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ORIGINAL RESEARCH

NWMN2330 May Be Associated with the Virulence of *Staphylococcus aureus* by Increasing the Expression of *hla* and *saeRS*

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Introduction: *Staphylococcus aureus* is an opportunistic pathogen that can cause life-threatening bloodstream infections such as sepsis and endocarditis. In recent years, the emergence and increase of methicillin-resistant and multidrug-resistant *S. aureus* has posed a great challenge to the antibiotic treatment of infectious diseases. Anti-virulence strategies targeting virulence factors are an effective new therapy for the treatment of *S. aureus* infections.

Results: In this study, we constructed a *NWMN2330* deletion mutant (*Newman-ΔNWMN2330*) and a complement (*Newman-ΔNWMN2330-C*) of *S. aureus Newman* to study the role of *NWMN2330* in the virulence of *S. aureus*. Through transcriptome sequencing, it was found that the expression of 224 genes in *Newman-ΔNWMN2330* was significantly different (>2-fold) compared with *S. aureus* Newman, and these differentially expressed genes were related to multiple functions of *S. aureus*. And we found that *NWMN2330* could positively regulate the expression of *S. aureus hla* gene. Therefore, the deletion mutant *Newman-ΔNWMN2330* exhibited lower hemolytic activity and lower α-toxin production than *Newman. Newman-ΔNWMN2330* also exhibited lower lethality and pathogenicity in worm survival experiments and nude mouse skin abscess model. RT-qPCR results showed that compared with the wild-type strain, the expression of *saeRS* and *hla* in *Newman-ΔNWMN2330* strain was significantly reduced at the mRNA level, which preliminarily indicated that *NWMN2330* promoted the expression of *hla* by up-regulating *saeRS*.

Discussion: In general, our results indicated that *NWMN2330* may be associated with the virulence of *Staphylococcus aureus* by increasing the expression of *hla* and *saeRS*.

Keywords: virulence, *NWMN2330*, *Staphylococcus aureus*, *hla*, *saeRS*

Introduction

Staphylococcus aureus is an asymptomatic human nostril colonizer, permanently colonizing approximately 30% of individuals.[1] And it is one of the most notorious and widespread bacterial pathogens, causing incalculable numbers of uncomplicated skin infections and possibly hundreds of thousands to millions of more serious invasive infections worldwide each year.[2,3] *S. aureus* infections are particularly problematic due to the frequent emergence of antibiotic resistance among *S. aureus* isolates, of which methicillin-resistant *S. aureus* (MRSA) is the most clinically important.[4] Resistance of *S. aureus* to other antibiotics is also common. For example, resistance to β-lactamase-sensitive traditional β-lactam antibiotics (penicillin and derivatives) is almost ubiquitous in *S. aureus*.[5] Furthermore, *S. aureus* typically exhibits resistance to nearly all available antibiotics in combination.[6] Vancomycin remains the antibiotic of last resort for MRSA infections, and highly vancomycin-resistant strains (VRSA) have emerged but have not spread, probably due to the greatly increased fitness costs imposed by vancomycin resistance genes.[7] However, some strains have acquired moderate resistance to vancomycin (VISA).[8] Available antibiotics are not sufficiently effective against multidrug-resistant *S. aureus* strains, and although several *S. aureus* vaccines have been tested in clinical trials, all vaccine candidates have so far failed clinical trials.[9,10] Overreliance on animal models and an incomplete understanding of

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S. aureus pathogenesis during human infection may explain this failure.[11] Based on the failure of all vaccines, people are forced to seek new treatments for *S. aureus* infections.

Since *S. aureus* produces a variety of virulence factors that play important roles in infection, including hemolysin, enterotoxins, plasma coagulase, and leukocidal toxins,[12,13] anti-virulence strategies for the treatment of *S. aureus* infections have received increasing attention. The anti-bacterial virulence strategy is to achieve the purpose of anti-infection by reducing the virulence of pathogens. It does not directly kill the bacteria, and it is not easy to cause drug resistance and destroy the beneficial microbiota. Current studies have found that α-hemolysin, SrtA and golden yellow pigment are good virulence targets.[14–18] In our previous study, our group found that after sub-inhibitory concentrations of resveratrol reduced the virulence of *S. aureus*, the expression of the *NWMN2330* gene was down-regulated in its transcriptome.[19] Therefore, we speculate that this gene may be closely related to the virulence of *S. aureus*. See Supplementary Material for *NWMN2330* gene sequence.

Our study mainly focuses on the role of *NWMN2330* in the virulence of *S. aureus*, which provides a theoretical basis for further understanding the role of *NWMN2330* in *S. aureus*, and also provides a new target for the treatment of *S. aureus* infection.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study were listed in Table 1. According to the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France), the VITEK-2 microbial analyzer was used to identify the isolates. *S. aureus Newman* and *S. aureus Newman NWMN2330* mutants were grown in tryptic soy broth (TSB)(BD) medium, supplemented in tryptic soy broth (TSB)(BD, NJ, United States) containing 10 mg/l chloramphenicol Grow in a medium, shake 220 rpm at 37°C. *Escherichia coli* was cultured in Luria broth (LB,)(Oxoid) medium containing appropriate antibiotics (ampicillin 100 mg/L and anhydrotetracycline 50 mg/L).

Construction of *S. aureus NWMN2330* Mutant Strain (*Newman-ΔNWMN2330*) and Its Complemented Strain (*Newman-ΔNWMN2330-C*)

The accession number of the *Newman2330* gene in the NCBI GenBank is BAF68602.1.

The specific function in *S. aureus Newman* is unclear. We blasted other *S. aureus* with this gene. In common *S. aureus*, such as USA300, the gene expresses hypothetical protein, and in some other *S. aureus* such as 16,405, the gene expresses GtrA family protein. The temperature-sensitive plasmid pKOR1 was used to construct the *NWMN2330* deletion mutant of *S. aureus Newman* through allelic replacement. Use primer sets *NWMN2330*-UP-F/*NWMN2330*-UP-R and *NWMN2330*-DOWN-F/*NWMN2330*-DOWN-R to amplify the upstream and downstream fragments of *NWMN2330* from the genomic DNA of *S. aureus Newman* (Table 2). The resulting amplicon was digested with XhoI and then ligated with T4 DNA ligase to generate a homology arm fragment with *NWMN2330* gene deletion, which was then cloned into pKOR1. The recombinant plasmid pKOR1-Δ*NWMN2330* was sequentially transferred to *E. coli* DH5α and DC10B competent cells, and finally electroporated into *S. aureus Newman*

Table 1 Bacterial Strains and Plasmids Used in This Study

Strains and Plasmids	Description	Reference or Source
Strains		
S. aureus Newman	CA-MSSA	NARSA[a]
Newman-ΔNWMN2330	Isogenic *NWMN2330* deletion mutant in Newman	This study
Newman-ΔNWMN2330-C	*NWMN2330* mutant complemented with pLI*NWMN2330*	This study
DH5α	*Escherichia coli* isolates, clone host strain	TransGen
DC10B	*Escherichia coli* isolates, clone host strain	TransGen
Plasmids		
pKOR1	Shuttle cloning vector, temp sensitive (Cm^R, Amp^R)	Addgene
pLI50	Shuttle cloning vector (Cm^R, Amp^R)	Addgene

Note: Italic font represents the name of the bacteria.
Abbreviations: *S. aureus, Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*; Cm^R, chloramphenicol resistance; Amp^R, ampicillin resistance; [a]NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.

Table 2 Primers Used in This Study

Primer	Primer Sequence (5'- 3')	Product Length	Annealing Temperature	Note
NWMN2330-F	CCGAGATGTTAGGCGACCA	485bp	60°C	
NWMN2330-R	TTGATGTTGAGGAGTTACAC		56°C	
NWMN2330-UP-F	CAAATCCGCTGAGTATCTTTATCCA	979bp	65°C	
NWMN2330-UP-R	CCGCTCGAGCGGAGTAGCGTAAACCACTAAAAAACGC		65°C	XhoI
NWMN2330-DOWN-F	CCGCTCGAGCGGTGTGTAACTCCTCAACATCAAAATA	988bp	58°C	XhoI
NWMN2330-DOWN-R	GGACTTTCCTGTGCACATTTTTATC		65°C	
NWMN2330-UP-F-*attB2*	GGGGACCACTTTGTACAAGAAAGCTGG GTCAAATCCGCTGAGTATCTTTATCCA	2354bp	65°C	*attB2*
NWMN2330-DOWN -R-*attB1*	GGGGACAAGTTTGTACAAAAAAGCAGG CTGGACTTTCCTGTGCACATTTTTATC		65°C	*attB1*
NWMN2330-RT-F	TAGCCAGGATACAAATACATA	145bp	56°C	
NWMN2330-RT-R	CCTACTTGGAGAAAATTCATTA		58°C	
NWMN2330-C-F	GGAATTCCCCGAGATGTTAGGCGACCA	712	60°C	EcoRI
NWMN2330-C-R	GGGGTACCCCTTGATGTTGAGGAGTTACAC		56°C	KpnI
gyrB-RT-F	ACATTACAGCAGCGTATTAG	345bp	56°C	
gyrB-RT-R	CTCATAGTGATAGGAGTCTTCT		60°C	
hla-RT-F	GTCATTTCTTCTTTTTCCCAATCG	116bp	63°C	
hla-RT-R	CACGTATAGTCAGCTCAGTAACA		63°C	
saeR-RT-F	GTCGTAACCATTAACTTCTG	153bp	60°C	
saeR-RT-R	ATCGTGGATGATGAACAA		60°C	
saeS-RT-F	TGTATTTAAAGTGATAATATGAG	145bp	62°C	
saeS-RT-R	CTTAGCCCATGATTTAAAACA		62°C	
sarA-RT-F	CGTACTTTCTATTATTTGTTACC	120bp	59°C	
sarA-RT-R	CAGTGCAGTTTTAATGATAAG		59°C	

Note: Italics represent gene names.

competent cells. The allelic replacement mutants were selected as described previously.[20] Briefly, we amplified the *NWMN2330* fragment using *Newman* genomic DNA as a template. The plasmid pLI50 and the gene fragment were digested with EcoRI and KpnI restriction enzymes, and the plasmid and fragment were ligated with T4 ligase. The ligated products were sequentially thermally transformed into *E. coli* DH5α and DC10B for amplification and modification. Finally, it was electroporated into *Newman-ΔNWMN2330*, and each step is verified by PCR and first-generation Sanger DNA sequencing.

Growth Assay

The strain *Newman*, the deletion mutant *Newman-ΔNWMN2330*, and the complementary strain *Newman-ΔNWMN2330-C* were inoculated on blood agar plates and incubated overnight at 37 ° C incubator. Pick a single clone and inoculate in 5mL of tryptic soy broth (TSB) (BD, NJ, United States) for shaking culture, and then inoculate in 50mL TSB for shaking culture at a ratio of 1: 200. All cultures were induced at 37 °C with shaking at 220 r.p.m. and the OD_{562} values were measured every hour for a total of 24 h. The assay was performed in triplicate.

RNA-seq and Identification of Differentially Expressed Genes

The wild strains of *S. aureus* Newman and *Newman-ΔNWMN2330* were cultured in TSB for 9h, and then the bacteria were collected by centrifugation at 12,000 g for 1 minute at 4 ° C. The method recommended by the manufacturer (QIAGEN, Berlin, Germany) for RNA extraction. Illumina HiSeq X platform and pe150 (150 bp double-stranded assay) strategy were used to analyze the tested RNA, and DEGseq software was used to analyze differentially expressed genes. log2 (fold-change) |> 1, and p <0.005 represents the difference in gene expression between samples.

Quantitative Enzyme-Linked Immunosorbent Assay for α-Toxin

The bacteria were cultured in TSB with shaking for 24 hours, after which the supernatant was collected by centrifugation at 6000 g for 2 minutes and filtered with a 0.22 μm filter. We used the *staphylococcus* alpha-toxin enzyme-linked immunoassay kit (Sigma-Aldrich, St. Louis, Missouri, USA) to detect alpha-toxin. The above-extracted supernatant was added to the detection plate, and then horseradish peroxidase (HRP) label was added to the plate, and the above mixture became an antibody-antigenase labeled antibody complex. After washing, TMB (3,30,5,50-tetramethylbenzidine) substrate solution was added, and finally sulfuric acid solution was added to stop the reaction, and the color change was measured by spectrophotometry at 450 nm wavelength. Use the standard curve y = ax + b to calculate the concentration of alpha toxin in each sample and then multiply it by the corresponding dilution factor. Each test was repeated three times.

Determination of Hemolytic Ring

Newman and *Newman-ΔNWMN2330* were inoculated on Columbia blood agar medium, *Newman-ΔNWMN2330-C* was inoculated on the plate containing chloramphenicol, and placed in a 37°C incubator for overnight culture. Bacteria were shaken overnight at 37°C in TSB liquid medium at 220 rpm. Centrifuge at 4000g at room temperature for 5 minutes, discard the supernatant, resuspend the bacterial solution in PBS, and centrifuge again at 4000g at room temperature for 5 minutes. After that, 10 μL of bacteria was dropped on Columbia blood agar medium, placed in a 37°C incubator for 24 hours, and the size of hemolytic ring was observed.

α-Toxin Activity Determination

The experimental method is slightly modified on the basis of the previous literature.[15] Briefly, *S. aureus* strains were cultured in TSB at 37 ° C, and the supernatant was extracted according to the above experimental method. Then add 100 μL supernatant to 875 μL hemolysis buffer, incubate with 25 μL defibrillated rabbit blood at 37 ° C for 1 h, and centrifuge at 6000 g for 2 minutes. Take the supernatant and measure the OD value of the supernatant at 450 nm. Triton X-100 was used as a positive control and 0.9% NaCl was used as a negative control. Each test was performed independently in triplicate.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

After shaking culture of *S. aureus* for 9h, RNA was extracted according to the above method. The primer pairs used in real-time RT-PCR are listed in Table 1. The total RNA was reversed to cDNA using Takara RNA PCR kit (Takara, Tokyo, Japan). PCR was performed in 20 μL reaction mixture using Luna Universal qPCR Master Mix (NEB, MA, United States). Each test was performed in triplicate.

Determination of Silkworms Survival Rate

This experiment mainly refers to previous studies with minor modifications.[21] Newman and *Newman-ΔNWMN2330* were inoculated on Columbia blood agar medium, *Newman-ΔNWMN2330-C* was inoculated on the plate containing chloramphenicol, and placed in a 37°C incubator for overnight culture. Pick a single clone and add it to 5mL TSB liquid medium, shake it at 220rpm, 37°C, and grow it in the late logarithmic growth period, about 8 hours. The bacteria were centrifuged at 5000 g for 5 min at room temperature. After discarding the supernatant, they were resuspended in PBS, centrifuged again and repeated three times. Adjust the bacterial solution to a consistent OD value, probably at OD_{562}=1.5. The experiment was divided into 4 groups, namely wild bacteria group (*Newman*), deletion mutant group (*Newman-ΔNWMN2330*), complementary strain group (*Newman-ΔNWMN2330-C*), and blank control group, which was injected with PBS. The experimental sample for each group is 12. Between the second and third gastropods of the silkworms, 8×10^8 CFU/100 μL of bacteria were injected into the body with an insulin needle, and PBS was used as a blank control. Placed in a 37°C incubator, record the survival time of the wax moth, and draw it with drawing software.

Mouse Skin Abscess Model

Animal research was approved by the Institutional Animal Care and Use Committee.

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 approved by the Ethics Committee of Shanghai pulmonary hospital, Tongji University School of medicine. In the study, 4 to 6 week old male BALB/c nude mice were used, divided into three groups of four. For the specific method, refer to the previously published article.[22] BALB/c mice were completely anesthetized with 1% (mass/volume) pentobarbital sodium (50 mg/kg body weight). Then, 100 μL of 5×10^8 CFU bacteria were inoculated subcutaneously on the back of the mice, and the mice injected with PBS were used as blank controls. The area evaluated by the maximum length and width of the skin damaged part is measured every day. Use the formula A = L × W to calculate the damage area, where L is the length and W is the width.

Statistical Analysis

Experimental data were analyzed using GraphPad Prism 6 software (version 6.00, La Jolla, CA, United States). A p-value less than 0.05 was considered to be statistically significant.

Result

NWMN2330 Did Not Affect the Growth of *S. aureus*

In order to test whether *NWMN2330* had an effect on the growth of *S. aureus Newman*, we measured the growth curves of *S. aureus Newman, Newman-ΔNWMN2330* and *Newman-ΔNWMN2330-C*. The results showed that the growth of the three strains was similar, with no statistical difference (Figure 1), indicating *NWMN2330* had no effect on the growth of *S. aureus Newman*.

NWMN2330 Participates in the Regulation of Multiple Genes Related to Virulence

Transcriptome data showed that *Newman-ΔNWMN2330* has a total of 224 gene expression changes (according to |log2 (Fold Change)| >1 and q value<0.05 criteria) relative to *Newman*, of which 91 genes are up-regulated and 133 genes are down-regulated (Figure 2). After bioinformatics analysis, it was found that these genes are involved in multiple metabolic pathways, including *S. aureus* infection, cell killing, carotenoid biosynthesis, two-component regulatory system, complement binding, and amino acid anabolism (Figure 3 and Table 3).

NWMN2330 Inhibits the Expression of Virulence Gene *hla*

Transcriptome data showed that the expression of *hla* in *Newman-ΔNWMN2330* was significantly down-regulated by 2^10.008-fold compared to *Newman*. We used RT-qPCR to verify the transcriptome data, the results showed (Figure 4) that *hla* expression in *Newman-ΔNWMN2330* was significantly down-regulated, indicating that *NWMN2330* may be linked with *hla* regulation.

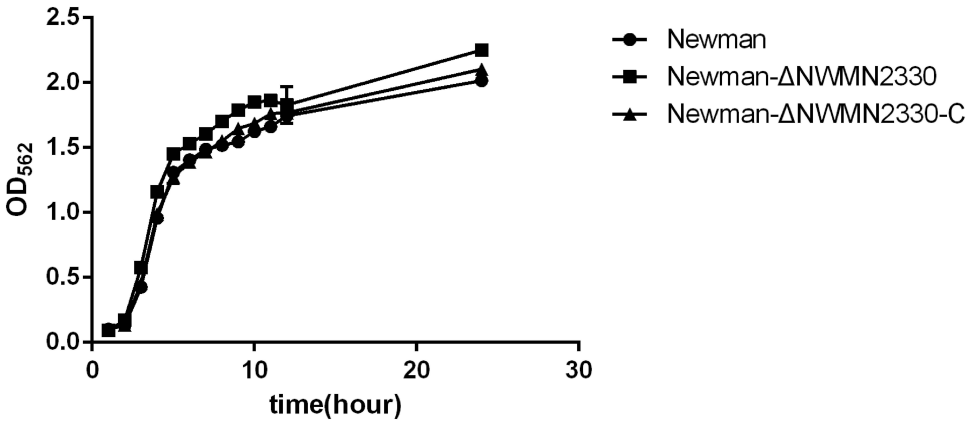

Figure 1 Growth curves of *Newman, Newman-ΔNWMN2330, Newman-ΔNWMN2330-C*. The data were analyzed by one-way ANOVA, and there was no difference in daily bacterial growth between *Newman* and *Newman-ΔNWMN2330*.

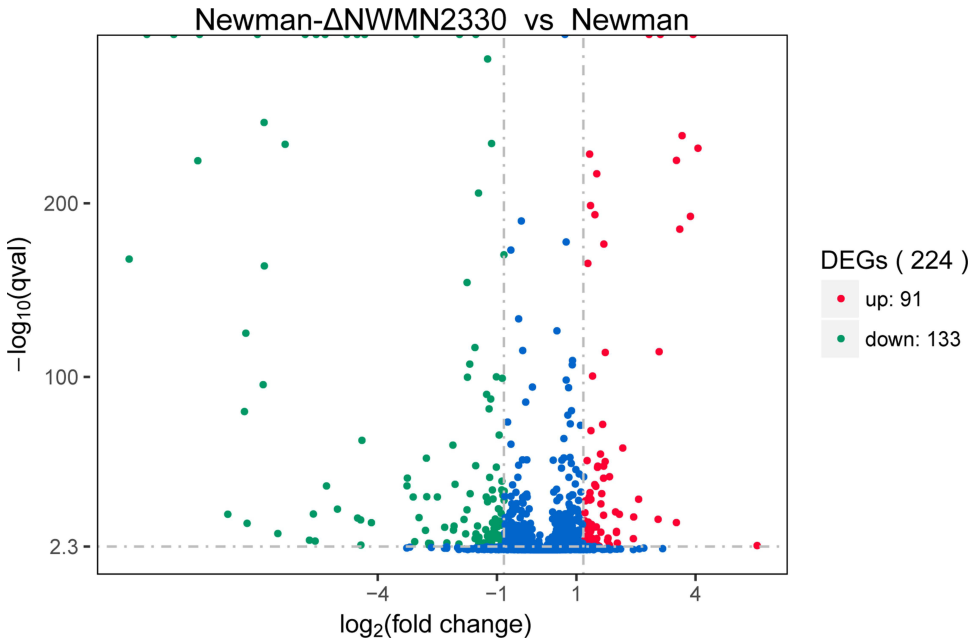

Figure 2 Volcano graph showing the difference in expression between *Newman* and *Newman-ΔNWMN2330*. The abscissa represents the fold change of gene expression in different samples; the ordinate represents the statistical significance of the difference in gene expression; the red dots in the figure indicate the up-regulated genes with significant differential expression, and the green dots indicate the down-regulation of significant differential expression gene.

Figure 3 Scatter plot of KEGG enrichment of differential genes. The vertical axis represents the name of the pathway, the horizontal axis represents the enrichment factor, the size of the dots indicates the number of differentially expressed genes in this pathway, and the color of the dots corresponds to different q value ranges.

Table 3 *Newman-ΔNWMN2330* vs *Newman* Genes with Significantly Changed Expression

Gene_id	Log2.Fold_Change	p value	q value	Gene Description
Staphylococcus aureus infection				
NWMN_1067	−3.3037	1.75E-32	1.71E-31	Inhibitory protein
NWMN_0055	−1.2103	4.23E-102	1.06E-100	Immunoglobulin G-binding protein A
NWMN_1069	−7.0507	4.32E-166	1.44E-164	Fibrinogen-binding protein
NWMN_1927	−6.527	2.18E-236	1.32E-234	Uncharacterized leukocidin-like protein 1
NWMN_1928	−7.0566	5.08E-249	3.56E-247	Uncharacterized leukocidin-like protein 2
NWMN_1872	−5.5207	0	0	65 kDa membrane protein
NWMN_1876	−4.9756	0	0	Staphylococcal complement inhibitor
NWMN_1877	−7.971	2.07E-22	1.48E-21	Chemotaxis inhibitory protein
NWMN_1070	−7.5518	5.32E-82	1.09E-80	Staphylococcal complement inhibitor
NWMN_2529	−1.8792	1.88E-109	5.01E-108	Clumping factor B
NWMN_2317	−9.331	0	0	Immunoglobulin-binding protein sbi
Two-component system				
NWMN_1822	1.1047	8.11E-18	4.97E-17	Response regulator protein VraR
NWMN_1823	1.6393	2.60E-44	3.53E-43	Sensor protein VraS
NWMN_0953	−1.1994	2.82E-08	9.56E-08	Putative cytochrome bd menaquinol oxidase subunit II
NWMN_0194	−1.2198	2.72E-08	9.28E-08	Sensor protein LytS
NWMN_0674	−4.5244	0	0	Histidine protein kinase SaeS
NWMN_0675	−4.7151	0	0	Response regulator SaeR
NWMN_0158	1.5292	4.52E-53	6.90E-52	Hexose phosphate transport protein
NWMN_1981	1.2022	8.82E-06	2.32E-05	Potassium-transporting ATPase B chain
NWMN_1982	1.6022	2.30E-08	7.96E-08	Potassium-transporting ATPase A chain
NWMN_1983	1.1459	1.09E-30	1.02E-29	Sensor protein KdpD
cell killing				
NWMN_1927	−6.527	2.18E-236	1.32E-234	Uncharacterized leukocidin-like protein 1
NWMN_1928	−7.0566	5.08E-249	3.56E-247	Uncharacterized leukocidin-like protein 2
NWMN_2318	−5.7469	0	0	Gamma-hemolysin component A
NWMN_2319	−7.2228	0	0	Gamma-hemolysin component C
NWMN_2320	−6.0229	0	0	Gamma-hemolysin component B
NWMN_1073	−10.008	0	0	Alpha-hemolysin
Amino acid metabolism				
NWMN_1348	−2.9716	4.71E-55	7.73E-54	L-threonine dehydratase catabolic TdcB
NWMN_1239	−1.7232	6.55E-11	2.79E-10	Aspartokinase 3
NWMN_2510	1.3361	1.07E-49	1.51E-48	Betaine aldehyde dehydrogenase
NWMN_2331	1.4893	7.89E-22	5.58E-21	Glycerate kinase
NWMN_1441	1.4886	2.01E-43	2.57E-42	Aminomethyltransferase
NWMN_1242	−1.3081	1.86E-17	1.11E-16	Homoserine kinase
NWMN_1241	−1.1079	1.13E-24	9.04E-24	Threonine synthase
NWMN_1240	−1.3098	1.39E-36	1.61E-35	Homoserine dehydrogenase

Note: Italics represent gene names.

NWMN2330 Enhances the Hemolytic Ability of *S. aureus*

The production of hemolysin is an important part of the virulence of *S. aureus*, and alpha toxin is the most important toxin in hemolysin. We tested the hemolytic ability of *S. aureus Newman, Newman-ΔNWMN2330* and *Newman-ΔNWMN2330-C*. The amount of α-toxin was quantitatively detected by ELISA, and it was found that the amount of *Newman-ΔNWMN2330* produced was significantly less than that of *Newman* (Figure 5A). And the hemolytic ring produced by *Newman* was significantly larger than that of *Newman-ΔNWMN2330*, and the complementary strain reached the level of *Newman* (Figure 5B). Because alpha toxin is a punch protein, it has the most obvious hemolytic effect on rabbit red blood cells. We used defibrillated rabbit blood to detect hemolytic activity, and used Triton as a positive control and normal saline as a negative control. It was found that the hemolytic

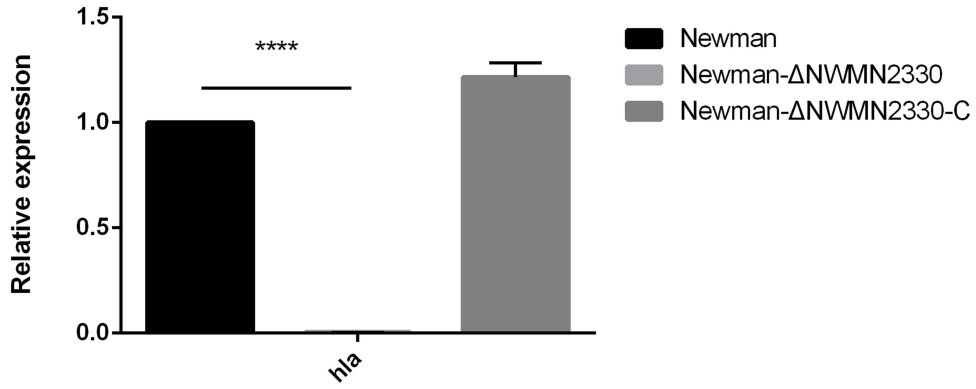

Figure 4 RT-PCR results of *hla. Newman* represents the wild strain, *Newman-ΔNWMN2330* represents the deletion mutant, *Newman-ΔNWMN2330-C* represents the reverted strain. Statistical significance was determined by the unpaired *t*-test, wild strain compared to deletion mutant strain, and ****Represents P<0.0001.

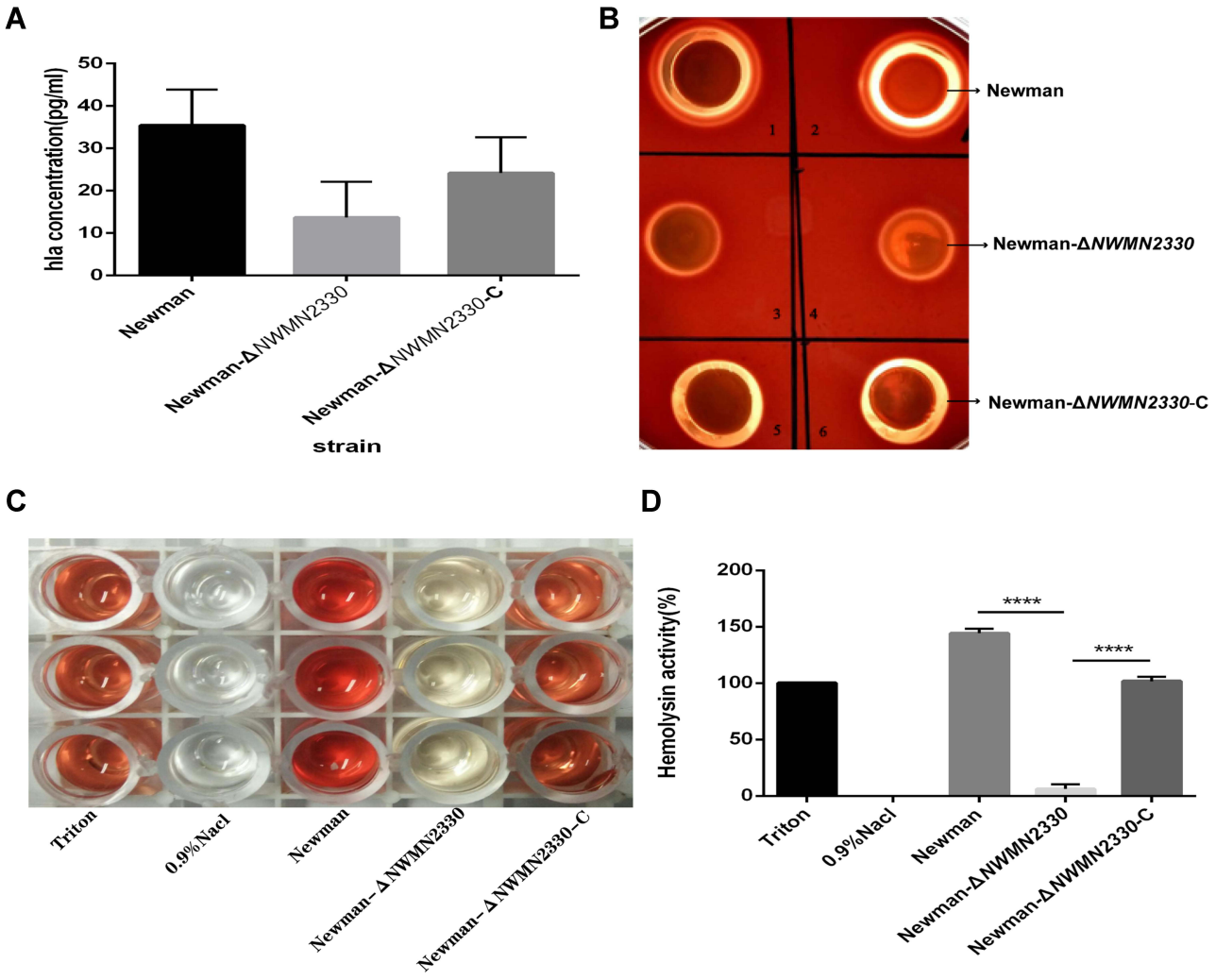

Figure 5 (**A**) ELISA detects the amount of α-toxin produced by each strain; (**B**) Colombian blood agar medium detects the hemolytic capacity of each strain; (**C** and **D**), detects the hemolytic activity of each strain.

Notes: (**C**) is a picture of hemolytic activity measurement, and (**D**) is a drawing software drawing after measuring the OD value and converting it with Triton as the 100% hemolysis rate. Newman represents a wild strain, *Newman-ΔNWMN2330* represents a deletion mutant, *Newman-ΔNWMN2330-C* represents a reverted strain, Statistical significance was determined by the one-way ANOVA test, wild strain compared to deletion mutant strain and complementary strains compared to deletion mutants, ****Represents P<0.0001.

activity of *Newman-ΔNWMN2330* was significantly lower than that of wild strain *Newman* (Figure 5C and D). The above results indicate that *NWMN2330* can positively regulate the expression of *S. aureus* hemolysin.

NWMN2330 Enhances the Lethality of *S. aureus* to Silkworms

Because the production of toxins is closely related to the pathogenicity of *S. aureus*, after observing the effect of *NWMN2330* on hemolysin, we speculate that *NWMN2330* may be related to the virulence and pathogenicity of *S. aureus*. So we measured the survival rate of the silkworms after infection with the three kinds bacteria. The results showed that the survival time of silkworms infected with *Newman* was significantly shorter than the time of injection of *Newman-ΔNWMN2330*, which initially indicated that *NWMN2330* could enhance the virulence of *S. aureus* (Figure 6).

Effect on Infection of Subcutaneous Abscess in Mice

S. aureus infection often causes skin abscesses.[23,24] We used skin abscess model in nude mice to investigate whether *NWMN2330* had an effect on the ability of *S. aureus* to cause skin abscesses. The results showed that the abscess area of mice infected with the mutant strain (4±0.76) was significantly reduced compared to the wild strain (130±16.16) (Figure 7). The above results indicate that *NWMN2330* is associated with the virulence and pathogenicity of *S. aureus* and can enhance the pathogenicity of *S. aureus*.

NWMN2330 May Promote *hla* Expression by Up-Regulating *saeRS*

The *saeRS* two-component regulatory system is a regulator that controls the expression of multiple virulences.[25–28] According to the transcriptome data, it is speculated that *saeRS* is also involved in the regulation of *hla* expression by *NWMN2330*. RT-PCR results showed that when *NWMN2330* was knocked out, the expression of *saeRS* was significantly down-regulated, and there was no downward trend in other related regulators *sarA* (Figure 8). The previous speculations have been further confirmed, indicating that *NWMN2330* may promote *hla* expression by up-regulating *saeRS*.

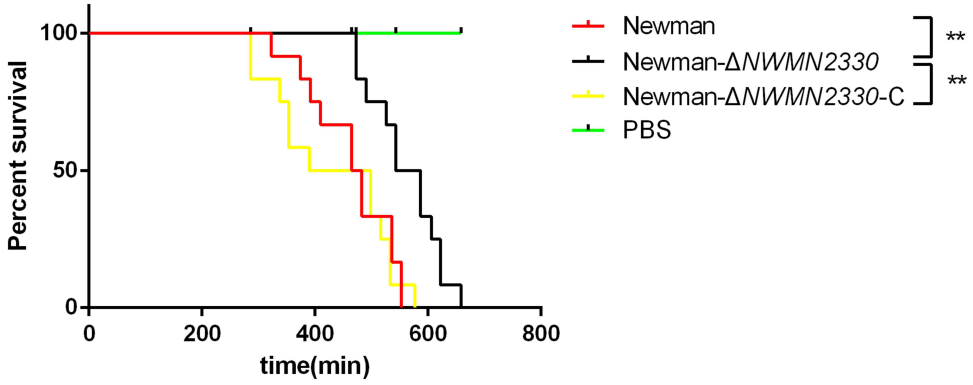

Figure 6 Determination of silkworms survival rate (N=12). PBS injection was used as a negative control. Statistical significance was determined by the Gehan-Breslow-Wilcoxon test, wild strain compared to deletion mutant strain and **Represents P<0.01.

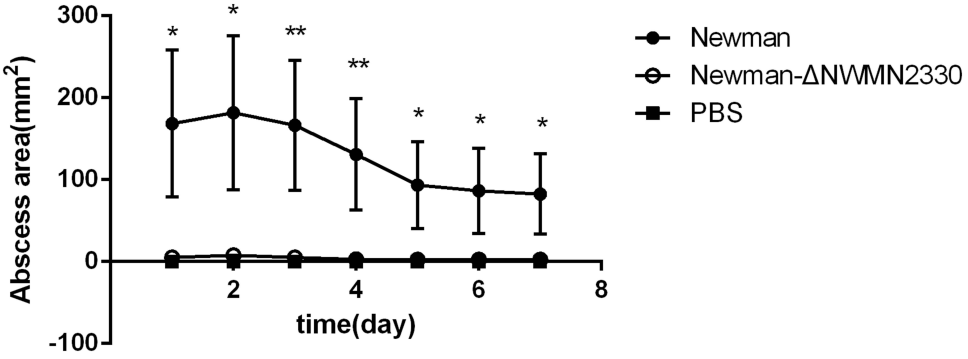

Figure 7 Results of nude mouse skin abscess model. PBS injection was used as a negative control. Statistical significance was determined by the unpaired *t*-test, wild strain compared to deletion mutant strain, *P < 0.05, **P < 0.01.

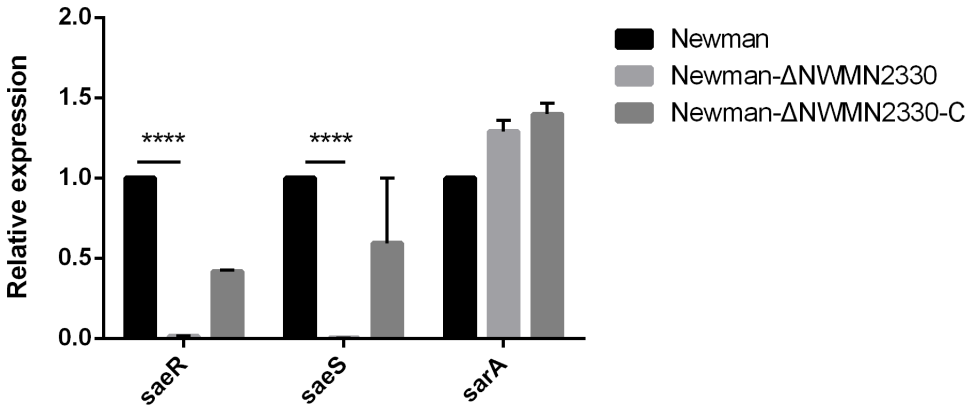

Figure 8 Relative expression of *saeRS* and *sarA* at the mRNA level. Statistical significance was determined by the unpaired *t*-test, wild strain compared to deletion mutant strain, ****Represents P<0.0001.

Discussion

Due to the increasing number of multidrug-resistant *S. aureus* in recent years, there is an urgent need for new antibacterial agents that can overcome these more robust *S. aureus* strains. The discovery of new antibiotics has slowed in recent years, and strategies to reduce the virulence of *S. aureus* to reduce its lethality have attracted great interest.[29] In recent years, there have been many studies on the use of anti-virulence strategies for the treatment of *S. aureus* infections,[30–32] but not enough for the rapidly evolving multidrug-resistant bacteria. Our previous study found that *NWMN2330* in *S. aureus* may be related to virulence,[19] but the specific role was not clear. The gene annotation of *NWMN2330* is "similar to functionally unknown protein", and its specific function is worth exploring.

In the present study, we mainly focused on the virulence control of *S. aureus* by *NWMN2330*. We successfully constructed the *S. aureus Newman* deletion mutant *Newman-ΔNWMN2330* and the complemented strain *Newman-ΔNWMN2330-C*. In recent years, transcriptome sequencing analysis has been frequently used to study the pathogenic and drug resistance mechanisms of *S. aureus*.[33,34] The results of transcriptome sequencing showed that compared with *Newman*, the expression of *hla* in the mutant strain was down-regulated very significantly. We also confirmed by real-time quantitative PCR that the expression of *hla* at the mRNA level was indeed significantly reduced in the mutant strain. We speculate that *NWMN2330* may regulate *S. aureus* virulence by enhancing *hla* expression.

Alpha-toxin (encoded by *hla*) is the most studied pore-forming toxin and plays an important role in *S. aureus* skin and soft tissue infections.[35–37] Alpha-toxin has an obvious hemolytic effect on red blood cells of various mammals, especially rabbit red blood cells.[38] Using the above characteristics of α-toxin, we detected the hemolytic activity, hemolytic ring and α-toxin production of *Newman, Newman-ΔNWMN2330* and *Newman-ΔNWMN2330-C* strains, respectively. The deletion mutant *Newman-ΔNWMN2330* showed lower hemolytic activity, lower α-toxin production and smaller hemolytic ring than the wild strain in the above three experiments. We know that *NWMN2330* also enhance the virulence and pathogenicity of *S. aureus* from survival experiments in silkworms and skin abscess model in nude mice. The production of *S. aureus* α-toxin is regulated by multiple regulatory systems, of which *saeRS* is considered to be the most important one because the *sae* locus is important for *hla* transcription.[39] The *S. aureus sar* locus also has alpha and beta hemolysins, and *SarA* directly increases α-toxin production.[40] We found by real-time quantitative PCR that the expression of *saeRS* decreased significantly in the deletion mutant, but the change of *sarA* was not significant. We speculate that *NWMN2330* may up-regulate the expression of *hla* by up-regulating the expression of *saeRS*, thereby enhancing the virulence of *S. aureus*.

Our research has many limitations. First, it is only tentatively speculated that *NWMN2330* up-regulates the expression of *hla* through *saeRS* to enhance the virulence of *S. aureus*, and has not been studied more precisely. Secondly, we only studied the regulatory effect of *NWMN2330* on the α-toxin of *S. aureus*, and there are many other functions that are not covered in this paper. We can see in the transcriptome sequencing data that *NWMN2330* also affects the expression of many other important virulence genes. Also, it has not been studied whether the gene has the same effect in other *S. aureus* strains.

In summary, our study shows that the gene *NWMN2330* can up-regulate the expression of *hla* and enhance the pathogenicity in *S. aureus Newman* strain. The gene *NWMN2330* may be an important target for limiting *S. aureus* infection in people colonised by this potential pathogen.

Ethics Statement

Animal research was approved by the Institutional Animal Care and Use Committee.

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 approved by the Ethics Committee of Shanghai pulmonary hospital, Tongji University School of medicine. The approved ethics review number is K20-151Y.

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Disclosure

No potential conflict of interest was reported by the authors.

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