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ORIGINAL ARTICLE

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cydA, *spdC*, and *mroQ* are novel genes involved in the plasma coagulation of *Staphylococcus aureus*

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Abstract

Coagulation is a critical pathogenic process in *Staphylococcus aureus*. Although the agglutination of *S. aureus* has been studied for a long time, the genes involved in this process are not completely clear. We performed tube agglutination and dynamic turbidimetry tests to identify novel genes involved in reduced plasma coagulation. A total of 15 genes were identified, including *coa*, *clfA*, *vwbp*, *saeS*, *agrA*, *trpC*, *spdC*, *mroQ*, *cydA*, *qoxC*, *sucC*, *pyrP*, *menH*, *threonine aldolase*, and *truncated transposase for IS1272*. The functions of these genes include bicomponent regulation, membrane transport, energy metabolism and biosynthesis, respectively. *cydA*, *spdC*, and *mroQ* genes were further studied by gene knockout and complementation. Results of gene knockout and complementation and real-time-qPCR proved that *cydA*, *spdC,* and *mroQ* genes are necessary for plasma coagulation. Furthermore, the survival ability of 7 day mice decreased significantly when *cydA*, *spdC*, and *mroQ* genes had been knocked out.

KEYWORDS
cydA, *mroQ*, plasma agglutination, *spdC*, *Staphylococcus aureus*

1 | INTRODUCTION

Staphylococcus aureus is an important bacterial pathogen that causes both local and severe systemic infections, such as skin and soft tissue infection, endocarditis, osteomyelitis, and sepsis.[1,2] Many virulence factors produced by *S. aureus*, such as coagulase, surface adhesive protein, and leukocidin assist in mediating the pathogenicity of *S. aureus* infections,[3] and these are associated with different pathogenic mechanisms. For example, coagulase acts as a mechanical barrier to antagonize leukocyte phagocytosis by promoting plasma hemagglutination.[4] *S. aureus* secretes two kinds of coagulases, free coagulase [5] and bound coagulase[6] (von Willebrand factor–binding protein [*vwbp*]). The free coagulase promotes agglutination of blood cells and plasma, forming the inner core of the coated vesicles,[5] whereas the bound coagulase forms the outer fiber network and cleaves the alpha and beta chains of fibrinogen.[6] With the help of

Abbreviations: clfA, clumping factor A; DTT, dynamic turbidimetry test; TCT, tube coagulase test; TSA, tryptic soy agar; TSB, tryptic soy broth; vwbp, von Willebrand factor–binding protein.

Dong Luo and Wei Wang contributed equally to this work.

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clumping factor A (*clfA*), *S. aureus* combines with the specific sites of soluble fibrin-forming complexes to protect *S. aureus* against immune clearance.[7] Previous studies have revealed that the lack of *clfa* gene in *S. aureus* assists in relieving the symptoms of invasive arthritis,[8] but no effect was observed when the ClfA antibody is used for treating staphylococcal sepsis.[9] This led to the confirmation that abscess formation is repressed when *S. aureus* lacks free coagulase or vWbp, and the ability is further restricted when both of these are absent.[8] Recent studies also confirmed that thickened cell wall could reduce plasma agglutination in *S. aureus*, which is probably a result of the inhibition of exocrinosity of plasma coagulase.[10] As described earlier, there might be some undetected genes involved in the formation of agglutination, except for some of the widely known genes such as *coa*, *vwbp*, and *clfA*.

To further investigate the genes in the coagulation process, the procoagulant genes were screened genomewide for the first time. We also constructed and screened a transposon library of *S. aureus* to identify novel genes involved in the agglutination process.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains, plasmids, antibiotics, and growth conditions

The *Escherichia coli* strain DH5α (TIANGEN Biotech Co., Ltd, Beijing, China) and the *S. aureus* strain RN4220 were used for DNA cloning. We used the *S. aureus* Newman strain as the parent strain for screening. Plasmids of pID408, pRB473, pRAB11, and pKOR1 were used for constructing the transposon library, genetic complementation, and homologous recombination.[11–13] Antibiotics with the following concentrations were used unless otherwise indicated: erythromycin, 20 μg/mL; ampicillin, 100 μg/mL; and chloramphenicol, 20 μg/mL (Solarbio C8050; Biotech Co., Ltd, Shanghai, China). Tryptic soy agar (TSA) and tryptic soy broth (TSB) were used for culturing *E. coli* and *S. aureus*, respectively.

2.2 | Construction of transposon mutant library

S. aureus transposon mutant library was constructed as follows: first, we prepared the *S. aureus* strains RN4220 and Newman from the log phase of the cultures grown in TSB, followed by collection of bacterial cells by centrifugation at 5000 g/min. Competent cells were then acquired by washing the bacterial cells three times with sucrose; next, the temperature-sensitive plasmid pID408 containing the transposon Tn917 was successively transformed into *S. aureus* strains RN4220 and Newman competent cells by electroporation (diameter of electrode cup = 0.2 cm, voltage = 2.5 kV, resistance = 100 Ω, capacitance= 25 μF) using Gene Pulser Xcell (BioRad). After resuscitation, the cells were spread on TSA plates (20 μg/mL erythromycin, 20 μg/mL chloramphenicol) and then incubated at 30°C. The Newman strain containing pID408 plasmid was cultured at 43°C, followed by shaking in an incubator at 250 rpm (875 g/min) for 18 h to induce transposon insertion. These induced clones were collected and incubated on TSA plates containing 20 μg/mL erythromycin with or without 20 μg/mL chloramphenicol. The transposon mutants were unable to survive on plates containing chloramphenicol because of missing pID408. The transposon mutagenesis bias was tested by inverse PCR and all the PCR products were confirmed by gene sequencing. Finally, about 10,000 mutant clones were picked and cultured in TSB and stored as transposon mutant library at −80°C until use.

2.3 | Tube coagulase test

The mutant *S. aureus* Newman strain was grown on plates containing 5% sheep blood (Kemajia Microbe Technology Co., Ltd) at 37°C for 24 h. Each colony was cultured in TSB (10 mL) at 37°C for 24 h. The *S. aureus* culture was centrifuged at 6000*g* for 30 min, the supernatant was discarded, and the pellet was resuspended in sterile normal saline to 4.0 McFarland units.

The tube coagulase test (TCT) was performed using 50 μL bacterial suspension and 450 μL mixed citrate plasma from healthy volunteers (no obvious hemolysis and jaundice) in small plastic tubes (13 mm diameter), followed by incubation of the tubes at 37°C after gentle mixing. After that, clot formation was examined every hour for direct and pelleted TCT at 35°C and 16 h after incubation at room temperature. An opaque clot was identified as a positive result.

2.4 | Dynamic turbidimetry test

The bacterial suspension was prepared as described above. The dynamic turbidimetry test (DTT) was performed in a 96 well plate with 10 μL of bacterial suspension and 90 μL of mixed citrate plasma from healthy volunteers (with no obvious hemolysis and jaundice). The agglutination reaction was detected using the reactive system (ELx808 enzyme labeling instrument; Berton Instruments Co., Ltd) at 37°C and 340 nm OD. Agglutination was evaluated every minute according to the instrument settings. Positive DTT results were identified if the OD_{340} value was greater than 0.05 when compared with the initial results.

2.5 | Identification of mutated genes

The transposon insertion sites were confirmed by detecting the resistance markers. First, each mutant's overnight grown culture with reduced coagulating ability in LB broth was

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lysed in a lysis buffer (Sangon Biotech Co., Ltd), followed by extraction of bacterial DNA by alkaline lysis (TIANGEN Biotech Co., Ltd). Next, *Eco*RI was used to digest the genome at 37°C for 12 h, and T4 DNA ligase was used to ligate the target fragment was transformed in the *E. coli* DH5α strain (TIANGEN Biotech Co., Ltd) by chemical transfection. The self-ligated fragments of plasmid on TSA plates containing 20 μg/mL ampicillin were screened and collected. *S. aureus* genome containing new plasmids was sequenced by designing primers based on the proximal repeat regions of the erythromycin-resistance gene Tn917. The sequencing results were compared with those of the standard sequence of the *S. aureus* Newman strain, and the transposon insertion site was recognized to determine whether specific genes were involved in plasma agglutination.

2.6 | Gene knockout and complementation

Furthermore, the pKOR1 plasmid was used to knockout the candidate genes associated with decreased ability to coagulate by homologous recombination as described previously.[14] *CydA* was amplified (primers used: *cydA*-UF, *cydA*-UR; *cyd A*-DF, *cydA*-DR, shown in Table 1) and cloned into the plasmid pKOR1. The recombinant knockout plasmid was transformed into RN4220 and then into the *S. aureus* Newman strain. Next, homologous recombination was carried out by a two-step procedure. In the first step, the plasmid was integrated into the chromosome of *S. aureus* by the selective pressure of high temperature (42°C) and 10 μg/mL chloramphenicol. In the second step, the culture was streaked on the plates containing 1 μg/mL anhydrotetracycline (20150831; National Institutes for Food

TABLE 1 PCR primers used in this study

Primer name	Sequence (5′–3′)	Endonuclease site
cydA-UF	GGGGACAAGTTTGTACAAAAAAGCAGGCTGACCAAATGCCTACAG	
cydA-UR	GCTATTGAACCTGGTATTATTAGAATAAAGCACGAAG	
cydA-DF	CTTCGTGCTTTATTCTAATAATACCAGGTTCAATAGC	
cydA-DR	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCTTTACCCATACTCG	
cydA-IF	CAGTTCATTGTGTTGTCATC	
cydA-IR	TTATAACACTGTTATACC	
pRB473-cydA-F	TGCTCTAGAGCATGGACAATCATTCTGCGT	*Xba*I
pRB473-cydA-R	CGGGGTACCCCGACCTGCCCCAAAATCTAT	*Kpn*I
spdC-FF	GGGGACAAGTTTGTACAAAAAAGCAGGCGATTGTTGTTCAGCACGTGT	
spdC-FR	TACTAGCAAGCGCTTTGTTATATATGTAACCTCCATTAGGT	
spdC-RF	ACCTAATGGAGGTTACATATATAACAAAGCGCTTGCTAGTA	
spdC-RR	GGGGACCACTTTGTACAAGAAAGCTGGGTGATTACATAAATATGGGAGGC	
spdC-IF	TTGGTTCACCTTGCTTGTA	
spdC-IR	GACATGACGCTGGGAATT	
spdC-PF	CGGGGTACCTATTTTGCTTGTTACCTAATGGAGG	*Kpn*I
spdC-PR	CGCGAGCTCCTTTGTTATTATTTGTTTTTATCTG	*Sac*I
MroQ-FF	GGGGACAAGTTTGTACAAAAAAGCAGGCAGCCTTCTTGAATCATTGC	
MroQ-FR	ATTCGCTTGTTCCGTGGCGATGCCCATAATCTTGTCAT	
MroQ-RF	ATGACAAGATTATGGGCATCGCCACGGAACAAGCGAAT	
MroQ-RR	GGGGACCACTTTGTACAAGAAAGCTGGGTCGAATAATGTGAAGCCAGACT	
MroQ-IF	TTAATAGGCGTCCAGTTCC	
MroQ-IR	TAATCAAGGTCAATGGCAAC	
MroQ-PF	CGGGGTACCACGATAAAATAAATTATAAACAAA	*Kpn*I
MroQ-PR	CGCGAGCTCCATGCTATTCTTATTTGTAAAGCGAAATAAAAA	*Sac*I

Note: Underline is the endonuclease site.

and Drug Control, China) to select *cydA* knockout mutant strains. The correct knockout strain was then verified by PCR amplification and subsequent sequencing. The candidate gene and its upstream promoters were amplified using *cydA*-IF and *cydA*-IR primers. The restriction endonucleases *Xba*I and *Kpn*I were used to cleave the plasmid pRB473 and the PCR-amplified product. Except for the complementary plasmid pRAB11, the knockout technique for *spdC* and *mroQ* was similar to that of the *cydA* gene. Detailed primer sequences and restriction sites are shown in Table 1.

2.7 | Real-time qPCR

Real-time (RT)-qPCR was used to assess the expression of *cydA*, *coa*, *vwbp*, and *clfA*, and *gyrB* was used as the reference gene. The *S. aureus* Newman wild-type, *cydA* knockout, and *cydA* complementary strains were incubated overnight in TSB at 37°C for 8 h. The obtained cultures were then centrifuged and washed two times with PBS. RNA isolation was performed according to the manufacturer's instructions (Sangon Biotech Co., Ltd). The primers were designed using the primer express software (Version 2.0, Applied Biosystems). After that, reverse transcription was performed using Super-Script III First-Strand Synthesis (Takara Bio). RT-qPCR was performed using SYBR Premix Ex Taq II (Takara Bio). The amplicon of *gyrB* gene was used as an internal control. The PCR conditions were as follows: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, and 60°C for 30 s. The relative expression levels were determined by the comparative threshold cycle ($2^{-\Delta\Delta C_t}$) method. All primers are listed in Table 1.

2.8 | Growth ability tests

In order to observe whether the decrease of plasma agglutination ability of *cydA*, *spdC*, and *mroQ* is due to the low number of strains, we cultured mutant, complementary, and wild-type strains of *cydA*, *spdC*, and *mroQ* overnight to compare the growth ability. The detailed steps are as follows: the bacteria were cultured overnight at 37°C and diluted into the fresh TSB medium at 1:50, shaken, and cultured on the shaker at 37℃. Then, 100 μL bacteria were taken every 1 h to measure OD_{600} within a 12 h period. The experiment was repeated three times.

2.9 | Survival tests

In order to understand whether the decreased plasma agglutination abilities of *cydA*, *spdC*, and *mroQ* mutations affect the virulence of the strain, the mouse systemic infection test was performed. The wild-type, mutant, and complementary strains of *cydA*, *spdC*, and *mroQ* genes in logarithmic growth phase were cultured and harvested. The precipitate was then centrifuged at 400 g/min and washed with PBS three times. Finally, 1×10^7 CFU of bacteria were injected into the tail vein of 4–6 weeks' old mice, and PBS was used as control. The death status of mice was observed within 7 days.

2.10 | Statistical analysis

SPSS (version 17.0; IBM, New York, New York) was used for statistical analysis. The dynamic agglutination values of different genes were expressed as mean ± SD. RT-qPCR expression of normal genes and their knockout or complementary strains were compared using the chi-square test.

3 | RESULTS

3.1 | Construction of transposon library and screening of clones with reduced capacity of plasma agglutination and functional analysis of insertion genes

The transposon library was constructed using the Tn917 transposon located in pID408.[15] DTT and TCT identified the clones that had disappeared or decreased agglutinating ability. About 10,000 clones were collected, of which 157 showed decreased or disappeared agglutinating ability. Some gene mutants (such as NWMN_1282, NWMN_1228, and NWMN_0756) had decreased agglutination ability, whereas in 12 gene mutants (such as NWMN_0674, NWMN_0166, NWMN_0952, NWMN_2236, NWMN_1939, NWMN_0928, NWMN_1946, NWMN_1319, NWMN_1155, NWMN_1110, NWMN_0914, and NWMN_0757) agglutination ability disappeared. The detailed results are presented in Figure 1. All well-known genes involved in plasma agglutination, such as *coa*, *vwbp*, and *clfA*, had been identified. Two-component regulatory system genes, biosynthesis genes, energy producing genes, and surface protein synthesis gene had also been identified. These genes involved in plasma coagulation are shown in Figure 2 and Table 2.

3.2 | Importance of *cydA*, *spdC*, and *mroQ* genes in plasma coagulation

To validate the transposon library results and further characterize the role of identified genes in plasma coagulation, the most frequently identified genes, *cydA*, *spdC*, and *mroQ* genes, were selected for further study. The agglutinating abilities of knockout strains for *cydA*, *spdC*, and *mroQ* were similar to the

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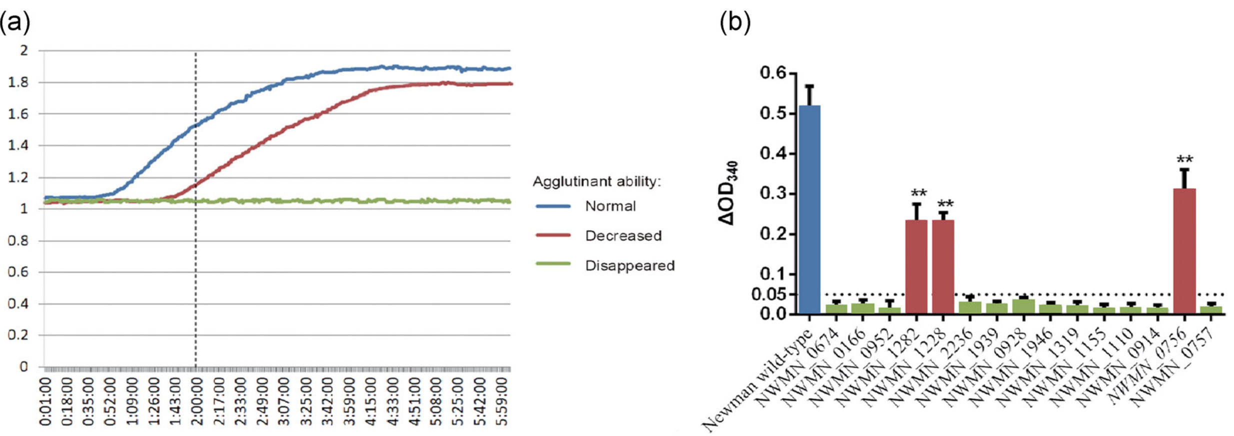

FIGURE 1 (a) The bacterial suspension was mixed with citrate plasma obtained from healthy volunteers (i.e. those with no obvious hemolysis and jaundice). The reactive system detected the agglutination reaction at 37°C and 340 nm OD. The agglutination reaction was evaluated every minute according to the instrument settings. Negative DTT results were identified if the OD_{340} value was less than 0.05 when compared with the initial results at 2 h. Decreased agglutination ability was observed when the OD_{340} value was above 0.05, and was significantly lower than that of the wild-type strain. (b) A total of 15 genes that showed association with plasma agglutination promotion in *Staphylococcus aureus* were screened, which included 3 genes related to decreased agglutination and 12 responsible for disappearance of agglutination. The experiments were performed with three biologically independent samples. Error bars were presented as means ± SD. $^{**}p < .001$

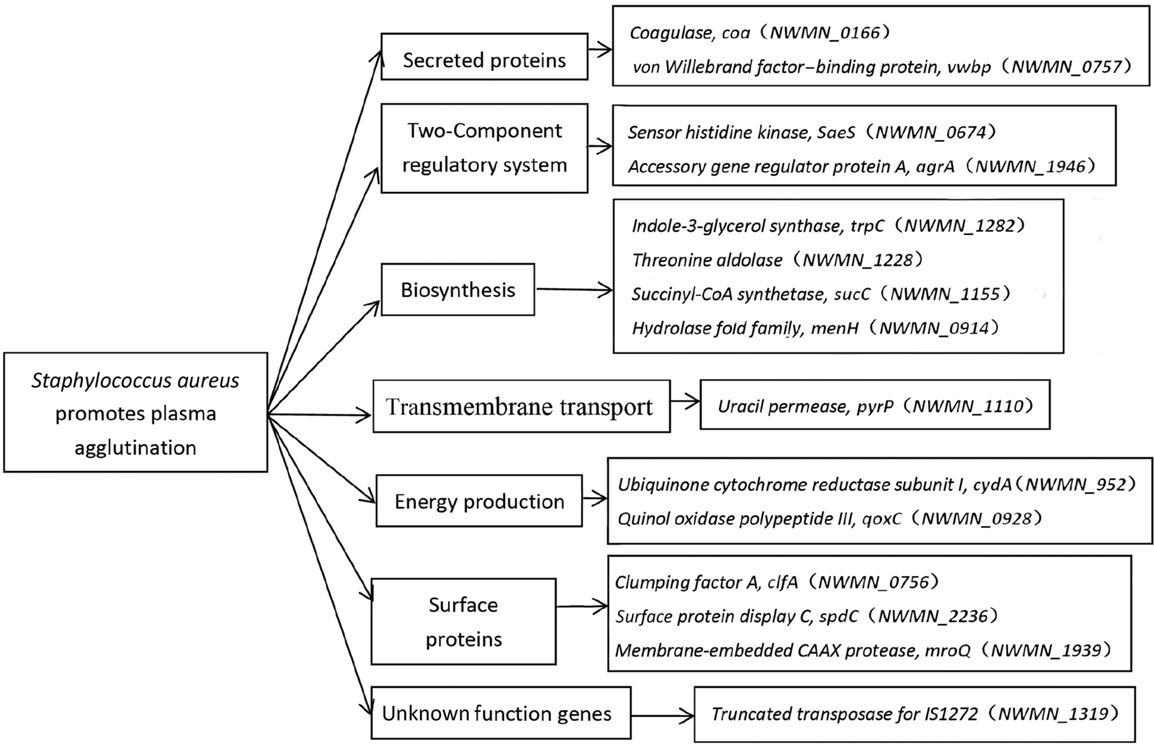

FIGURE 2 The proposed pathways involved in promoting plasma agglutination of novel genes in *Staphylococcus aureus*

mutants of *cydA*, *spdC*, and *mroQ*. This further proved that *cydA*, *spdC*, and *mroQ* genes are involved in the agglutination process of *S. aureus*. However, the results of complementary strains showed some discrepancies. The agglutinating abilities of *spdC* and *mroQ* complementary strains were restored, but not those of *cydA* (Figures 3 and 4). The RT-qPCR results revealed that the expression level of *cydA* in the complementary strain was much higher than that of the wild-type strain (Figure S1), which suggested that the expression of *cydA* itself might have no contribution to agglutination. We also found that the expressions of *coa*, *vWbp*, and *clfA* were significantly inhibited when the function of *cydA* was lost (Figure S2), indicating that the

TABLE 2 The biological information of the mutated genes

Quantity	Agglutinant ability	Located gene	Predictive function
12	Disappeared	*NWMN_0674*	Sensor histidine kinase, *SaeS*
10	Disappeared	*NWMN_0166*	Coagulase, *coa*
16	Disappeared	*NWMN_0952*	Ubiquinone cytochrome reductase subunit I, *cydA*
8	Decreased	*NWMN_1282*	Indole-3-glycerol synthase, *trpC*
1	Decreased	*NWMN_1228*	Threonine aldolase
14	Disappeared	*NWMN_2236*	Surface protein display C, *spdC*
13	Disappeared	*NWMN_1939*	Membrane-embedded CAAX protease, *mroQ*
4	Disappeared	*NWMN_0928*	Quinol oxidase polypeptide III, *qoxC*
5	Disappeared	*NWMN_1946*	Accessory gene regulator protein A, *agrA*
10	Disappeared	*NWMN_1319*	Truncated transposase for IS1272
6	Disappeared	*NWMN_1155*	Succinyl-CoA synthetase, *sucC*
6	Disappeared	*NWMN_1110*	Uracil permease, *pyrP*
8	Disappeared	*NWMN_0914*	Hydrolase fold family, *menH*
4	Decreased	*NWMN_0756*	Clumping factor A, *clfA*
1	Disappeared	*NWMN_0757*	von Willebrand factor–binding protein (*vwbp*)

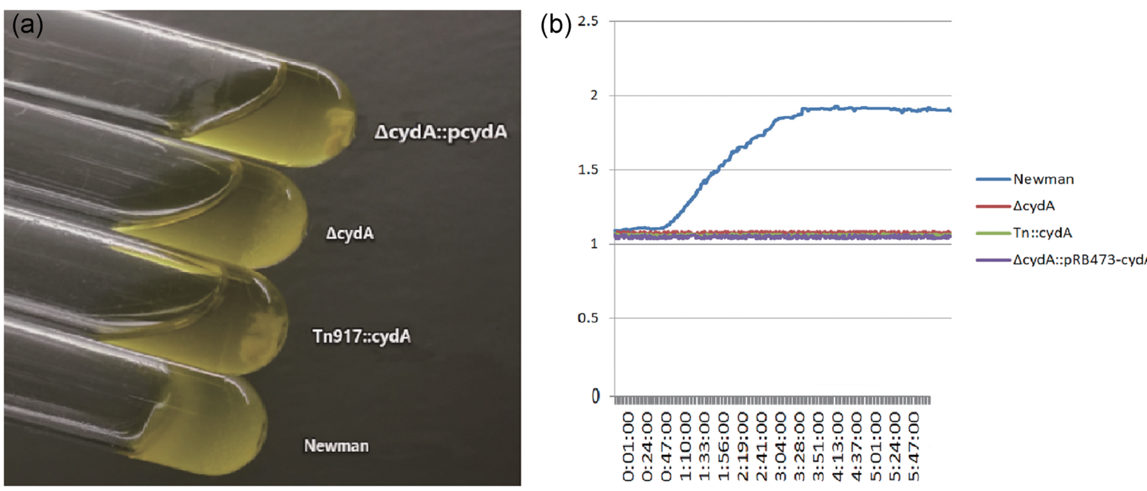

FIGURE 3 The *CydA* gene is necessary for plasma coagulation. (a) Tube plasma agglutination test proved that the *Staphylococcus aureus* Newman wild-type strain had plasma agglutination ability, but the *CydA* mutant, *cydA* knockout, and *cydA* complementary strains had no plasma agglutinating ability. (b) The results of dynamic turbidimetry also proved that the *S. aureus* Newman wild-type strain had plasma agglutination ability, but the *cydA* mutant, *cydA* knockout, and *cydA* complementary strains showed no change in the OD value

cydA mutant may present decreased agglutination ability by affecting the expression of other agglutination-associated genes.

3.3 | Impact of *cydA*, *spdC*, and *mroQ* on the bacteria growth and survival ability

To further understand the effects of *cydA*, *spdC*, and *mroQ* on plasma agglutination, we performed a growth rate and survival test. Our results showed that *cydA*, *spdC*, and *mroQ* gene detentions did not affect bacterial growth ability, and the growth status was the same as that of the wild-type strain within 12 h (Figure S3). Furthermore, the systemic infectious tests showed that deletion of the three genes could significantly improve mice's survival ability, and so the pathogenicity of the mutant strains was significantly reduced (Figure S4).

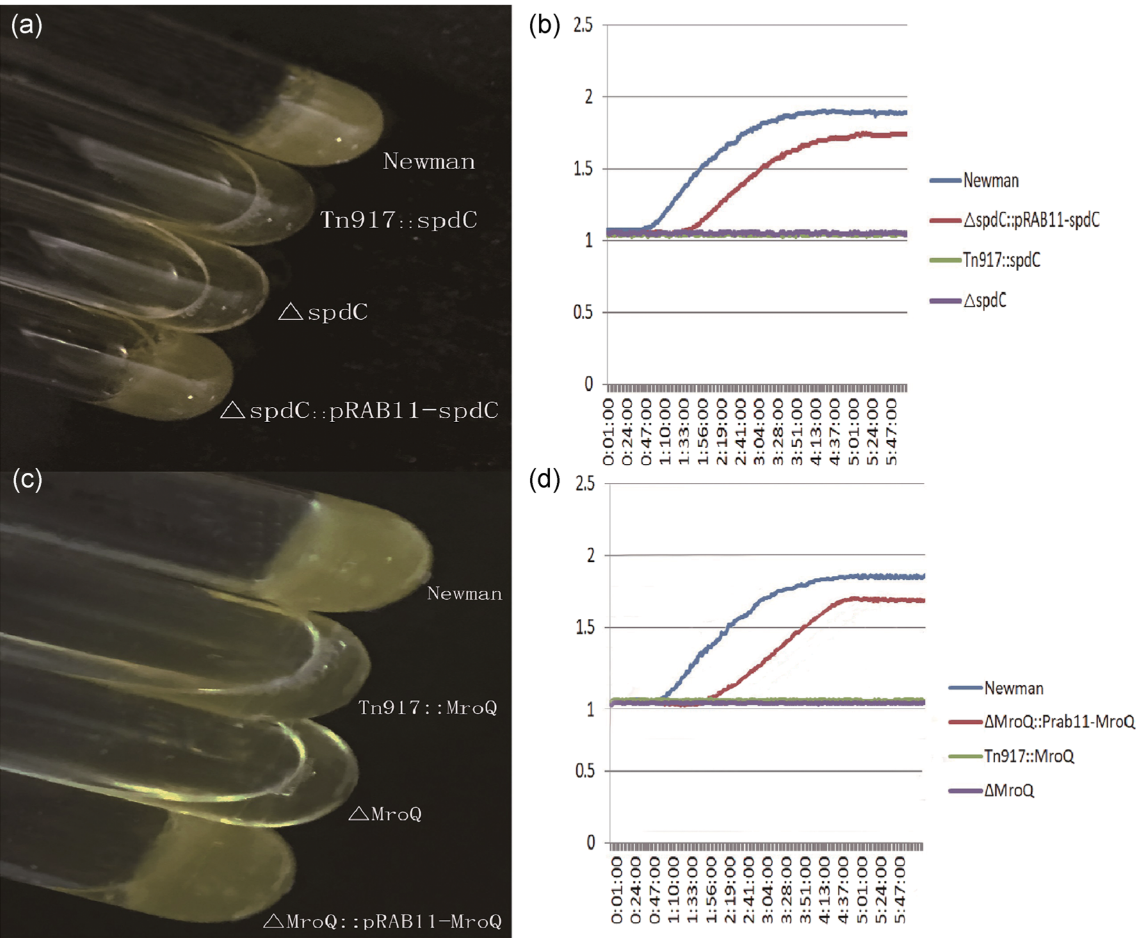

FIGURE 4 *SpdC* and *mroQ* genes are necessary for plasma coagulation. (a, c) Tube plasma agglutination test proved that the *Staphylococcus aureus* Newman wild-type strain and the *SpdC* or *mroQ* complementary strain had plasma agglutinating ability. (b, d) The results of DTT also proved that the *SpdC* or *mroQ* mutant strain and the *SpdC* or *mroQ* knockout strain demonstrated no change in the OD value

4 | DISCUSSION

Agglutination is considered a vital factor in *S. aureus* pathogenicity and is associated with infections at various sites. Investigation into the detailed coagulation mechanism can benefit the development of novel therapeutic strategies. In this study, we screened the *S. aureus* Newman mutant library and identified 15 genes, among which 7 were reported previously and 7 were not reported. The functions of the seven novel genes included biosynthesis, energy metabolism, surface proteins, and the insertion of sequences. To further understand these genes, we focused on *cydA*, *spdC*, and *mroQ* genes by constructing new knockout mutants and complementary strains. Our results proved that the freshly constructed *cydA*, *spdC*, and *mroQ* knockout mutants also similarly decreased plasma coagulation while the growth ability remained unchanged. Our results also demonstrated that these genes were indeed involved in promoting plasma coagulation by adopting gene complementary tests, and plasma coagulation was important risk factor for mice survive by adopting mice survival tests.

The *cyd* gene consists of *cydA* and *cydB*, and encodes ubiquinone cytochrome oxidase in the bacterial electron transfer chain.[16] Few studies suggest that electron transfer is involved in coagulase production, although the genes associated with energy production may affect the expression of virulence factors.[17,18] Energy metabolism inevitably changed the intracellular c-di-AMP concentration, an important secondary messenger related to the expression of virulence factors.[19] *cydA*-deleted transcriptome sequencing results obtained in this study showed that the expression of *saeRS* in *cydA*-deleted strains was at least five times lower than that observed in the Newman wild-type strains (data not shown). Furthermore, the known plasma-agglutinating functional genes under the control of *saeRS*, such as *coa*, *vwbp*, and *clfA*, showed significant downregulation (data not shown). Our results also showed that *cydA* complementary strains did not restore plasma agglutination. This can be explained as follows: the *cyd* gene is a four-subunit structure with a long nucleotide distance, and therefore, it is difficult to electroporate all the nucleotide sequences that encode the cyd gene into engineering plasmid strains.[20] As a result, the *cydA* mutant used in this study did not express the *cydB* gene, as the *cydA*

gene alone could not complement the phenotype.[21] *spdC* encodes abortive infection protein and can control histidine kinase involved in signal transduction.[22] In this study, the plasma coagulation function was not observed when the *spdC* function was diminished. WalKR, a two-component system, is mainly involved in controlling cell wall metabolism, which could positively regulate *spdC* through regulation of autolysin production.[22] At the same time, *spdC* could have a negative effect on WalKR regulon genes, thus synergistically regulating downstream virulence genes.[22] The coding product of *mroQ* is membrane-embedded CAAX protease, which belongs to the M79 protease-family protein. With the knockout of this gene, the plasma agglutination ability is lost. The possible mechanism involves hydrolysis of auto-inducing peptide by *mroQ* coding synthase, resulting in the inactivation of *agrC*. This subsequently inactivated the function of downstream RNAII and RNAIII, and the regulated genes, including coagulase, showed no expression.[23,24]

In summary, we found that *cydA*, *spdC*, and *mroQ* genes were associated with plasma coagulation of *S. aureus*. The findings of this study provide insights into the agglutinating mechanisms of *S. aureus* and offer new targets for the prevention of *S. aureus* infections.

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DISCLOSURE

The authors declare that there are no conflicts of interests.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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