A Single Point Mutation in GraS Drives Co-Evolution of Vancomycin Resistance and Virulence in *Staphylococcus aureus*

Zhen Hu †, Yifan Rao †, Lu Liu, Zuwen Guo, Yuting Wang, Weilong Shang, Huagang Peng *

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Article

A Single Point Mutation in GraS Drives Co-Evolution of Vancomycin Resistance and Virulence in *Staphylococcus aureus*

Zhen Hu [1,†], Yifan Rao [2,†], Lu Liu [1], Zuwen Guo [1], Yuting Wang [1], Weilong Shang [1] and Huagang Peng [1,*]

[1] Key Laboratory of Microbial Engineering under the Educational Committee in Chongqing, Department of Microbiology, College of Basic Medical Sciences, Army Medical University (Third Military Medical University), Chongqing 400038, China

[2] Department of Emergency Medicine, the Second Affiliated Hospital, Army Medical University, Chongqing 400037, China

* Correspondence: huagangpeng@tmmu.edu.cn

† These authors contributed equally to this work.

Abstract

The emergence of vancomycin-intermediate *Staphylococcus aureus* (VISA) threatens the efficacy of this last-line antibiotic. The GraSR two component system is frequently mutated in VISA strains. Here, we demonstrate that the GraS(T136I) point mutation, identified in the clinical VISA isolate XN108, is a key determinant of reduced vancomycin susceptibility. Introducing this mutation into the susceptible strain Newman increased the vancomycin MIC from 1.5 to 4 mg/L, while its reversion in XN108 decreased the MIC from 12 to 8 mg/L. The mutation conferred common phenotypes, including thickened cell wall, decreased autolysis, and reduced cell surface negative charge via upregulation of the *dltABCD* operon and *mprF*. Notably, GraS(T136I) mutation also upregulated virulence genes (*efb*, *hlb*, *sbi*, *hld*) and enhanced hemolytic activity. Interestingly, despite this hypervirulence profile, the mutant showed impaired long term survival within macrophages. Our study reveals that a single GraSR mutation can co-regulate vancomycin resistance and virulence, offering new insights into the adaptation of *S. aureus* to antibiotic pressure.

Keywords: vancomycin-intermediate *Staphylococcus aureus*; GraSR; vancomycin resistance; virulence

1. Introduction

Staphylococcus aureus is a versatile human pathogen responsible for a wide spectrum of infections, ranging from mild cutaneous and soft tissue manifestations to life-threatening conditions, including bacteremia, endocarditis, sepsis, and toxic shock syndrome [1,2]. The emergence and global spread of methicillin-resistant *S. aureus* (MRSA) have rendered vancomycin a critical last-line therapy for severe staphylococcal infections [3,4]. In recent decades, however, the rising prevalence of vancomycin-intermediate *S. aureus* (VISA) and heterogeneous VISA (hVISA) strains has posed a serious clinical challenge, often correlating with treatment failure and poor patient outcomes [5]. Thus, a comprehensive understanding of the bacterial mechanisms underlying vancomycin resistance is critical for developing novel strategies to combat these life-threatening infections.

Since the first documentation of VISA in 1997, extensive research has been devoted to unraveling the molecular drivers of reduced vancomycin susceptibility [6]. VISA strains typically display conserved phenotypic traits, such as thickened cell walls, diminished autolysis, altered cell surface charge, and attenuated virulence [7–9]. Genomic comparisons between vancomycin-susceptible *S.*

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aureus (VSSA)/VISA isolates have revealed recurrent mutations in regulatory genes,[10,11]. especially those encoding two-component systems (TCSs) such as *vraSR* , *graSR* , and *walKR* [12–14]. Additional mutations have identified in genes involved in cell wall metabolism (*sle1*), central metabolism (*cmk*, *fdh2*), and transcriptional regulation (*rpoB*) [15–17].

TCSs are key bacterial signaling pathways that enable adaptation to environmental stresses, including antibiotic exposure [18]. In *S. aureus*, GraSR TCS plays a central role in modulating both antimicrobial resistance and virulence [19,20]. GraSR activates the *dltABCD* operon, which mediates D-alanylation of wall teichoic acids (WTAs) and lipoteichoic acids (LTAs),and the *mprF* gene, which catalyzes lysinylation of membrane phospholipid [21,22]. These modifications enhance the positive charge on the bacterial surface, reducing the binding of cationic antimicrobial peptides (CAMPs) and contributing to CAMP resistance. Clinically, mutations in the *graSR* have been repeatedly identified in VISA strains. For instance, the GraR(N197S) substitution was shown to convert an hVISA strain into a fully VISA phenotype, highlighting its direct role in resistance development [23]. Beyond resistance, GraSR also governs the expression of multiple virulence determinants, including α-hemolysin, enterotoxins, and adhesins, thereby influencing host colonization, immune evasion, and disease progression [20].

In a previous study, we characterized an ST239 SCCmec-III VISA strain XN108, which exhibits a vancomycin MIC of 12 mg/ml [24]. Genome sequencing identified several non-synonymous mutations, including WalK(S221P), GraS(T136I), and RpoB(H481N), that were hypothesized to contribute to its VISA phenotype [25]. In this study, focus on the role of GraS(T136I). Using allelic exchange, we introduced this mutation into the VSSA strain Newman and reversed it in XN108. We demonstrate that GraS(T136I) is both necessary and sufficient to confer reduced vancomycin susceptibility, accompanied by hallmark VISA phenotypes: thickened cell wall, decreased autolysis, and reduced cell surface negative charge. Moreover, this mutation upregulateskey virulence genes and enhances hemolytic activity, indicating a dual role in antibiotic resistance and pathogenicity. Our data establish GraS(T136I) as a critical determinant linking vancomycin resistance and virulence in *S. aureus*.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

The VISA strain XN108 was isolated from a burn patient with a severe *S. aureus* infection [24]. The VSSA strain Newman was provided by Prof. Lu Yu (Jilin University). The restriction modification-deficient *S. aureus* strain RN4220 was used as an intermediate host for plasmid construction. *Escherichia coli* DH5α was used for general cloning. The *E. coli-S. aureus* shuttle vector pBT2 served as the allelic replacement vector. *S. aureus* strains were routinely cultured in brain heart infusion (BHI, Oxoid, Hampshire, UK) at 37℃ or 30℃ with shaking. When required, chloramphenicol (20 μg/mL) was added. *E. coli* strains were grown in Luria–Bertani (LB) medium supplemented with ampicillin (100 μg/mL) as needed.

2.2. Genetic Manipulation of S. Aureus

The GraS(T136I) mutation was introduced into the VSSA strain Newman using a previously described allelic replacement method [25]. Briefly, the *graS* gene carrying the T136I mutation was amplified by PCR from XN108 genomic DNA using primers pBT2-GraS-5 (CCGGAATTCACTAAATGATATTGGGTGATATGG) and pBT2-GraS-3 (GCGGGATCCGTATATCAGATAATTCCTTGTTTG) and cloned into the temperature-sensitive shuttle vector pBT2. The resulting plasmid, pBT2-GraS(T136I), was electroporated into *S. aureus* RN4220, purified, and subsequently electroporated into Newman. Homologous recombination and plasmid curing were performed to generate the mutant strain Newman-GraS(T136I). The reciprocal revertant XN108-GraS(I136T) was constructed in a similar manner, using the wild-type *graS* allele from Newman. All constructs were verified by PCR amplification and Sanger sequencing.

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2.3. Antibiotic Susceptibility Assay

Vancomycin MICs were determined by E-test on BHI agar plates according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Fresh bacterial suspensions were adjusted to approximately 1×10^6 CFU/mL, and 100μL was spread evenly onto BHI agar. A vancomycin E-test strip (bioMérieux) was placed onto the center of the inoculated plate. Plates were incubated at37℃ for 24 h at, and the MIC value was read at the point where the inhibition ellipse intersected the strip.

2.4. Triton X-100-Stimulated Autolysis Assay

Autolysis was performed as previously described with some modifications [26]. Strains were grown in BHI to an optical density at 600 nm (OD600) of 1.0,collected by centrifugation, and washed twice with ice-cold 0.05 M Tris–HCl buffer (pH 7.5). Bacterial cells were resuspended in the same buffer containing 0.05% (v/v) Triton X–100 and incubated with shaking at 37℃. The decrease in OD600 was measured every hour using a microplate reader.

2.5. Transmission Electron Microscopy (Tem)

S. aureus cells were grown in BHI to the exponential growth phase, harvested, and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) overnight at 4 °C as previously described [27]. Samples were washed, post fixed with 1% osmium tetroxide, dehydrated in a graded ethanol series, and embedded in epoxy resin. Ultrathin sections (approximately 70 nm) were cut, stained with uranyl acetate and lead citrate, and examined with a Philips TECNAI 10 transmission electron microscope (The Netherlands) operated at 120 kV inChengdu Lilai Biotechnology Co. Ltd. Cell wall thickness was measured from 15 randomly selected cells per strain using Image J software.

2.6. Cytochrome C Binding Assay

Cell surface charge was assessed via the cytochrome C binding assay as previously described [28]. Briefly, mid-logarithmic phase bacteria were washed twice with 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (pH7.0) and adjusted to an OD600 of 14 in the same buffer. Cytochrome C (Macklin) was added to a final concentration of 0.5 mg/mL. After 10 min incubation at room temperature, samples were centrifuged at 18,000 × g for 6 min., and the absorbance of the supernatant was measured at 530 nm. A control without bacteria was used to determine 100% unbound cytochrome C. The percentage of bound cytochrome C was calculated from three independent experiments, each performed in triplicate.

2.7. Rna Extraction and Rt-Qpcr Determination

Total RNA was extracted from mid-logarithmic phase bacterial cultures using SV Total RNA Isolation System (Promega). Cells were lysed in 0.5 mL nuclease free water with 0.1 mm silica beads in a FastPrep 24 homogenizer (MP Biomedicals). RNA (500 ng) was reverse transcribed with random primers using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative PCR (qPCR) was performed with SsoAdvanced™ Universal SYBRR Green Supermix (Bio-Rad) on a CFX96™ Real-Time PCR Detection System (Bio-Rad,). The primers used are listed in Table 1. The housekeeping gene *pta* was used for normalization. The assays were repeated with three independent biological samples. Statistical analyses were performed based on the Student's *t*-test to determine the significance of the gene expression levels, where $P < 0.05$ was considered statistically significant.

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Table 1. Primers used in RT-qPCR.

Primer	Oligonucleotide (5′-3′)
RT-*graS*-F	CGTCAAATCTCAGCGCACAAAG
RT-*graS*-R	TGTTTTCTTTCTTGATTTTTTTCTTGATC
RT-*graR*-F	AATGGGATTTTAATGTTGCTGGTATT
RT-*graR*-R	GATCCATTGGATTATCACGAGATGAT
RT-*dltB*-F	AAGTACATGGTTAGGTGGACATCAGA
RT-*dltB*-R	GTCCAGATGAAATCGTTGGGAAG
RT-*mprF*-F	CTGCACTTTAGTGTCGTGTGTTGAAT
RT-*mprF*-R	CGGTACAAAATAGTACGCAAAACG
RT-*efb*-F	GCACGTCCACAATTTAATAAACCA
RT-*efb*-R	TCAATTCGCTCTTGTAAGACCATT
RT-*hlb*-F	GGTGGGACAAAACTGAAGGTAGC
RT-*hlb*-R	TGCTATCATTATCGAATCCACAACC
RT-*sbi*-F	GGGGAAGCAAAAGCGAGTG
RT-*sbi*-R	TGCACGTTCTGGGTGTTCG
RT-*hld*-F	TTATTTTTTAGTGAATTTGTTCACTGTGTC
RT-*hld*-R	ATGAGTTGTTTAATTTTAAGAATTTTTATCTT
RT-*pta*-F	AAAGCGCCAGGTGCTAAATTAC
RT-*pta*-R	CTGGACCAACTGCATCATATCC

2.8. Hemolytic Activity

Hemolytic activity was performed as previously described [29]. Overnight cultures of *S. aureus* XN108 and its derivatives were diluted 1:1,00 in fresh BHI and grown at 37℃for 16 h. Supernatants were obtained by centrifugation (14,000 ×g, 1 min). A 100-μL aliquot of each supernatant was mixed with 900μL of PBS containing 3% (v/v) sheep red blood cells and incubated at 37℃ for 20 min. PBS alone and ddH_2O containing 3% sheep red blood cells served as negative and positive controls, respectively. After centrifugation(5,000×g, 4℃, 10 min), the OD543 value of the supernatant was measured. Hemolysis percentage was calculated as [(ODsample – ODnegative control) / (ODpositive control – ODnegative control)] × 100.

2.9. Macrophage Infection Assay

Macrophage infection assay was performed as previously described [30]. Briefly, RAW264.7 macrophages were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific) supplemented with 10% (v/ v) fetal bovine serum at 37℃ with 5% CO_2. For infection, exponentially grown bacteria were washed twice with PBS and resuspended in PBS. RAW264.7 cells were infected with *S. aureus* at a multiplicity of infection (MOI) of 10 in 24-well plates. After 4 h of incubation at 37℃ with 5 % CO_2, the supernatant was removed, and the cells were washed with PBS. Fresh DMEM containing lysostaphin (1 mg/mL) and gentamicin (50 μg/mL) was added to eliminate extracellular *S. aureus*. After lysis with PBS containing 1% Triton X-100, Serial dilutions of the lysates were plated on BHI agar for CFU enumeration.

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2.10. Statistical Analysis

All experiments were repeated at least three times, and the data are expressed as mean ± standard deviation (SD). Statistical analyses (Student's *t*-test or two-way ANOVA) were conducted using GraphPad Prism 9.0. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Gras(t136i) Mutation Confers Reduced Vancomycin Susceptibility in S. Aureus

We have previously characterized a VISA strain, XN108, which exhibits a vancomycin MIC of 12 mg/L [24]. Whole-genome sequencing of XN108, compared with the reference strain TW20, identified 46 mutations across 33 genes. Among these, mutations in WalK(S221P), GraS(T136I), and RpoB(H481N) were speculated to contribute to the VISA phenotype of XN108. Our previous work demonstrated that allelic replacement of WalK(S221P) in XN108 with the wild-type WalK(P221S) considerably decreased vancomycin resistance, confirming the the critical role of WalK(S221P) in mediating the reduced vancomycin susceptibility observed in XN108[25]. To specifically assess the impact of the GraS(T136I) point mutation on vancomycin resistance, iwe introduced plasmids harboring this mutation into the vancomycin-susceptible *S. aureus* (VSSA) strain Newman to generate the mutant strain Newman-GraS(T136I) through homologous recombination. Compared to the parental Newman strain (vancomycin MIC = 1.5 mg/L), Newman-GraS(T136I) exhibited a marked increase in vancomycin resistance, with an MIC of 4 mg/L (Figure 1A, B). Conversely,we introduced the wild-type GraS(I136T) allele from the VSSA strain Newman into XN108, generating the revertant strain XN108-GraS(I136T). This revertant displayed a reduced vancomycin MIC of 8 mg/L, compared to 12 mg/L for the parental XN108 strain (Figure 1C, D). These complementary genetic experiments confirm that the GraS(T136I) single-point mutation is directly responsible for the observed alterations in vancomycin MIC.

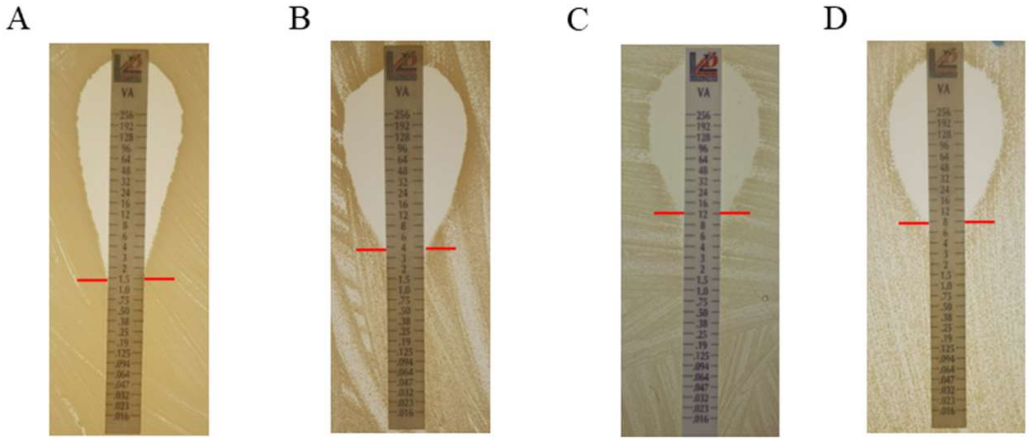

Figure 1. GraS(T136I) mutation reduces vancomycin susceptibility in *S. aureus*. Vancomycin susceptibilities of S. aureus strain Newman (A), Newman-GraS(T136I) (B), XN108 (C) and XN108-WalK(P221S) (D) were determined by E-test. Strains were grown overnight in BHI medium prior to MIC determination.

3.2. Impact of Gras(t136i) Mutation on Typical Visa Phenotypes

Previous studies have established that increased vancomycin resistance in *S. aureus* is frequently associated with common phenotypic changes, such as thickened cell walls and reduced autolytic activities[10]. To investigate whether GraS(T136I) confers these phenotypes, we performed TEM on exponentially growing cells. The cell walls of the revertant strain XN108-GraS(I136T) were significantly thinner than those of the parental XN108 strain (Figure 2A, B). Quantitative measurements confirmed this observation, with mean cell wall thicknesses of 40.28 ± 3.78 nm for

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XN108 and 29.08±2.72 nm for XN108-GraS(I136T). Given that cell wall thickening is generally accompanied with decreased autolysis, we assessed the autolytic activity using the Triton X-100-induced autolysis assay. The autolysis rate of XN108 was significantly lower than that of the XN108-GraS(I136T) revertant (Figure 2C).

Beyond cell wall remodeling, alterations in cell surface charge represent another key mechanism of vancomycin resistance. Vancomycin, a positively charged glycopeptide, binds less efficiently to bacterial surfaces with increased positive charge. To verify this mechanism, we detected relative cell surface charge using a cytochrome C binding assay. Strain XN108 bound significantly less cytochrome C than the XN108-GraS(I136T) revertant (Figure 2D), indicating that XN108 possesses a less negatively charged cell surface. This result supports the model where GraS(T136I)-mediated reduction in surface negative charge contributes to decreased vancomycin affinity and enhanced resistance.

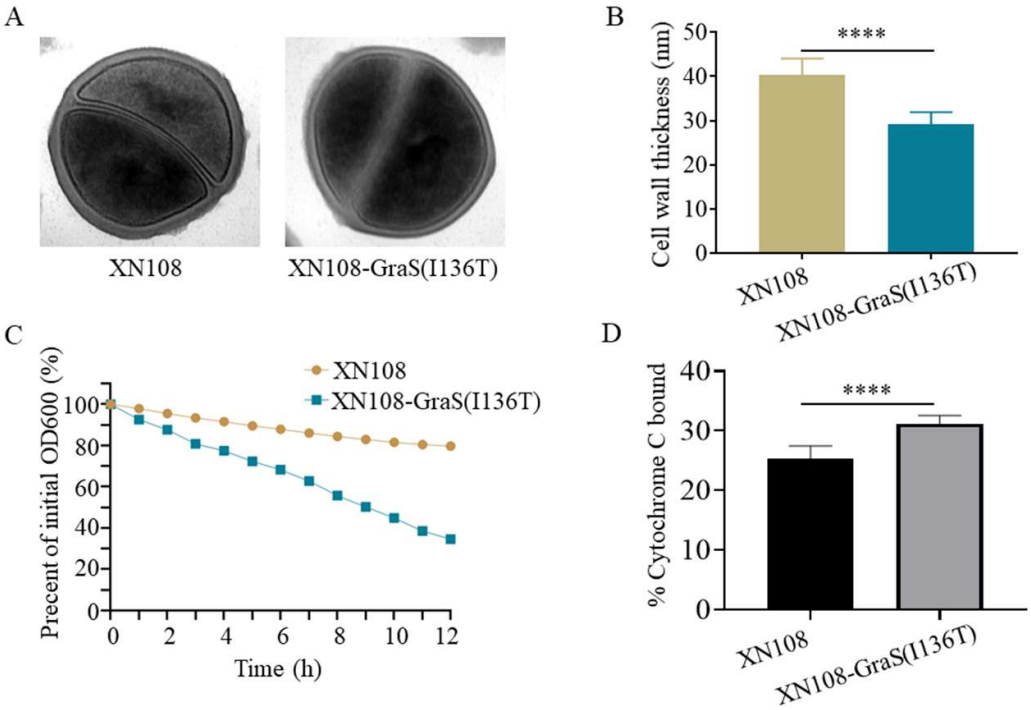

Figure 2. Effects of the GraS(T136I) mutation on typical VISA phenotypes. (A) TEM images of XN108 and the reverse mutant XN108-GraS(I136T). (B) Comparison of cell wall thickness between XN108 and XN108-GraS(I136T) (n = 15 for each strain). (C) Autolysis assay stimulated by Triton X-100. The autolysis rate was measured in XN108 and XN108-GraS(I136T) as described in Materials and methods. Results are from three independent experiments, each performed in triplicates. (D) Surface charge assessed by cytochrome C binding assay. Data are presented as mean ± SD. Statistical significance was determined using a two-tailed Student's t-test, with XN108 as the reference. ****$p < 0.001$.

3.3. Gras(t136i) Alters Expression of Genes Controlling Cell Surface Charge and Virulence

The GraSR two-component system is known to be upregulated in VISA isolates [31]. Activation of GraSR enhances vancomycin resistance primarily by directly binding to and upregulating the *mprF* and*dltABCD* operon, which are involved in modulating cell surface charge modification and peptidoglycan metabolism [28]. To investigate whether the GraS(T136I) mutation affects the expression of these genes, we quantified relative transcript levels in XN108 and the XN108-GraS(I136T) revertant using RT–qPCR. The transcript levels of *graS*, *graR*, *dltB* and *mprF* were significantly higher in XN108 compared to the revertant strain (Figure 3A). This result aligns with the findings of the cytochrome C binding assay, confirming that the GraS(T136I) mutation activates

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the GraSR system, leading to the upregulation of *dltABCD* and *mprF*. This transcriptional reprogramming subsequently modulates cell surface charge and culminates in increased vancomycin resistance.

The GraSR system also modulates *S. aureus* pathogenesis [20]. Previous reports indicate that virulence-related genes, such as *efb*, *hlb*, *sbi*, and *hld*, are under GraSR regulation, as their expression was downregulated in a *graSR* deletion mutant. To examine the impact of the GraS(T136I) mutation on virulence, we determined the transcript levels of these genes. The results indicated that all tested virulence genes were significantly upregulated in XN108 relative to the XN108-GraS(I136T) revertant (Figure 3B). This result indicates that the GraS(T136I) point mutation not only promotes vancomycin resistance but also concurrently upregulates the expression of key virulence determinants.

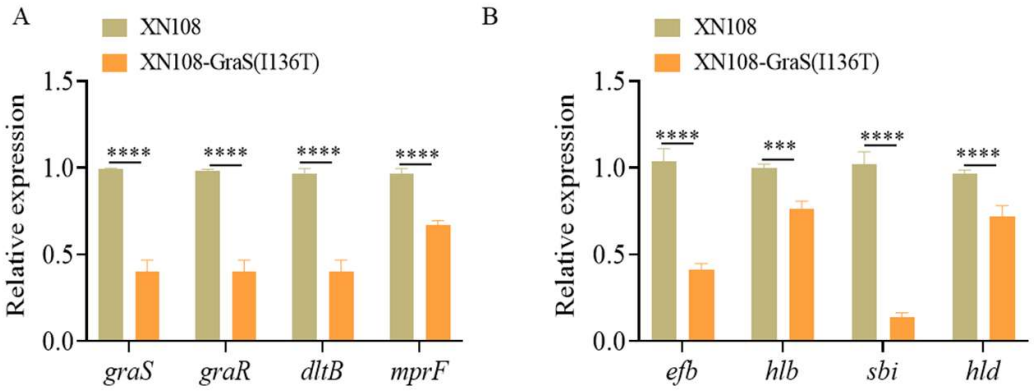

Figure 3. GraS(T136I) influences the expression of genes associated with cell surface charge and virulence. (A) Transcript levels of *graS*, *graR*, the *dlt* operon, and *mprF* in XN108 and XN108-GraS(I136T) during the mid-logarithmic phase, as determined by qRT-PCR. (B) Transcript levels of virulence-related genes. Data are presented as mean ± SD. Statistical significance was analyzed with a two-tailed Student's *t*-test, using XN108 as the reference. ***p < 0.001, ****$p < 0.0001$.

3.4. Gras(t136i) Enhances Hemolytic Activity and Impairs Intracellular Survival in Macrophages

Given the pronounced upregulation of hemolysin genes in XN108, we compared the hemolytic activities of XN108 and XN108-GraS(I136T). As shown in Figure. 4A, the XN108 strain exhibited significantly higher hemolytic activity compared to the XN108-GraS(I136T) revertant. Macrophages serve as a primary defense against bacterial invasion. To detect whether GraS(T136I)-mediated upregulation of virulence genes affects bacterial survival within host cells, we assessed the intracellular survival of both XN108 and XN108-GraS(I136T) strains within RAW264.7 macrophages. After 4 h of co-culture, the survival rates of XN108 and XN108-GraS(I136T) were comparable. However, after 12 h of culture, the intracellular survival of XN108 was markedly reduced compared to the XN108-GraS(I136T) revertant (Figure 4B). These results illustrate that while the GraS(T136I) mutation enhances virulence factor expression and hemolytic activity, it paradoxically attenuates the ability of S. aureus to survive long-term within macrophages, potentially impairing sustained cellular invasion..

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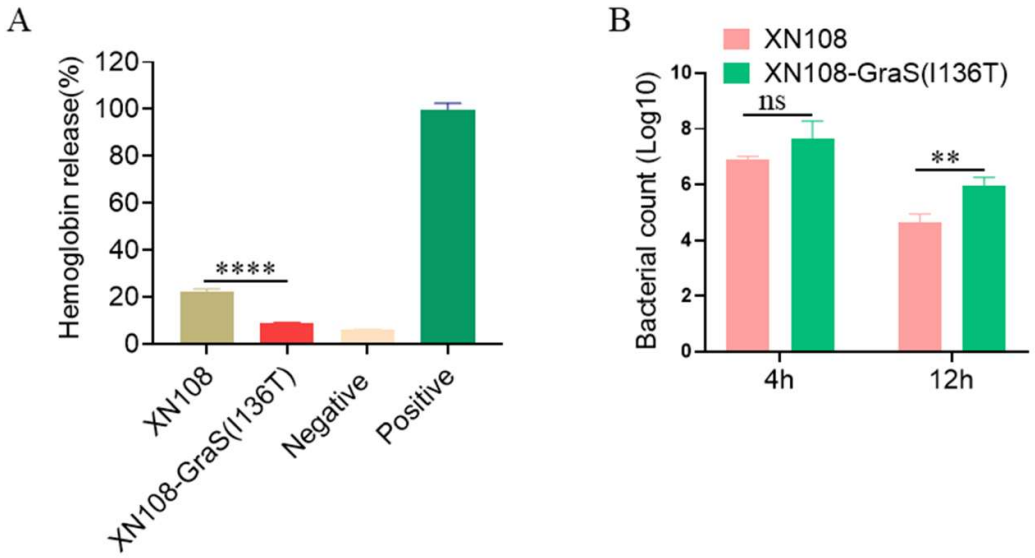

Figure 4. GraS(T136I) mutation enhances *S. aureus* virulence and impairs host cell invasion. (A) Hemolytic activity of XN108 and XN108-GraS(I136T) strains determined after 1 h incubation with 3 % sheep red blood cells. PBS- and ddH_2O-treated sheep red blood cells served as negative and positive controls, respectively. (B). Bacterial counts of XN108 and XN108-GraS(I136T) strains 4 and 12 h post phagocytosis by RAW264.7 macrophages. Data represent the mean ± SD (n = 3). Statistical significance was calculated by two-way ANOVA. * $p < 0.05$, **** $p < 0.001$, and ns indicates no significance.

4. Discussion

S. aureus is a remarkably adaptable pathogen capable of evolving resistance to last-line antibiotics while maintaining or even enhancing its virulence potential [32–36]. WSince 1980s, the widespread use of vancomycin has driven the emergence of VISA and hVISA strains, posing a major global public health threat [16,37]. Although comparative genomics has identified numerous mutations associated with the VISA phenotype, only a subset has been functionally investigated. Among these, mutations in the GraSR regulatory system have emerged as a recurrent theme [6]. The GraR(N197S) substitution in the clinical VISA strain Mu50, for example, was shown to convert the hVISA strain Mu3 into a VISA phenotype upon genetic complementation [23]. However, the mechanistic basis of how specific *graSR* mutations affect vancomycin resistance and other bacterial traits remains incompletely defined.

In this study, we show that the GraS(T136I) point mutation, which was identified in the clinical VISA isolate XN108, is sufficient to elevate vancomycin resistance in the otherwise susceptible strain Newman, converting it to a VISA phenotype. Conversely, reverting this mutation in XN108 to the wild-type GraS(I136T) significantly reduced resistance (Figure 1). Notably, the vancomycin MIC of Newman-GraS(T136I) remained lower than that of parental XN108. This finding underscores the polygenic nature of VISA development, wherein multiple mutations collectively fine-tune cell wall homeostasis and surface properties to reduce drug access [25].

The GraSR is a TCS associated with glycopeptide resistance [28]. Primarily, GraSR enhances cell envelope charge through transcriptional control of the *dltABCD* operon and *mprF* [28]. These genes promote the incorporation of D-alanine into teichoic acids and lysine into membrane lipids, respectively, thereby increasing the positive charge of the cell surface [21,22]. Consistent with earlier reports [28,31], we found that GraS(T136I) activates the GraSR regulon, upregulating *graS*, *graR*, *dltB* and *mprF* (Figure 3a). Correspondingly, the XN108 strain exhibited a less negatively charged surface than the GraS(I136T) revertant, as measured by cytochrome C binding (Figure 2d). Since vancomycin is a positively charged glycopeptide, this charge repulsion likely contributes to reduced drug binding and higher MICs. In parallel, GraS(T136I) promoted cell wall thickening and

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diminished autolytic activity (Figure 2A-C), two canonical features of VISA that may further restrict vancomycin access to its target.

Beyond its role in resistance, the GraSR system also modulates *S. aureus* virulence, though the phenotypic consequences of specific *graS* mutations have been less thoroughly explored [20]. Here, we show that GraS(T136I) significantly upregulates the expression of virulence-associated genes (*efb*, *hlb*, *sbi*, *hld*) and enhances hemolytic activity (Figure 3B, 4A). This suggests that the mutation not only aids bacterial survival under antibiotic pressure but may also augment pathogenic potential. Interesting, however, the enhanced virulence profile did not translate into improved intracellular survival in macrophages. Instead, XN108 showed markedly reduced persistence in RAW264.7 cells compared to the GraS(I136T) revertant after 12 h of infection (Figure 4B). These findings imply that while GraS(T136I) upregulates certain toxin genes, it may simultaneously impair adaptations required for long-term intracellular niche establishment, a trade-off that warrants further investigation.

5. Conclusions

In summary, this study demonstrates how a single nucleotide substitution in the GraS sensor kinase can simultaneously alter vancomycin susceptibility and virulence in *S. aureus*. The GraS(T136I) mutation is sufficient to confer a VISA phenotype, characterized by thickened cell wall, reduced autolysis, and decreased net negative surface charge, changes mediated through GraSR-dependent upregulation of *dltABCD* and *mprF*. In parallel, the same mutation enhances the expression of key virulence genes and increases hemolytic activity, indicating a coordinated remodeling of both resistance and pathogenicity programs. These findings illustrate the multifunctional role of GraSR in *S. aureus* adaptation and highlight how point mutations in global regulators can have pleiotropic effects on bacterial fitness, resistance, and virulence. Unraveling such connections may inform new strategies for countering the concurrent evolution of antibiotic resistance and virulence in this formidable pathogen *S. aureus*.

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