcoI	CATGccatggGCTCCAGAGGTAACTATACGC	Expression of WalK
WalK-exp-R-XhoI	CCGctcgagTTCATCCCAATCACCGTCTTCAATG	Expression of WalK
WalR-exp-F-NcoI	CATGccatggCTAGAAAAGTTGTTGTAGTTG	Expression of WalR
WalR-exp-R-XhoI	CCGctcgagCTCATGTTGTTGGAGGAAATATCCA	Expression of WalR
RT-*atlA*-F	AGCACCAACGGATTAC	qRT-PCR
RT-*atlA*-R	CATACTCAGCACTGTCT	qRT-PCR
RT-*sle1*-F	CACCAGTATTCAGTCACCAA	qRT-PCR
RT-*sle1*-R	CAGTTATTAGCATTCCACCAAT	qRT-PCR
RT-*lytN*-F	TGACACCATTAGTAGAACCAA	qRT-PCR
RT-*lytN*-R	AACATTGCCATCCATAACTAC	qRT-PCR
RT-*oatA*-F	GGATATAATTCTAATGACGCTCAA	qRT-PCR
RT-*oatA*-R	GTTGTTGTAGTAGTATGTGTTGT	qRT-PCR
RT-*murD*-F	CTAATCTCTGAAGCACCAATC	qRT-PCR
RT-*murD*-R	ATGACGACAACTCTGTAACTA	qRT-PCR
RT-*ddl*-F	GGTCAAGTGTAGGTATCAGTA	qRT-PCR
RT-*ddl*-R	GTCGCTTCAGGATAGTCAT	qRT-PCR
RT-*femA*-F	ACTTACTGCTGTACCTGTTAT	qRT-PCR
RT-*femA*-R	CCTGTAATCTCGCCATCAT	qRT-PCR
RT-*mgt*-F	AATGGAAGATGAACGATT	qRT-PCR
RT-*mgt*-R	AACTTGTTGTGTAATGGT	qRT-PCR
RT-*dltA*-F	GTTCATTCAAGGTCGTATT	qRT-PCR
RT-*dltA*-R	CATTGTCCGTAACTTCAG	qRT-PCR
RT-*spa*-F	AAGTGCTAACCTATTGTCAGA	qRT-PCR
RT-*spa*-R	AACCATTGCGTTGTTCTTC	qRT-PCR
RT-*agrA*-F	TTATGGCGATTGACGACAA	qRT-PCR
RT-*agrA*-R	GGCAGTAATTCAGTGTATGTT	qRT-PCR
RT-*rot*-F	AAAGGTTCTATGACGCTTAAAG	qRT-PCR
RT-*rot*-R	CATTGCTGTTGCTCTACTTG	qRT-PCR
RT-*efb*-F	AGATGCGAGCGAAGGATA	qRT-PCR
RT-*efb*-R	GCTGCTGGTTTATTAAATTGTG	qRT-PCR
RT-*clfB*-F	AGACAGCGACTCAGACTC	qRT-PCR
RT-*clfB*-R	TTGGTGGTGTAACTCTTGAAT	qRT-PCR
RT-*cspC*-F	GAATAACGGTACAGTTAAATGG	qRT-PCR
RT-*cspC*-R	CCTTCAACGATGTCGAATT	qRT-PCR
RT-*lrgA*-F	CTGTTAAGTTAGGCGAAGTC	qRT-PCR
RT-*lrgA*-R	AATGGTGCTTGGCTAATGA	qRT-PCR
RT-*clfA*-F	TCCTGAACAACCTGATGAG	qRT-PCR
RT-*clfA*-R	TGAATCTGAACCACTATCTGA	qRT-PCR
RT-*lip*-F	CGGTCGTGTAGATTATGGT	qRT-PCR
RT-*lip*-R	CGCAGTAATTCTTCTAGTTGA	qRT-PCR
RT-*capA*-F	GCAGTCTGTTAATACATATACG	qRT-PCR
RT-*capA*-R	GCTGTTGAATACTGACCATC	qRT-PCR
RT-*splE*-F	GTCCGTTATTCAGGTCAAGA	qRT-PCR
RT-*splE*-R	GGTTCTGGATAGCCAACAA	qRT-PCR
RT-*fnbA*-F	ACGCAACACAAGTAACAAC	qRT-PCR
RT-*fnbA*-R	CGCTTCTTCCTTAACTACCT	qRT-PCR
RT-*pta*-F	AAAGCGCCAGGTGCTAAATTAC	qRT-PCR
RT-*pta*-R	CTGGACCAACTGCATCATATCC	qRT-PCR
p*oatA*-F	ACATTCAATGAAGCTTTATT	EMSA
p*oatA*-R-biotin	AATTAAATATCCTCCCAATA	EMSA
p*sle1*-F	TTAGATATTGTAAAATCTAA	EMSA
p*sle1*-R-biotin	TTTAAAATCCTCCTCTTGCT	EMSA
p*mgt*-F	TATAACAATATTTGACATGATTAA	EMSA
p*mgt*-R-biotin	GCGTTTGCTCCTTCTTTTAA	EMSA
hu-F	AAAAAGAAGCTGGTTCAGCAGTAG	EMSA
hu-R	TTTACGTGCAGCACGTTCAC	EMSA
lacZ-oatA-F-EcoRI	CCGgaattcGAAATATTACATTACTTCTG	*lacZ* report
lacZ-oatA-R-BamHI	CGCggatccggTGTAACGATTTTTTTCATA	*lacZ* report
lacZ-mgt-F-EcoRI	CCGgaattcGAGAAATCGTTAAACAATTAC	*lacZ* report
	CGCggatccggCCTATCGCTTCTTTTCATG	*lacZ* report

(*continued on next page*)

Table 5 (*continued*)

Primer	Oligonucleotide (5′-3′)[a]	Application
lacZ-mgt-R-BamHI		
gfp-oatA-F-EcoRI	CCGgaattcGAAATATTACATTACTTCTG	*gfp* report
gfp-oatA-R-XbaI	CTAGtctagaTTAAATATCCTCCCAATAATC	*gfp* report
gfp-mgt-F-EcoRI	CCGgaattcGAGAAATCGTTAAACAATTAC	*gfp* report
gfp-mgt-R-XbaI	CTAGtctagaGTTTGCTCCTTCTTTTAAAAC	*gfp* report
gfp-sle1-F-EcoRI	CCGgaattcGCGTCCTACAAGCAAAGTTC	*gfp* report
gfp-sle1-R-XbaI	CTAGtctagaTAAAATCCTCCTCTTGCTT	*gfp* report

[a] Lowercase letters: restriction endonuclease recognition sites.

vancomycin-resistant enterococci (VRE) conjugative plasmid or transposition of Tn*1546* from the VRE plasmid into a staphylococcal plasmid (Perichon and Courvalin, 2009; Zhu et al., 2010). Although VRSA displays a higher resistance to vancomycin, the limit of reported cases is associated with the high fitness cost conferred by *vanA*-type transposon (Foucault et al., 2009). Therefore, the growing and widespread VISA globally has attracted more attention in recent years.

While the majority of VISA were reported to evolve from hospital associated MRSA, which display a similar genetic background of clonal complex 5 and 8 (Hafer et al., 2012), the MSSA-derived VISA from clinical or laboratory isolates are reported rarely (Pillai et al., 2009; Wang et al., 2017). In this study, we generated a VISA strain SVNM from MSSA strain Newman, and identified a novel point mutation, WalK (I237T), located in the TCS *walKR*. By using allelic exchange experiment, strain NMK1 contained the same single mutation of *walK* as strain SVNM. The vancomycin MIC of NMK1 was increased to 4 mg/liter in MH medium, in line with the standards of isolate classification of VISA by Clinical and Laboratory Standards Institute (CLSI) (Institute, 2006). Our results reveal that the substitution of WalK (I237T) in Newman decreases the susceptibility to vancomycin, and NMK1 displays the phenotypes of a thickened cell wall and decreased autolytic activity, which are supposed to be the common characteristics of VISA (Boyle-Vavra et al., 2003; Cui et al., 2003). Consistent with such changes, previous studies find the expression of genes associated with autolysis (*atlA*, *ssaA*, *isaA*, *sle1*, and *lytM*) and cell wall synthesis or modification (*dltA*, *mprF*, *murG*, and *glmU*) is altered in VISA (Howden et al., 2011; Peng et al., 2017). To get a comprehensive understanding of genes altered in NMK1, we performed RNA sequencing and qRT-PCR, our results indicate that the transcriptional levels of *atlA*, *sle1*, *lytN*, *oatA*, and *lrgA* are significantly decreased, while *murD*, *femA*, *mgt*, and *dltA* are increased in VISA. Besides, the expression of virulence genes such as *spa*, *rot*, *efb*, *clfA*, *cspC*, and *lip* was found to be significantly decreased, which is consistent with previous report (Howden et al., 2008a). Meanwhile, there were some other virulence genes including *capA*, *splE*, and *fnbA* that upregulated in NMK1, suggesting the complicated influences by point mutation of WalK (I237T). Taken together, our results indicated the contribution of WalK (I237T) to the VISA emergence in MSSA, which is associated with altered genes expression.

While the emergence of VISA strains is always considered as a stepwise process, one report shows that VSSA strain N315ΔIP can acquire complete VISA phenotype by sequentially introducing mutations into six genes (Katayama et al., 2016). We noticed the difference of VISA phenotype between NMK1 and SVNM, suggesting the possible participation of other genes involved in the generation of VISA. Besides WalK (I237T), there are other 9 mutations in 8 genes that were identified by comparative genome analysis. Among these mutations, VraS (A314 V) is another novel mutation located on the TCS *vraSR* and may play an important role on the development of VISA resistance. Several reports reveal that mutations of VraS (S329 L) (Katayama et al., 2009), VraS (234Δ) (Gardete et al., 2012), and VraS (L114S + D242 G) (Berscheid et al., 2014) confer the vancomycin resistance in VSSA directly, while VraS (I5N) and VraS (G45R) can cooperate with other mutations of GraR (N197S) and Stp1 (Q12ΔC)-YjbH (K23ΔC), respectively, to promote VISA resistance (Cui et al., 2009; Renzoni et al., 2011). So it is our future work to verify the function of VraS (A314V) and other mutations during

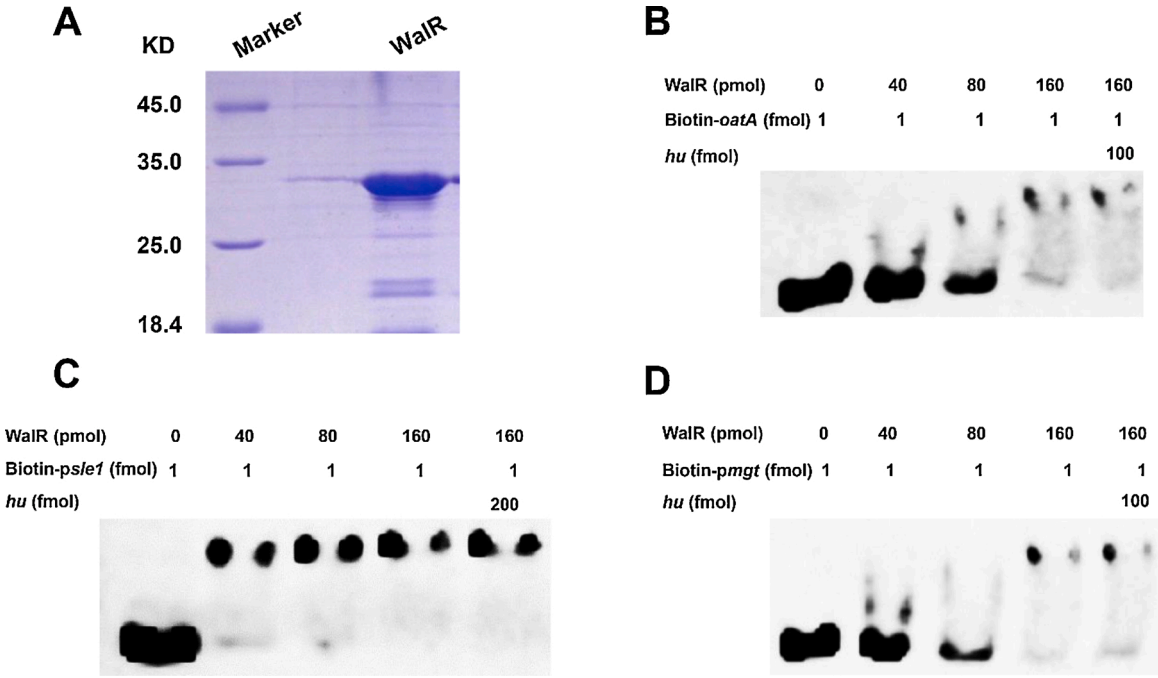

Fig. 3. EMSA represents the binding abilities of purified WalR to the promoter regions of *sle1*, *oatA*, and *mgt*. (A) SDS-PAGE analysis of WalR purified from the pET28a (+) expression vector. The gel was stained with Coomassie blue. (B-D) EMSA of the purified WalR with probe of *oatA*, *sle1*, and *mgt* promoter labeled with biotin. Increasing concentrations of purified WalR and 1 fmol of the biotin-labeled probe was used in the reactions. The unlabeled DNA fragment of the *hu* ORF region was added as a nonspecific competitor (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

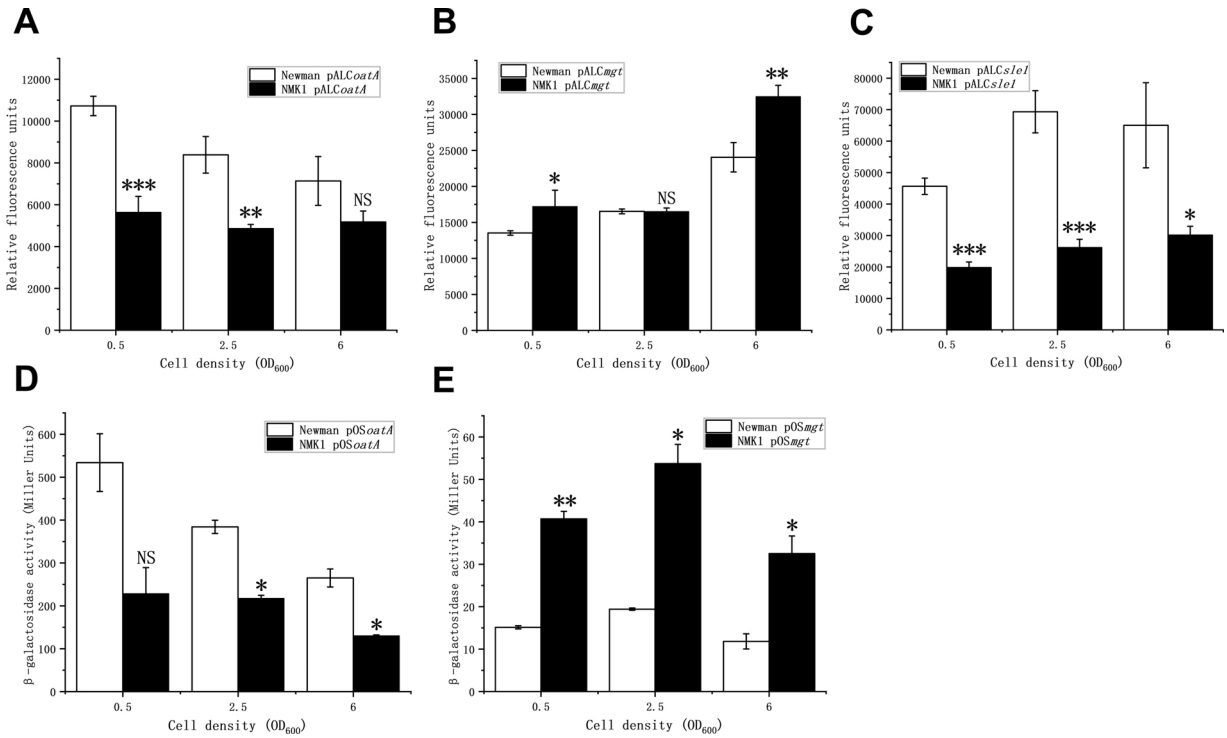

Fig. 4. Detection of fluorescence-based and β-galactosidase activities of the *oatA*, *mgt*, and *sle1* promoter. Cells were grown in TSB containing 15 μg/mL chloramphenicol and collected in early exponential ($OD_{600} = 0.5$), mid-logarithmic ($OD_{600} = 2.5$), and early stationary ($OD_{600} = 6$) phases. The GFP fluorescence was analysed by CLARIOstar, and the β-galactosidase activity was detected with *o*-nitrophenyl-β-D-galactopyranoside (ONPG). (A-C) Newman and NMK1 strain with a plasmid pGFP*oatA*, pGFP*mgt* or pGFP*sle1* were measured at the indicated time points. (D-E) Newman and NMK1 strain with a plasmid pOS*oatA* or pOS*mgt* were measured at the indicated time points. Error bars represent SD (n = 3). Statistically significant differences calculated by the unpaired two-tailed Student's *t* test are indicated: NS, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the generation of VISA to fully illuminate the mechanism underlying vancomycin resistance in SVNM.

WalKR system regulates the expression of *ssaA*, *isaA*, and *lytM*, by directly binding to the consensus sites upstream from these genes (Dubrac and Msadek, 2004). Our EMSA results reveal that WalR can bind to the promoter regions of *oatA*, *sle1*, and *mgt*. While the *oatA* codes the homolog of SsaA in Newman, we firstly reported the directly binding of WalR to *sle1* and *mgt* promoters. Meanwhile, two reports studying the regulatory mechanism of *walKR* mutations in VISA strains show that WalK (G223D) and WalK (S221P) decrease the binding activities of WalR to the *altA* and *lytM* promoter, respectively, by reducing the autophosphorylaton of WalK, and then reducing phosphorylation of the WalR (Hu et al., 2015; Peng et al., 2017). Like WalK (G223D) and WalK (S221P), the WalK (I237T) mutation identified in SVNM is located in the same HAMP domain which is responsible for signal transduction (Hulko et al., 2006). It strongly implies that the WalK (I237T) may share a similar pathway to regulate the expression of genes. However, the expression of WalK (I237T) protein is failed with various kinds of plasmids, strains, and culture conditions to perform autophosphorylaton assay. In this study, we designed fluorescence-based promoter activity and β-galactosidase assays, and results showed that WalK (I237T) can alter promoter activities of *oatA*, *mgt*, and *sle1* to regulate the expression of genes. Besides, the expression of *walK* and *walR* in the strain Newman and NMK1 were measured and results revealed no significant difference among two strains at indicated phases (Fig. S1), which eliminate the impact of different transcriptional level of *walKR*. However, the expression and phosphorylation analysis of WalK (I237T) may help us explain more details of regulatory network by WalKR system.

In conclusion, we have identified a novel mutation of WalK (I237T) from the MSSA-derived VISA strain SVNM and verified that the introduction of WalK (I237T) confers vancomycin resistance to Newman with common characteristics of VISA, including thickened cell walls, reduced autolysis, and attenuated virulence. Our findings suggest that the molecular mechanism underlying VISA resistance from MSSA or MRSA share some features in common. In addition, our study reveals that while WalR directly binds to the promoter regions of *oatA*, *sle1*, and *mgt*, WalK (I237T) can alter promoter activities of *oatA*, *mgt*, and *sle1*, thereby regulating genes expression. These results provide new insights into the regulatory network by WalKR system and mechanisms underlying the generation of VISA resistance.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2021.151473.

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