

Functional Insights of *MraZ* on the Pathogenicity of *Staphylococcus aureus*

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Introduction: In recent years, multidrug-resistant methicillin-resistant *Staphylococcus aureus* has become increasingly prevalent, which raised a huge challenge to antibiotic treatment of infectious diseases. The anti-virulence strategy targeting virulent factors is a promising novel therapy for *S. aureus* infection. The virulence mechanism of *S. aureus* was needed to explore deeply to develop more targets and improve the effectiveness of anti-virulence strategies.

Results: In this study, we found *mraZ* was highly conserved in *S. aureus*, and its production is homologous with the *MraZ* of *Escherichia coli*, a transcriptional regulator involved in the growth and cell division of *E. coli*. To investigate the function of *mraZ* in *S. aureus*, we constructed a MW2 *mraZ* deletion mutant and its complementary mutant for virulence comparison. Although no remarkable influence on the growth, the *mraZ* deletion mutant led to significantly reduced resistance to human neutrophils and decreased virulence in *Galleria mellonella* model as well as mouse skin and soft tissue infection models, indicating its essential contribution to virulence and immune evasion to support the pathogenicity of *S. aureus* infection. RNA-Seq and quantitative RT-qPCR revealed that *MraZ* is a multi-functional regulator; it involves in diverse biological processes and can up-regulate the expression of various virulence genes by *agr* and *sarA*.

Conclusion: *mraZ* plays vital roles in the pathogenicity of *S. aureus* via regulating many virulence genes. It may be an attractive target for anti-virulence therapy of *S. aureus*.

Keywords: *Staphylococcus aureus*, virulence, *mraZ*, *agr*, *sarA*

Introduction

Staphylococcus aureus is a common opportunistic pathogen that usually colonizes in human skin and nasal mucosa, which can cause a variety of infections, ranging from mild skin lesion to severe invasive diseases such as endocarditis, osteomyelitis, pneumonia, and even life-threatening septic shock.¹⁻³ Moreover, with the emergence of multidrug-resistant methicillin-resistant *S. aureus* (MRSA) strains in recent years, *S. aureus* infections rise a serious concern in public health. A report from Centers for Disease Control and Prevention from USA showed that the mortality rate caused by MRSA infection was 2.88 per 100 000 population per year, which leads to approximately 100 trillion dollars in damage to the global economy.⁴ It is obvious that the problem of antibiotic resistance in bacteria has reached the crisis stage.

A key characteristic in pathogenicity of *S. aureus* that promote its infection is the production of a large array of virulence factors, such as hemolysins, pore-forming leukocidins, enterotoxin, and plasma-coagulase,⁵⁻⁸ which allow them to resist

clearance by the host, to invade and gain access to deeper tissues, and to damage host cells. Currently, most treatments of infectious diseases in clinical are based on the ability of antibacterial agents to kill pathogens quickly. However, the harmful effects of bacterial virulence factors are almost inevitable. Furthermore, treatment of infections caused by MRSA strains has become more difficult because of the emergence of multidrug-resistant strains. As conventional antibiotics become less valid, the anti-virulence strategies are drawing increasing attention. One of the advantages of the anti-virulence strategies is that it can specifically block the virulence expression of bacteria without killing or inhibiting the growth of bacteria pathogens, and avoid the selective pressure from antibiotics treatment, which will be less likely lead to the emergence of drug resistance.^{9,10} Therefore, the investigation of more unidentified virulence factors of *Staphylococcus aureus* and its pathogenic mechanism can provide more directions for the anti-virulence strategies of *Staphylococcus aureus*.

The *mraZ* gene is highly conserved in many bacterial species. In some Gram-positive bacteria, *mraZ* is associated with the cell division and cell wall biosynthesis, which plays a vital role in the growth of bacteria.^{11–13} A previous study demonstrated that the overproduction of *Escherichia coli* *MraZ* could inhibit cell division and was lethal in rich medium at high induction levels and in minimal medium at low induction levels.¹¹ Notably, the *MraZ* from *E. coli* and *S. aureus* shares 36% sequence identity. However, limited studies have evaluated the role of *mraZ* gene in *S. aureus*. The biological functions of the *mraZ* gene in *S. aureus* still require further investigation.

In this study, to better understand the function of *MraZ* in *S. aureus*, we constructed the MW2 *mraZ* deletion mutant and its complementary mutant, and investigated the effects of *MraZ* on the bacterial growth, the virulence and immune evasion capacity. We found that *MraZ* is a multi-functional regulator, especially plays a crucial part in *S. aureus* virulence and immune evasion by regulating the expression of extensive virulence genes.

Materials and Methods

Bacterial Strains and Culture Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Given MW2 is a typical community-acquired MRSA isolate with enhanced virulence, and its genetic background has been well established, it was used for the construction of *mraZ* knockout and complementation. *E. coli*

Table 1 The Bacterial Strains and Plasmids Used in the Present Study

Bacterial Strains and Plasmids	Description	Source
Strains		
<i>S. aureus</i> MW2	CA-MRSA strain	Laboratory stock ^a
$\Delta mraZ$	<i>mraZ</i> deletion mutant using MW2 as parental strain	This study
$\Delta mraZ$ -C	$\Delta mraZ$ mutant complemented with plasmid pRB473 harboring <i>mraZ</i> gene	This study
<i>E. coli</i> DH5 α	Clone host strain	Laboratory stock ^a
<i>E. coli</i> DC10B	<i>dam</i> ⁺ <i>Δdcm</i> ⁻ <i>ΔhsdRMS</i> <i>endA1</i> <i>recA1</i> ; clone host strain	Laboratory stock ^a
Plasmids		
pKOR1	Temperature-sensitive <i>E. coli</i> (Amp ^r) <i>Staphylococcus</i> shuttle (Cm ^r) vector	Laboratory stock ^a
pKOR1- $\Delta mraZ$	Recombinant plasmid	This study
pRB473	Shuttle plamid (Cm ^r)	Laboratory stock ^a
pRB473- <i>mraZ</i>	Shuttle plamid (Cm ^r), <i>mraZ</i> gene with its own promoter was cloned into plasmid pRB473	This study

Note: ^aAll these strains were stored in The First Affiliated Hospital of Wenzhou Medical University.

DH5 α and *E. coli* DC10B was used for staphylococcal cloning host. *E. coli* strains were cultured in Luria-Bertani (LB) medium, and *Staphylococcus* strain were grown at tryptic soy broth (TSB) medium. All strains were shaken in an incubator at 37 °C, ~220 rpm (adjusted appropriately). The medium was supplemented with an appropriate antibiotic (ampicillin 100 mg/L, chloramphenicol 10 mg/L and anhydrous tetracycline 50 μg/L) as needed.

Construction of *mraZ* Knockout Mutant and Complemented Strain

A knockout mutant of *mraZ* was constructed using the temperature-sensitive plasmid pKOR1 based on the homologous recombination.¹⁴ Briefly, the upstream and downstream fragments of *mraZ* were amplified, linked

by the T4 enzyme, and ligated to the vector pKOR1, resulting in recombinant pKOR1- $\Delta mraZ$. This recombinant plasmid was consecutively transferred into *Escherichia coli* DH5 α for amplification, into DC10B for bypass the *S. aureus* restriction barriers, and then electrotransferred into MW2. Under the dual pressure of high temperature and antibiotics, $\Delta mraZ$ was generated by the homologous recombination of the upstream and downstream homologous arm in the pKOR1- $\Delta mraZ$ with the MW2 genome, as previously described.¹⁴ The deleted mutant $\Delta mraZ$ was verified by PCR with DNA sequencing and quantitative reverse transcription-PCR (qRT-PCR).

To further verify whether the biological alteration of the deletion mutant was caused by the absence of *mraZ*, we constructed the complementary mutant strain $\Delta mraZ$ -C using the shuttle plasmid pRB473. Briefly, the *marZ* with its promoter regions was amplified and inserted into pRB473. The pRB473-*mraZ* was transferred into $\Delta mraZ$ by electroporation. The primers used above are listed in Table 2.

RNA Extraction and qRT-PCR

Total RNA of *S. aureus* was extracted using RNA extraction kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. Briefly, overnight cultures were diluted 1:200 into fresh TSB and incubated at 37°C. After 9 h, the prepared lysostaphin and lysozyme solution were mixed with bacterium suspension for disrupt the cell walls, and then RNA extracted was followed the kit instructions. A PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA)

was used to get purified RNA, and then cDNA was obtained using a PrimeScript RT reagent kit (TaKaRa, Japan). The housekeeping gene *gyrB* was used as an internal reference gene, and relative quantification of Expression levels were calculated by the $2^{-\Delta\Delta C_t}$ method.¹⁵ The primer pairs used in qRT-PCR are listed in Table 3.

Growth Assay

S. aureus strains were grown to stationary phase (12h) and then diluted (1:200) in TSB medium. All the cultures were incubated at 37°C with shaking at 220rpm. The OD600 value was measured hourly for 24h for bacterial growth curves. Meanwhile, the cells were plated in serial dilutions on TSB agar at 1h intervals for 24h, and the colony-forming units (CFU) were counted for viable bacterial count at indicated time points. The assay was performed in duplicate.

Analysis of Pigments Production

To evaluate pigment production, bacteria were incubated into 3mL TSB medium at 37°C with shaking. After 24h, the cultures were centrifuged at 8000 rpm for 10 min, and pigment production was assessed visually by the color of centrifuged precipitation.

Hemolytic Capacity

The hemolytic capacity of *S. aureus* isolates was evaluated based on the zone of clearing around colonies plated on sheep blood agar plates. Briefly, *S. aureus* strains were cultivated to stationary phase (12h), the cells were collected, washed twice with PBS, and adjusted to OD600=1;

Table 2 Primers Used for Construction of $\Delta mraZ$ and $\Delta mraZ$ -C

Primer	Primer Sequence (5'-3')	Underlined
<i>mraZ</i> -F	ATGTTTCATGGGAGAATACGATCATC	
<i>mraZ</i> -R	TAAAAATCAAATCTATTAATCTT	
<i>mraZ</i> UP-F	GGGGACCACTTTGTACAAGAAAGCTGGGT GCTTGAATGCGATGAT	attB2
<i>mraZ</i> UP-R	GGAATTCACCTCACCTTATATATAATTACC	EcoRI
<i>mraZ</i> DOWN-F	GGAATTCGGGAGGAATTGAAGTGTTCATCATA	EcoRI
<i>mraZ</i> DOWN-R	GGGGACAAGTTTGTACAAAAAAGCAGGCT GCTTCGTGCTCT	attB1
<i>mraZ</i> HF-F	CGGAATTCGAATGGGAGGATTACAAGA	ECORI
<i>mraZ</i> HF-R	GGGGTACCCCCACCTAGCGTACAGTCA	KPNI

Table 3 Primers Used in qRT-PCR

Primer	Primer Sequence (5'-3')
<i>gyrB</i> -RT-F	ACATTACAGCAGCGTATTAG
<i>gyrB</i> -RT-R	CTCATAGTGATAGGAGTCTTCT
<i>mraZ</i> -RT-F	TGATTATACCGTCCAAGT
<i>mraZ</i> -RT-R	CACCAGAGAAGAACATAC
<i>sarA</i> -RT-F	AAACCCTGAATTTGAATG
<i>sarA</i> -RT-R	GATATTACATCTGCTCCT
<i>agrA</i> -RT-F	TCCAGCAGAATTAAGAACTCG
<i>agrA</i> -RT-R	ATATCATCATATTGAACATACACT
<i>RNAlII</i> -RT-F	CATGGTTATTAAGTTGGGATGGC
<i>RNAlII</i> -RT-R	GAAGGAGTGATTTCAATGGCACA
<i>hld</i> -RT-F	GAAGGAGTGATTTCAATG
<i>hld</i> -RT-R	TGTCGATAATCCATTTTAC
<i>sec4</i> -RT-F	TCAGCAACTAAAGTTAAG
<i>sec4</i> -TR-R	GCAGTTTACATAGTAATTTG

then 10ul each culture suspension was spotted on sheep blood agar plates. The zones of hemolysis (diameter) were measured after 24h of 37°C incubation. The experiments were conducted in triplicate.

Neutrophil Killing Assays

Neutrophils with >95% purity and viability were isolated from the venous blood of healthy volunteers using the Human Neutrophil Isolation Kit (Haoyang, Tianjin, China). In brief, *S. aureus* isolates were grown to mid-logarithmic growth phase, collected, washed, and resuspended in PBS. After the opsonization of 5% normal human serum, 1.0×10^5 CFU bacterial suspension were mixed with 1.0×10^6 neutrophils in RPMI 1640 medium at 37°C with gentle rotation. Samples were taken in 1h, 2h, and 3h for CFU enumeration. 0.25% TritonX-100 was added to each sample to ensure lysis of phagosomes before diluting and plating on TSB agar. The experiments were done in triplicate.

Galleria mellonella Infection Model

As previously described, larvae of *G. mellonella* weighing 250–350mg (Tianjin huiyude Biotech, Tianjin, China) were stored in the dark at 10°C and used within a week of delivery. Overnight cultures of *S. aureus* were collected,

washed, and resuspended in PBS. *G. mellonella* was injected with 3.0×10^6 CFU/larvae, via the last right proleg using Hamilton syringe. Each experimental group contained 10 larvae. Larvae with injected sterile PBS and uninjected larvae as control. The treated larvae were placed in clean, sterile plastic petri dish and incubated at 37°C. The survival rate was monitored every 12h for 3 days. The results were analyzed by GraphPad Prism 6 software. The experiment was repeated three times with similar results.

Mouse Model of Skin Abscess

The mouse skin infection model was performed as described previously. Female BALB/C nude mice aged 4 to 6 weeks old and weighing about 22g were used for the experiments. In brief, all mice were administered with sterile feed, pure water one week before the time of use to adapt to the experimental environment. *S. aureus* strains were grown to mid-logarithmic phase as described above. The mice were injected with 100μL PBS containing 1.0×10^7 CFU *S. aureus*, and mice injected with 100μL PBS as control. Each experimental group contained 10 mice. Abscess formations were observed and recorded daily for 7 days. The length (L) and width (W) were measured to calculate the size of the abscesses follow the formula: $A = \pi (L \times W) / 2$. All animals were sacrificed 7 days post infection.

Cytokine Expression in Bacteremia Mouse Model

BALB/C nude mice were immunodeficient animals with reduced cellular immunity and humoral immune function, which might affect the production of inflammatory cytokines. Therefore, BALB/C female mice with complete immunity capacity aged 4 to 6 weeks old were used for the bacteremia model and were tested for the expression levels of cytokine. Briefly, mice were randomly divided into four groups, and each group contains 10 mice. The mice were infected by intraperitoneal injection with 100ul PBS containing 1.0×10^7 CFU *S. aureus*, and mice injected with 100μL PBS as control. They were raised under the same conditions for 3L days, and then 7 mice were randomly selected for further analysis (exclude the death mice). Blood samples were obtained, and the level of cytokines in the sera of each mouse was detected by ELISA method.

RNA-Sequencing and Data Analysis

S. aureus strains were inoculated for 9h in TSB medium at 37°C, and total RNA was extracted as above. Qualitative RNA samples were analyzed by RNA-seq (transcriptome sequencing) using an Illumina HiSeq X platform and the pe150 (150 bp double-stranded assay) strategy. Differential expression analysis was performed by DEG-seq software on the RNA sequencing reads. Gene was considered significantly differentially regulated with \log_2 (fold change) > 1 and $P < 0.005$.

Statistical Analysis

Statistical comparisons were performed using SPSS (version 16.0) and GraphPad Prism 8. Two groups comparison was analyzed using unpaired *t*-test. Three or more groups comparison was analyzed using one-way or two-way ANOVA. The survival curve was analyzed by the Log rank test (Mantel-Cox). The threshold for statistical significance adopted in all analyzes was $P < 0.05$.

Ethics Statement

All procedures involving human participants were performed in accordance with the ethical standards of First Affiliated Hospital of Wenzhou Medical University. All volunteers gave written informed consent in accordance with the Declaration of Helsinki prior to donating blood. All animal assays were carried out in accordance with The Regulation on the Management of Laboratory Animals for the welfare of the laboratory animals, and the protocol was approved by the Institutional Animal Care and Ethics Committee at First Affiliated Hospital of Wenzhou Medical University.

Results

Construction of *mraZ* Deletion Mutant Strains

In the genome of *S. aureus* MW2 (GeneBank accession number: GCF_000011265.1), *mraZ* is a 432bp gene encoding a 143 amino acid protein with contained SpoVT-AbrB DNA-binding domains, and it is located downstream of the putative cysteine ligase encoding gene *bshC* and upstream of the 16S rRNA methyltransferases encoding gene *rsmH*.

The temperature-sensitive shuttle plasmid pKOR1 was used to generate the $\Delta mraZ$ mutant in the MW2 strain. The complementary strain of $\Delta mraZ$ mutant named $\Delta mraZ-C$ was constructed using the vectors pRB473. All mutants were verified by PCR with sequencing and qRT-PCR. As

shown in Figure 1, the $\Delta mraZ$ mutant and $\Delta mraZ-C$ were successfully constructed.

MraZ Has No Effect on the Growth and Pigment Production, While Enhances the Hemolysis Capacity of *S. aureus*

In order to investigate the potential impact of the *mraZ* deletion on *S. aureus* growth rate, the growth curves and the viable bacterial counts of strains MW2, $\Delta mraZ$ and $\Delta mraZ-C$ were determined. Compared with MW2 and $\Delta mraZ-C$, no substantial differences in the growth curve or the viable bacterial count were found in $\Delta mraZ$, indicating that the absence of *mraZ* gene did not affect the growth of *S. aureus* (Figure 2A and B). We also found that there is no difference in pigment production between $\Delta mraZ$ and MW2 or $\Delta mraZ-C$ (Figure 2C). We further examined the hemolysis ability of the bacteria, one of the most important virulence phenotypes of *S. aureus*, and found that the hemolytic capability of $\Delta mraZ$ was significantly reduced (Figure 2D).

MraZ Makes a Significant Contribution to Virulence of *S. aureus* in the *G. mellonella* Infection Model

To investigate whether the absence of *mraZ* can lead to less virulence, we first compared the survival rates of the wide-type strain MW2, $\Delta mraZ$ deletion mutant and $\Delta mraZ-C$ in *G. mellonella* infection model. As shown in Figure 3, the survival rate of *G. mellonella* infected with $\Delta mraZ$ deletion mutant was significantly higher than the

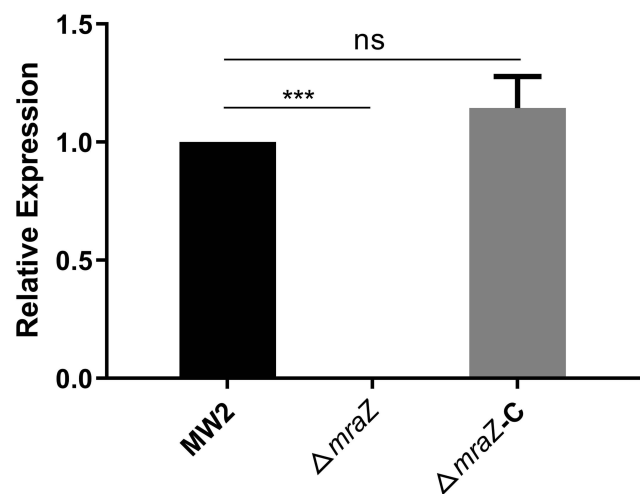


Figure 1 Validation of the *mraZ* expression level of the knockout strain and complementary strain. The *mraZ* expression level of the wild strain used as a control, was regarded as 1. *** $p < 0.001$; ns, $p \geq 0.05$.

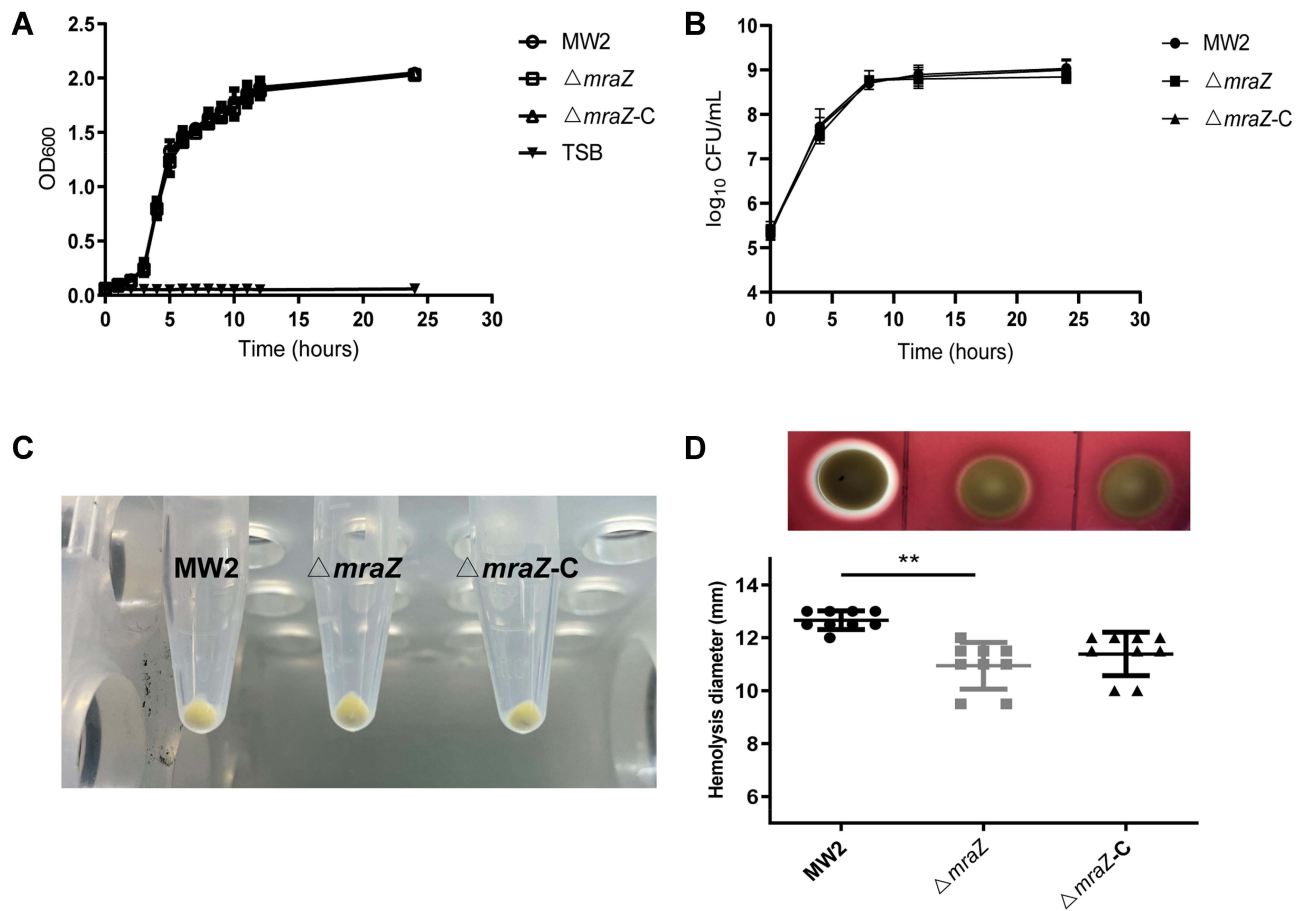


Figure 2 The role of MrzA in *S. aureus* MW2, $\Delta mraZ$ and $\Delta mraZ-C$ phenotypes. **(A)** The growth curve by measuring the OD₆₀₀. **(B)** Viable bacteria count by measuring CFU on TSB agar plates. **(C)** Production of pigments by MW2, $\Delta mraZ$ and $\Delta mraZ-C$. **(D)** Analysis of the hemolysis capacity by the zone of Hemolysis on sheep blood agar plates. ** $p < 0.01$.

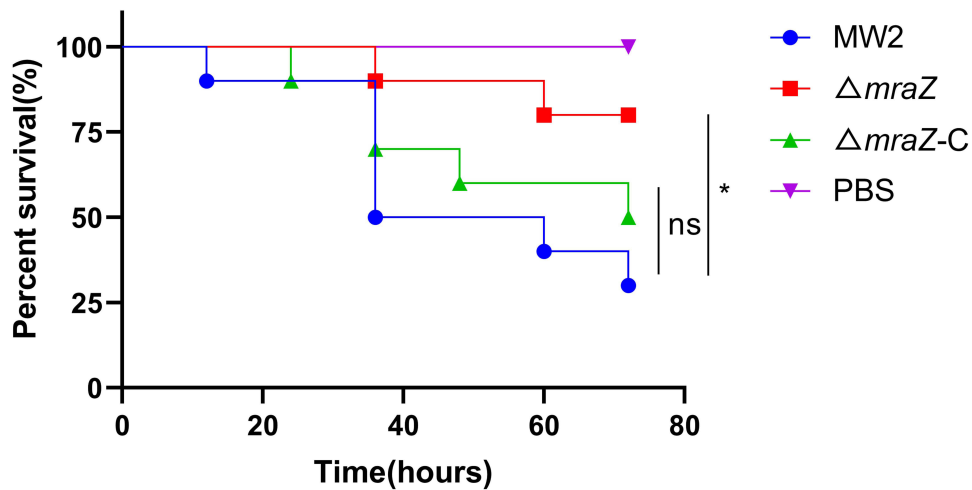


Figure 3 *Galleria mellonella* infection model. The survival rates of the *G. mellonella* after infection with MW2, $\Delta mraZ$ and $\Delta mraZ-C$ were expressed by the Kaplan-Meier survival plot. * $p < 0.05$; ns $p \geq 0.05$.

wide-type strain, while the virulence of $\Delta mraZ$ -C was restored to the wild-type level.

MraZ of *S. aureus* Promotes the Formation of Skin Abscesses in Mouse

To further verify the role of *mraZ* in the invasiveness and acute virulence potential of *S. aureus*, we established mouse models of skin abscess infection against MW2, $\Delta mraZ$ and $\Delta mraZ$ -C and compared their ability to cause pathological lesions or abscesses. As shown in Figure 4, all the mice infected with these three strains were found skin lesion; however, the abscess area of the $\Delta mraZ$ was significantly smaller than the MW2, indicating that MraZ plays a considerable role in the process of abscess formation.

MraZ of *S. aureus* Helps Evade the Elimination of Human Neutrophils

To determine the role of *mraZ* in *S. aureus* immune evasion, we evaluated the survival rates of the wide-type strain MW2, $\Delta mraZ$ and $\Delta mraZ$ -C in human neutrophils. As shown in Figure 5, the survival rate of $\Delta mraZ$ was lower than the parent strain after 1 h of incubation ($p < 0.05$). After incubated for 2h, a significant reduction of the

survival rate of $\Delta mraZ$ deletion mutant was found compared with the wide-type strain MW2. It indicated that the presence of *mraZ* gene could help resist clearance by the host innate immune system, thereby likely contributing to the promotion of virulence.

The *mraZ* Deletion Mutant Has Lower Pro-Inflammatory Cytokine Expression Levels in Mouse Bacteremia Model

Cytokines play major roles in the innate immune system by the interaction of pro-inflammatory cytokines such as TNF- α and IL-6 and anti-inflammatory cytokines such as IL-10 and IL-4. Therefore, we injected the bacteria into abdominal cavity of mice and determined the cytokines levels of MW2, $\Delta mraZ$ and $\Delta mraZ$ -C to evaluate the role of *mraZ* in the inflammatory response in *S. aureus* infection. As shown in Figure 6, the levels of TNF- α and IL-6 in $\Delta mraZ$ were both significantly lower than the other two strains ($p < 0.01$), while IL-4 and IL-10 have no significant difference in these strains, suggesting that the presence of *mraZ* can stimulate the production of pro-inflammatory cytokines and promote the inflammatory response to *S. aureus*.

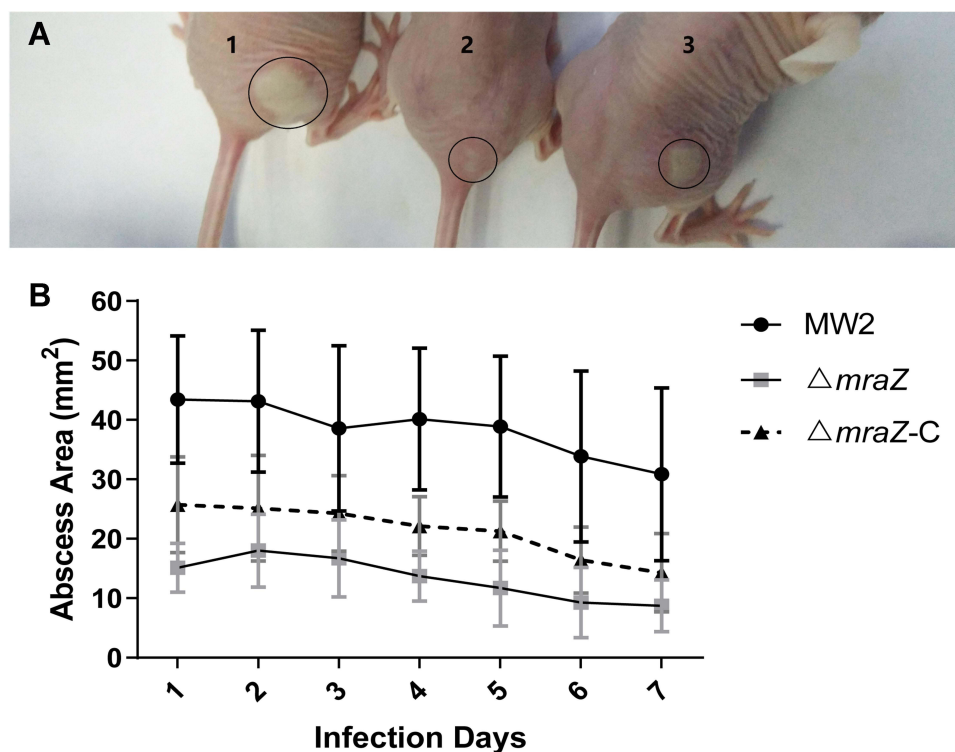


Figure 4 Comparison of infected area of mouse skin abscess. (A) The picture of representative abscesses on days 2 after infection. The No. 1 indicated an abscess after infection with a wild-type MW2 strain, No. 2 was a $\Delta mraZ$ strain, and No. 3 was a $\Delta mraZ$ -C strain. (B) A plot of the area changes of abscesses during skin infections in mice every day.

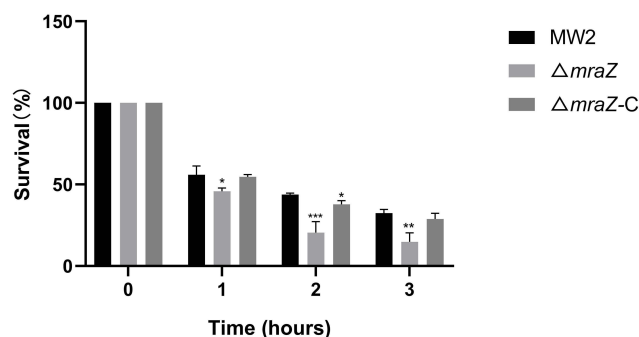


Figure 5 Survival rates of *S. aureus* MW2, $\Delta mraZ$ and $\Delta mraZ-C$ after incubated with human neutrophils. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Transcriptome Comparison of MW2 and $\Delta mraZ$

With the involvement of the *marZ* gene in virulence and immune evasion of *S. aureus*, we speculated *MraZ* might involve in regulating the expression of various genes. Therefore, we performed RNA-Seq analysis on MW2 and MW2 $\Delta mraZ$. Compared with the wild strain of MW2, there were 335 genes in the $\Delta mraZ$ mutant strain with differential expression ≥ 2 -fold, of which 187 were up-regulated and 148 were down-regulated (Figure 7A). Furthermore, The Go enrichment analysis revealed that the differentially expressed genes (DEGs) are related to multiple functions, mainly the decomposition and anabolism of multiple substances, DNA replication and repair, gene expression regulation, ribosome synthesis, material transport, cell division and infection (Figure 7B). Therefore, *MraZ* can act as a multi-functional regulator and affect the expression of various genes.

In the *mraZ* deletion mutant, the transcription levels of the two-component system *agr* was down-regulated 8.51-fold ($2^{3.09}$ -fold). The expression of *dlt*, an operon mediates d-alanine incorporation into teichoic acid and

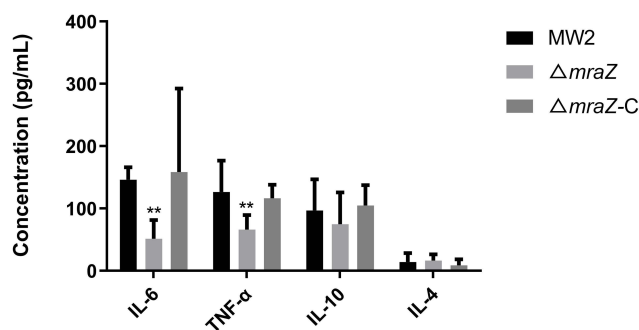


Figure 6 Comparison of Cytokine expression levels in mice after infection with MW2, $\Delta mraZ$ and $\Delta mraZ-C$, respectively. ** $p < 0.01$.

a determinant of defensin resistance that is essential for resistance to human neutrophil killing and virulence in mice,¹⁶ was down-regulated 2.58-fold ($2^{1.37}$ -fold) in MW2 $\Delta mraZ$. In addition, the transcription of *clfA* encoding fibrinogen-binding MSCRAMM clumping factors A was significantly down-regulated 2.16-fold ($2^{1.11}$ fold), which might associated with the decreased virulence of *S. aureus*¹⁷ (Table 4). Although the expression of Staphylokinase encoding gene *sak*, superantigen-like protein encoding gene *ssl3* were up-regulated, the transcription of the beta-class phenol-soluble modulins (PSM) encoding gene *psm β* , alpha family PSM encoding gene *psma 3* and *hld*, Alpha-hemolysin encoding gene *hly/hla*, and enterotoxin type C encoding gene *sec4* were down-regulated 61.82-fold ($2^{5.95}$ fold), 50.21-fold ($2^{5.65}$ fold), 33.13-fold ($2^{5.05}$ fold), 2.13-fold ($2^{1.09}$ fold) and 3.12-fold ($2^{1.64}$ fold), respectively (Table 4). These data suggested that the decreased virulence and compromised immune phenotype resulted from the absence of *mraZ* was associated with the down-regulated expression of *agr*, *dlt*, *clfA*, *psma*, *psm β* , *hly/hla*, *hld* and *sec4*.

The Transcription Levels of Well-Established Virulence Factors and Regulatory Genes in the MW2, $\Delta mraZ$ and $\Delta mraZ-C$

According to the absence of *mraZ* contributed to the virulence and immune evasion phenotype, we next measured the expression levels of several well-established virulence factors and regulator genes in detail in the MW2, $\Delta mraZ$ and $\Delta mraZ-C$ by qRT-PCR. As shown in Figure 8, the expression of *hld* and *sec4* were significantly down-regulated in $\Delta mraZ$ strain ($p < 0.05$); hemolysin and enterotoxin encoded by these two genes were the topical exotoxins contributed to the virulence of *S. aureus*, confirming that the *mraZ* affected the expression of virulence factors such as hemolysin or enterotoxin in *S. aureus*. The *agr* system consists of two adjacent transcripts RNA II and RNA III. RNA III can drive expression of most *agr* dependent virulence factors, such as the Alpha-hemolysin and delta-hemolysin, and *agrA* can promote the transcription of PSM genes.¹⁸ In agreement with the transcriptome data, the expression level of the *agrA* and RNAIII in $\Delta mraZ$ were significantly lower than the MW2 strain ($p < 0.05$). Furthermore, we also investigated the expression of the major *S. aureus* virulence regulatory locus *sarA* under the same conditions. We found that the expression

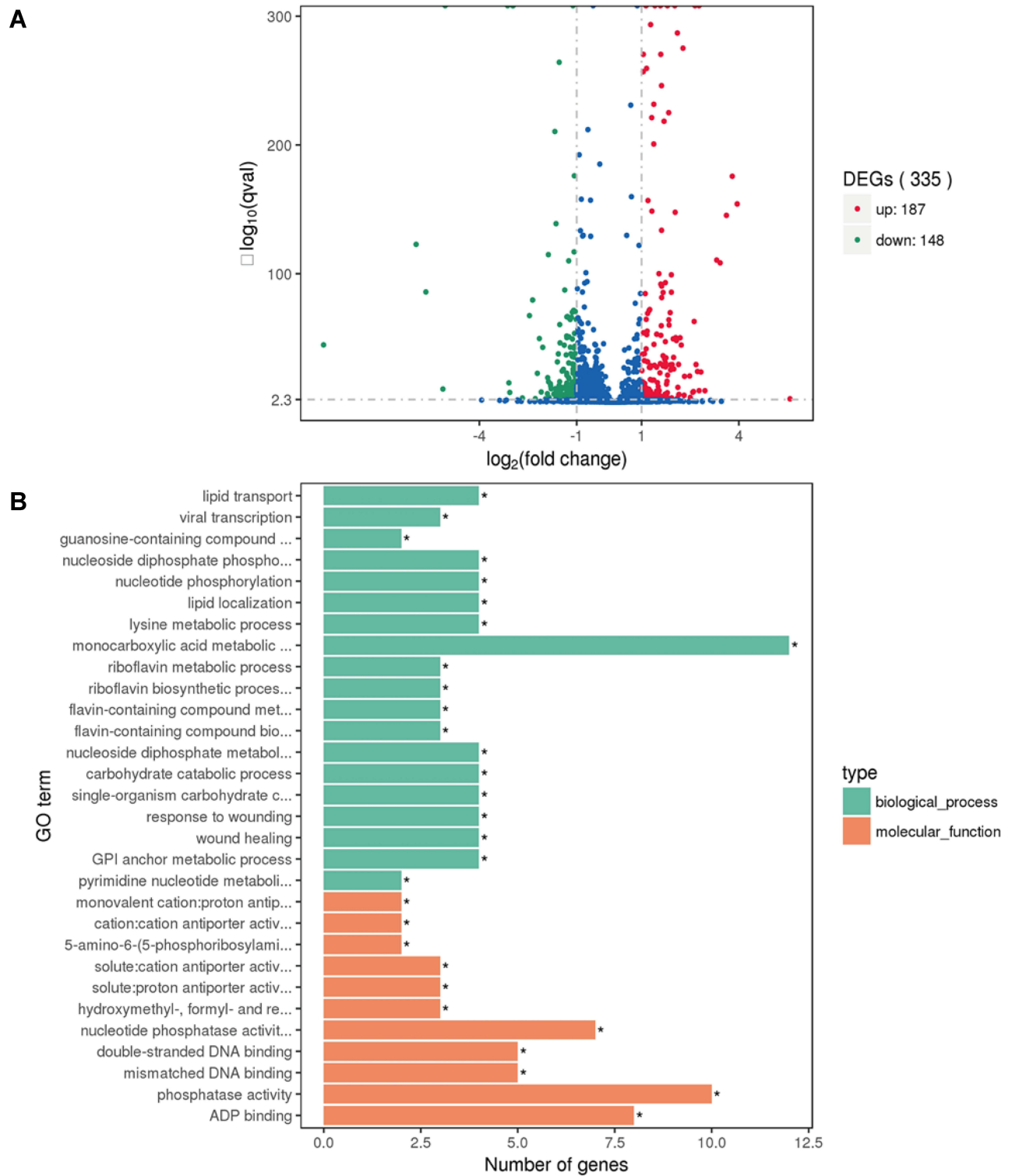


Figure 7 (A) Volcano plot of differences in gene expression between $\Delta mraZ$ and MW2. The abscissa refers to the fold-change in the two samples; the ordinate refers to the statistically significant difference in gene expression; red dots indicate a significant difference in up-regulated genes and green dots indicate down-regulated genes. A gene with $|\log_2(\text{Fold Change})| > 1$ and $q \text{ value} < 0.005$ between MW2 and $\Delta mraZ$ was considered to be significantly differentially expressed. **(B)** $\Delta mraZ$ vs MW2 differential gene GO enrichment histogram. The ordinate is the enriched GO term, and the abscissa is the number of differential genes in the term. Different colors are used to distinguish biological process and molecular function, with *For enriched GO term.

Table 4 Gene Expression Changes Associated with Virulence or Immune Evasion

Gene_ID	log ₂ Fold	p value	q value	Description
Two-component system				
MW_RS03395	-1.1112	6.42E-26	2.81E-25	Bacitracin ABC transporter permease VraG
MW_RS10660	-2.9599	0	0	Accessory gene regulator AgrB
MW_RS10665	-3.0897	1.94E-16	6.07E-16	Accessory gene regulator AgrD
MW_RS03390	-1.3327	5.76E-14	1.60E-13	ABC transporter ATP-binding protein VraF
MW_RS10675	-2.9722	0	0	Accessory gene regulator AgrA
Staphylococcus aureus infection				
MW_RS03395	-1.1112	6.42E-26	2.81E-25	Bacitracin ABC transporter permease VraG
MW_RS04415	-1.3686	3.27E-89	4.98E-88	D-alanyl-lipoteichoic acid biosynthesis protein DltB
MW_RS04425	-1.1376	6.26E-72	7.81E-71	D-alanyl-lipoteichoic acid biosynthesis protein DltD
MW_RS04410	-1.2454	5.98E-112	1.09E-110	D-alanine-poly(phosphoribitol) ligase subunit DltA
MW_RS03390	-1.3327	5.76E-14	1.60E-13	ABC transporter ATP-binding protein VraF
MW_RS04155	-1.1143	0	0	MSCRAMM family adhesin clumping factor ClfA
MW_RS04125	-1.6362	5.94E-141	1.38E-139	Staphylococcal enterotoxin type C SeC4
MW_RS10280	1.7703	1.06E-06	1.68E-06	Staphylokinase Sak
Cell killing				
MW_RS05625	-1.0928	2.14E-73	2.77E-72	Alpha-hemolysin
MW_RS10655	-5.0549	0	0	Delta-hemolysin
Toxin biosynthetic process				
MW_RS02670	-1.0167	3.37E-19	1.20E-18	PIN/TRAM domain-containing protein
Pathogenesis				
MW_RS10280	1.7703	1.06E-06	1.68E-06	Staphylokinase Sak
MW_RS05645	1.8343	0.0022839	0.0024122	Superantigen-like protein SSL13
MW_RS04125	-1.6362	5.94E-141	1.38E-139	Staphylococcal enterotoxin type C2 SeC4
MW_RS05690	-5.9501	7.81E-125	1.57E-123	Beta-class phenol-soluble modulins beta1
MW_RS10660	-2.9599	0	0	Accessory gene regulator AgrB
MW_RS05695	-5.1239	1.69E-11	3.99E-11	Beta-class phenol-soluble modulins beta2
MW_RS14485	-5.6492	1.02E-87	1.53E-86	Alpha-3 family phenol-soluble modulin
Cell adhesion				
MW_RS13825	-2.2759	0.0010466	0.0011656	Hypothetical protein
MW_RS04155	-1.1143	0	0	MSCRAMM family adhesin clumping factor ClfA

level of *sarA* in $\Delta mraZ$ also significantly decreased ($p < 0.05$). Additionally, except *sec4*, the expression of most above measured virulence genes and regulatory genes in the MW2 $\Delta mraZ$ -C were approximately recovered to the wild-type strain MW2 (Figure 8). Therefore, it is likely that the *mraZ* can activate the expression of the virulence factors by up regulating several key regulatory genes such as *agr* and *sarA*.

Discussion

In recent years, the anti-virulence strategies targeting multiple virulence factors of *S. aureus* have been proved to be an effective alternatives to antibacterial drugs in the treatment of bacterial infection; it is reported that clonal antibody against α -hemolysin could serve as an effective target for monoprophylaxis or adjuvant therapy for *S. aureus* pneumoniae;¹⁹ Diapophytoene desaturase CrtN,

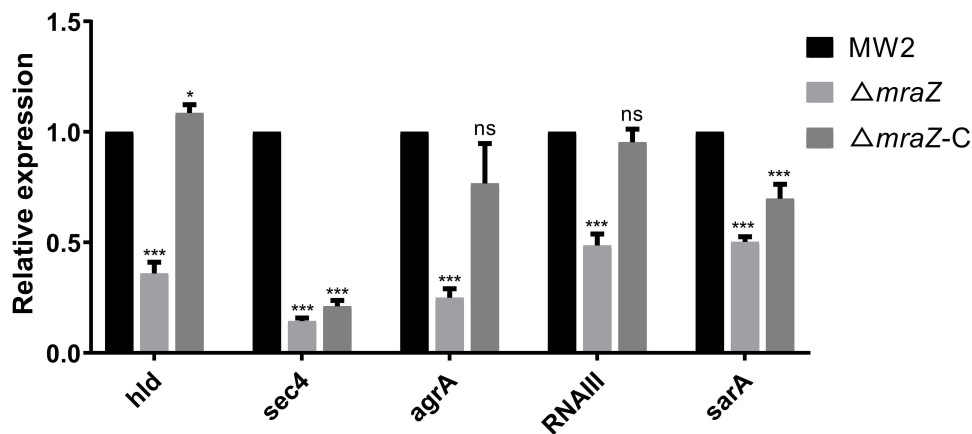


Figure 8 Relative transcription of various genes in MW2, $\Delta mraZ$ and $\Delta mraZ-C$ detected by RT-qPCR. Data are mean \pm SD of triplicate experiments and analyzed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test compared with the wide-type strain MW2. * $p < 0.05$; *** $p < 0.001$.

a key catalytic enzyme of the golden pigment biosynthesis pathway, can also effectively block the virulence of bacteria by targeting drugs.²⁰ However, *S. aureus* strains usually contain various virulence genes and display heterogeneous virulence profiles. In this study, for the first time, we revealed that MraZ highly conserved in *S. aureus* is a multi-functional regulator, and elucidated that it plays an essential role in the virulence and the innate host defense of *S. aureus*. Our research suggested a promising target for anti-virulence strategy for *S. aureus* infection.

Studies involving *mraZ* in *E. coli*, *Corynebacterium glutamicum* and other Gram-negative bacteria have described its position at the head of the division cell wall (*dcw*) operon (*mraZ-mraW-ftsL-ftsI-murE-murF-mraY-murD-ftsW-murG-murC-ftsQ-ftsA-ftsZ-envA*), which responsible for cell division and cell wall synthesis. MraZ can as regulator to control the expression of the first 11 genes of *dcw* operon and excessive production of MraZ inhibits cell division in *E. coli*.¹¹⁻¹³ We found *mraZ* was highly conserved in *S. aureus*, while it is located downstream of the *bshC* gene and upstream of the *rsmH* gene, which is different from other reported Gram-negative bacteria. Unsurprisingly, in our study, the MraZ in *S. aureus* have no effect on the bacterial growth, indicating the species-specific functions of MraZ homologs in bacteria.

We found that the MraZ protein of *S. aureus* MW2 contains SpoVT-AbrB DNA-binding domains, which existed in some toxin-antitoxin system of bacteria.^{21,22} Moreover, the downstream gene *rsmH* was proved to be related to the virulence of *S. aureus* in the silkworm infection model.²³ Therefore, we assumed the *mraZ*

might related to the *S. aureus* pathogenicity. We first found the hemolysis capability of the *mraZ* deletion mutant was significantly reduced, which may weaken the ability to induce acute infections such as abscess and pneumoniae.²⁴ We evaluated the killing ability of the wide-type strain and the mutants in the *G. mellonella* infection model, and we found the *mraZ* deletion mutant was less virulent. Given that *S. aureus* often cause skin and soft tissue infections (SSTIs) in human, mouse skin and soft tissue infection model was performed. Our results indicated that MraZ play an essential role in SSTIs and invasion infections. Moreover, the observed impact of *mraZ* on neutrophil killing revealed that MraZ contributed to immune evasion, which help explain how MraZ contribute to acute infection. Inasmuch as the success of a *S. aureus* infection depends on the effective evasion of host defenses and neutrophils represent the cornerstone of human innate defenses.^{25,26} The golden pigment of *S. aureus* serves as an antioxidant and is protective in killing by neutrophils, while our results indicating that the immune evasion mediated by MraZ was not associated this mechanism.²⁶ Furthermore, our results of the cytokine TNF- α and IL-6 level from mouse bacteremia model were also verified the effect of *mraZ* on the immune defense. These findings indicated that MraZ might was a virulence factor of *S. aureus*, and play an important role on the virulence and immune evasion in *S. aureus* infections.

Our RNA-seq analysis suggested that MarZ was a regulator of expression of extensive genes and many of which were related to *S. aureus* infection and immune evasions. It is evident that the initiates and maintains of

S. aureus infection involves an astounding array of virulence determinants.²⁵ The MSCRAMM surface proteins A (*clfA*) that mediates attachment and invasion of tissue cells was decreased in $\Delta mraZ$.¹⁷ The α -hemolysin is an important virulence factor in *S. aureus* infections; it can cause pore formation in a series of target cells and epithelial and endothelial breach to help systemic infection.²⁴ We observed that the expression of α -hemolysin were decreased in $\Delta mraZ$. Additionally, PSMs and δ -hemolysin were also markedly decreased in $\Delta mraZ$; these cytolytic toxins can drive attack and eliminate immune cells such as neutrophils and promote immune evasion. Moreover, after *S. aureus* have been managed to ingest by neutrophils, it has reported have several mechanisms providing resistance to antimicrobial peptides (AMPs) with positively charge.¹ Among them, the *dlt* operon can mediate the introduction of positive charge, increase the net charge of the cell surface, and inhibit neutrophil killing.^{16,27} Consistently, our data revealed that the deletion of *mraZ* can significantly reduce the expression of *dlt*. These findings confirmed that *mraZ* involved in virulence mechanisms of *S. aureus* and further explained that the significant effect of *mraZ* on virulence phenotype.

S. aureus has evolved a complex regulatory network to control virulence. It has well known that *agr* quorum-sensing system, some TCS systems *saeRS*, *srrAB* and *arlRS* and the assistant regulator *sarA*.¹⁸ Among them, *agr* can positively regulate α -hemolysin gene (*hla*), PSM genes (*pmsA1*-*pmsA4*, *psm β 1* and *psm β 2*) and δ -hemolysin gene (*hld*);²⁸ *sarA* can enhance the accumulation of many virulence factors via positive regulation of *agr* activity.^{18,29} In this study, we found that the deletion of *mraZ* resulted in decreasing expressions of *agr* and *sarA*, indicating that the *mraZ* can lead to the high expression of many virulence genes by upregulating *agr*, *sarA*. This notion is further supported by the reduced production of *agr*-dependent virulence factors in the mutant with reduced.

However, there are still some limitations in our study. Most of the phenotypic experiments of the complementary strain have not completely recovered to the level of its parent strain. The probable reason may be associated to the genes that only exists on the plasmid and did not restore in their chromosome. Although we confirmed that *mraZ* plays an important role in the virulence of *S. aureus*, and regulating some virulence genes and regulators, the regulatory mechanisms of *mraZ* in relation to virulence requires further investigated.

Conclusion

Our work demonstrates the *mraZ* plays vital roles in regulating many virulence genes via modulation of *agr* and

sarA expression in *S. aureus*. *MraZ* is an attractive target for anti-virulence therapy of *S. aureus*.

Author Contributions

FY and YG conceived and drafted the work. BW, JD, YJ designed of the work and analyzed and interpreted of data for the work. QZ, YX, HZ, XW and LR participated in the experimental work and data analysis. FY agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

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Disclosure

The authors report no conflicts of interest in this work.

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