capB2 Expression Is Associated with Staphylococcus aureus Pathogenicity

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CapB2 is recognized as a tyrosine kinase and is likely a vital factor in extracellular polysaccharide synthesis in Staphylococcus aureus, but its pathogenicity function and regulatory mechanism remain obscure. Here, we demonstrate that CapB2 enhances bacterial virulence in a murine model. Mice infected with the wild type SA75 strain exhibited significantly larger ($P < 0.05$) skin lesions from days 4 to 7 of infection than those challenged with the capB2 mutant strain. The effect on virulence was reverted by restoring the capB2 mutation to the wild type. The related components of the wild type SA75 cell wall in the capB2 mutant strain (SA75ΔcapB2) were thinner than wild type SA75 strain and the capB2 mutant complemented strain (SA75ΔcapB2-C), which was determined by the transmission electron microscopy. The survival percentages of the wild type strain SA75 and SA75ΔcapB2-C were significantly higher relative to SA75ΔcapB2. The results of qRT-PCR studies also indicated that mutations in regulatory gene sarA led to a drastic increase in capB2 gene transcription, with a 326-fold increase of growth at 6 h compared with the wild type strain, suggesting that sarA is a major negative regulator of capB2 expression. Taken together, these results demonstrate that the expression of CapB2 promotes S. aureus virulence in a mouse model of skin infection, and that capB2 gene transcription is regulated negatively by SarA.

Keywords: Staphylococcus aureus, capB2, sarA, virulence factor, regulation

INTRODUCTION

Staphylococcus aureus is a common bacterial pathogen which causes a wide range of human diseases, ranging from relatively benign skin and tissue infections to complicated life threatening diseases such as osteomyelitis, septic arthritis, and bacteremia. S. aureus pathogenicity is mediated by bacterial components and secreted virulence factors such as surface-associated adhesins, capsular polysaccharide (CP), and exotoxins. Studies have shown that CP is an important virulence factor in S. aureus, and it is believed to enhance microbial virulence (Nilsson et al., 1997; Thakker et al., 1998) in host immune evasion (Nanra et al., 2013).

More than 90% of S. aureus strains express CP, and 13 capsular serotypes have been described to date (Arbeit et al., 1984; Karakawa et al., 1985; von Eiff et al., 2007). Polysaccharide capsules corresponding to serotypes 1 and 2 (CP1 and CP2) are associated with heavily encapsulated...
mucoid strains, but are rarely encountered among clinical isolates (Luong T.T. et al., 2002; Luong et al., 2003). By contrast, CP5 and CP8 are both predominant among clinical infection isolates (Sompolinsky et al., 1985; O’Riordan and Lee, 2004), and are associated with microencapsulated strains exhibiting non-mucoid colony morphology. The cap5 and cap8 operons both contain 16 open reading frames, a vital factor in the synthesis and/or export of extracellular (Grangeasse et al., 2012), indicating that CapB2 is probably a regulator of chain length in CP biosynthesis of CapB1. However, despite CapB1 sharing more than 70% sequence identity with CapB2, no kinase activity could be detected for CapB1 in vitro. Of interest, the tyrosine kinase activity of CapB2 is more efficiently activated by the transmembrane kinase activity (Olivares-Illana et al., 2008). Whole genome sequencing of S. aureus has demonstrated that the gene products of capA2/capB2 (located elsewhere in the genome) are highly similar to those of capA1/capB1 (Kuroda et al., 2001; Soulat et al., 2006). However, despite CapB1 sharing more than 70% sequence identity with CapB2, no kinase activity could be detected for CapB1 in vitro. Of interest, the tyrosine kinase activity of CapB2 is more efficiently activated by the transmembrane stimulatory protein CapA1 than by the corresponding CapA2 activator (Kuroda et al., 2001; Soulat et al., 2006). Once CapB2 has been activated, its tyrosine cluster is phosphorylated (Grangeasse et al., 2012), indicating that CapB2 is probably a vital factor in the synthesis and/or export of extracellular polysaccharides.

The Staphylococcal accessory regular (sar) locus encodes a DNA binding protein, SarA, which is a key pleiotropic transcriptional regulator for the synthesis of extracellular virulence proteins (Cheung and Zhang, 2002). The sarA gene can regulate virulence expression dependently of agr (Cheung and Zhang, 2002). And it can also activate certain virulence genes by binding the promoter of the target gene directly (Cheung and Zhang, 2002).

In the present study, we analyzed the role of capB2 in the virulence of S. aureus by constructed an allelic replacement mutant of S. aureus 75. Our results show that a capB2 mutant of S. aureus is less virulence in the mice model of skin infection. In addition, we investigated the capB2 expression regulated by SarA.

### MATERIALS AND METHODS

#### Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are described in Table 1. S. aureus strain SA75 was isolated from a patient with a purulent skin infection at the First Affiliated Hospital in Wenzhou. Unless otherwise stated, Escherichia coli strains were cultured in Luria-Bertani medium, and S. aureus strains were grown in tryptic soy broth (TSB) medium, at 37°C with shaking at 200 rpm. The culture media were supplemented with appropriate antibiotics when required (ampicillin at 100 mg/l, chloramphenicol at 10 mg/l and anhydrotetracycline at 50 ng/ml).

### DNA Manipulations

Genomic DNA extraction was performed as described previously (Yu et al., 2014). Plasmid DNA extracted from E. coli strains was purified according to the manufacturer’s instruction (Axygen, Union City, CA, USA). To extract plasmid DNA from S. aureus strains, 20 μl lysostaphin (2 mg/ml) was used with suspension buffer S1 to re-suspend the collected cells from 10 ml overnight culture, incubated at 37°C for 1 h, and then processed following the manufacturer’s instruction (TIANGEN, Beijing, China).

### Construction of S. aureus capB2 Mutant Strain by Allelic Replacement

In order to generate a capB2 mutant from SA75, genomic DNA was extracted from S. aureus SA75 and the two sets of primers listed in Table 2 were used to amplify DNA fragments 1.5-kb upstream and downstream of capB2 gene, for capB2 mutagenesis (Cm' Amp').

#### TABLE 1 | Bacterial strains and plasmids used in this study.

| Strains and plasmids | Description | Source |
|----------------------|-------------|--------|
| **Strains** | | |
| S. aureus SA75 | Wild type, CP8-positive, clinical MRS strain isolated from an abscess | Laboratory stock |
| S. aureus SA75 ΔsarA | Isogenic sarA deletion mutant in SA75 | Laboratory stock |
| S. aureus SA75 ΔcapB2 | Isogenic capB2 deletion mutant in SA75 | This study |
| S. aureus SA75 ΔsarA-C | sarA mutant complemented with pRBsarA | This study |
| S. aureus SA75 ΔcapB2-C | capB2 mutant complemented with pRBcapB2 | This study |
| E. coli DH5α | Clone host strain | Laboratory stock |
| E. coli DC10B | Dam-RecA1, ΔompT1 ΔhisRΔMS endA1 ΔrecA1; clone host strain | Laboratory stock |
| **Plasmids** | | |
| pKOR1 | Shuttle cloning vector, temperature sensitive (Cm' Amp') | Laboratory stock |
| pKcapB2 | pKOR1 containing fragments 1500-bp upstream and 1500-bp downstream of capB2 gene, for capB2 mutagenesis (Cm' Amp') | This study |
| pRB473 | Shuttle cloning vector (Cm') | Laboratory stock |
| pRBsarA | pRB473 with sarA and its promoter (Cm') | This study |
| pRBcapB2 | pRB473 with capB2 and its promoter (Cm') | This study |

aCm' Amp', chloramphenical and ampicillin resistance.
Capsular Genotype Detection

Capsular genotype was detected by PCR as described previously (Verdier et al., 2007). Genomic DNA from strain SA75 was used as template, with primers cap5k-F and cap5k-R for detection of capsular type 5, and primers cap8I-F and cap8I-R for detection of capsular type 8. PCR product sizes were 361 and 173 bp for capsular types 5 and 8, and were confirmed by sequencing.

Growth Curve

*Staphylococcus aureus* wild type strain SA75 and its derivative strains were incubated overnight in 5 ml TSB at 37°C with shaking at 200 rpm. The overnight cultures were diluted in 30 ml TSB to obtain the same starting optical density (OD) at 562 nm. The growth of each strain was monitored by Microplate Manager 6 (Bio-Rad, USA) software at 1 h intervals for a total of 12 h.

S. aureus Survival Analyze in Human Blood

Blood samples from healthy donors were collected in 10 mg/ml heparin anticoagulation tubes. The ability of *S. aureus* SA75 and SA75ΔcapB2 to survive in human blood was determined under post-logarithmic growth phase. The strains were analyzed after washed twice and suspended to an OD of 0.6 at 562 nm. Then, 1 ml of heparinized human blood was inoculated with 100 µl aliquot of these suspensions and was incubated at 37°C. After 7 h, bacteria were appropriate diluted to detect the endpoint numbers of CFU. The survival rate of the bacteria cells was determined by comparing to the initial inoculum.

Mouse Model of Skin Infection Assay

Animal studies were approved by the Institutional Animal Care and Use Committee at Wenzhou Medical University. We confirm that all animal care and methods were performed in accordance with the guidelines and regulations approved by the Administration of Affairs Concerning Experimental Animals in China. The skin abscess model was performed as described previously (Li et al., 2012). Female, 4- to 6-week old, BALB/C-nu mice were used in the study. The mice were housed for 7 days before inoculation with food and water *ad libitum*. *S. aureus* cultures were grown to post-exponential phase, washed with sterile phosphate-buffered saline (PBS), and then suspended in PBS to achieve a concentration of 1.0 × 10^5 CFU/100 µl. The bacterial numbers were confirmed by plate counts. After the mice were anesthetized with diethyl ether, each mouse was injected subcutaneously with 100 µl aliquot of either *S. aureus* suspension or PBS. Skin lesions developing on test animals were observed daily, and the abscess area calculated using the formula length (L) × width (W). The values of L and W for each infected mouse were determined by caliper. Dermonecrosis was observed and recorded for 10 days, following which all animals were euthanized.

Table 2: Primers used in this study.

| Primers | Oligonucleotide (5’→3’)* |
|----------|--------------------------|
| capB2-up-F-BamHI | CGgagatccGACGCGTGTGGTGTAAAG |
| capB2-up-R-HindIII | CGCACGCGTGTGGTGTAAAG |
| capB2-down-F-HindIII | CGCACGCGTGTGGTGTAAAG |
| capB2-down-R-XhoI | CGCACGCGTGTGGTGTAAAG |
| UP-F-attB2 | GGACACCACTTTGCAAGAAGAGCCTTTG |
| Dn-R-attB1 | GGACACCACTTTGCAAGAAGAGCCTTTG |
| SW-cp-F | ATATTTTGATAGGAGACTG |
| XW-cp-R | TACAGGAAATACGAGAAGAG |
| Cp-BD-F | TACAGGAAATACGAGAAGAG |
| Cp-BD-R | TACAGGAAATACGAGAAGAG |
| Pstl-sarA-F | AAAAACgtcgATAAACCAATGCTAACCC |
| BamHI-sarA-R | CAGCGATCCATACGAGAAGAG |
| Pstl-cap-19F | AAAAACgtcgATTGTATAGGCTAACCC |
| BamHI-cap-19R | CAGCGATCCATACGAGAAGAG |
| cap5K-F | GTTACGAGTATAGGCTAACCC |
| cap5K-R | GTTACGAGTATAGGCTAACCC |
| cap8I-F | GCCGGAATAGGCTAACCC |
| cap8I-R | GCCGGAATAGGCTAACCC |
| gyrB-F | ACATTACAGCGCGGATAGG |
| gyrB-R | ACATTACAGCGCGGATAGG |
| cap-XF | GCTTCGGAATAGGCTAACCC |
| cap-XR | GCTTCGGAATAGGCTAACCC |
| sarA-qtF | GTTACGAGTATAGGCTAACCC |
| sarA-qtR | GTTACGAGTATAGGCTAACCC |
| Pstl | CGCggatccAGTGCCATTAGTGCAAAAC |
| capB2 | CGCggatccAGTGCCATTAGTGCAAAAC |
| cap8I | CGCggatccAGTGCCATTAGTGCAAAAC |
| cap5k | CGCggatccAGTGCCATTAGTGCAAAAC |
| up-R | CGCggatccAGTGCCATTAGTGCAAAAC |
| down-F | CGCggatccAGTGCCATTAGTGCAAAAC |
| down-R | CGCggatccAGTGCCATTAGTGCAAAAC |
| BamHI | CGCggatccAGTGCCATTAGTGCAAAAC |
| Pstl | CGCggatccAGTGCCATTAGTGCAAAAC |

*Restriction sites are indicated by lowercase letters; underlined sequences denote recombination sites.
Electron Microscopy Study on Abscess Tissue
Overnight cultures of *S. aureus* SA75, capB2 mutant and the complemented strains were harvested and washed twice with sterile PBS, and then suspended to achieve a concentration of $1.0 \times 10^8$ CFU/100 µl. Mouse model of subcutaneous abscess was performed as above. The abscess tissue was obtained at the fourth day of infection. Samples were fixed and processed further for transmission electron microscopy.

RNA Extraction, cDNA Synthesis, and Quantitative Reverse Transcription-PCR (qRT-PCR)
The quantitative Real-Time PCR was performed according to the MIQE guidelines. For RNA isolation, *S. aureus* strains were diluted 1:100 in TSB from overnight cultures and grown to post-exponential phase at 37°C with shaking at 200 rpm. Total RNA was isolated and purified using a PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA); 2 µg purified RNA was then used to obtain cDNA using a PrimeScript RT reagent kit (Bio-Rad, USA) with the Bio-Rad CFX96 Manager software. Oligonucleotide primers used are listed in Table 2. Wild type strain SA75 was used as a control (relative expression = 1), and gyrB was used as a reference gene to investigate genes of interest. RNA transcript levels were calculated by the method of delta delta Ct ($\Delta \Delta$Ct) (Montgomery et al., 2012). Data analysis was carried out using Bio-Rad CFX software. Each reaction was performed in triplicate.

Statistical Analysis
Skin abscesses in the mouse infection model were compared with one-way analysis of variance (ANOVA) using SPSS Statistics 17.0. Results were considered statistically significant if P-values were < 0.05. Relative expression levels resulting from qRT-PCR were analyzed using GraphPad Prism 5.0 software.

RESULTS

Capsular Genotype and Growth Curve Analysis
In order to rule out the potential influence of the capB2 deletion on bacterial growth rate, the growth curves of strains SA75, SA75ΔcapB2, and SA75ΔcapB2-C were monitored hourly. The growth curves of the three strains were measured in TSB medium at the same starting OD of 562 nm, with TSB medium used as a blank control. There were no substantial differences in growth curves among all three strains. The capB2 gene was expressed at the indicated time point (3, 6, 9, 12 h) and assessed in strain SA75, with expression shown to be highest at the 6 h time point.

Deletion of capB2 in *S. aureus*
The capB2 deletion was introduced by allelic replacement into *S. aureus* strain SA75, yielding the mutant strain SA75ΔcapB2. High-efficiency cloning strain *E. coli* DC10B was used as an intermediate host instead of *S. aureus* RN4220 in the study in order to improve the transformation efficiency. To confirm the deletion of capB2SA75ΔcapB2 was amplified with primers SW-cp-F and XW-cp-R using LA Taq DNA polymerase. The band size in strain SA75ΔcapB2 reflected a 693 bp deletion compared to the wild type strain. The relative expression of capB2 in strain SA75ΔcapB2 was dramatically decreased relative to that of strain SA75 (Figure 1).

Absence of capB2 Decreased *S. aureus* Viability in Human Blood
To determine the contribution of capB2 in the pathogenicity of *S. aureus*, we compared the relative viability of *S. aureus* SA75 and SA75ΔcapB2 in human blood. After 7 h incubation, the survival percentages of the wild type strain SA75 and the complemented strain SA75ΔcapB2-C were significantly higher relative to SA75ΔcapB2 (Figure 2).

Deletion of capB2 Decreases Virulence of *S. aureus* SA75 in a Skin Infection Model
To determine the role of capB2 in the pathogenesis of *S. aureus*, 4-to-6 week old BALB/C-nu mice were subcutaneously injected with $1.0 \times 10^5$ CFU of strains SA75, SA75ΔcapB2, or SA75ΔcapB2-C. As shown in Figure 3b, the resulting abscess sizes in mice inoculated with the capB2 mutant strain were smaller than the lesion sizes among animals infected with SA75 or the complemented strain SA75ΔcapB2-C. The lesions remained until the end point of the experiment (Figure 3a). The overall rates of skin lesions were similar between the wild type and the complemented strain. As shown in Figure 3a, animals challenged with $1.0 \times 10^5$ CFU of SA75 had significantly larger ($P < 0.05$) skin lesions from the fourth day to the seventh day of infection than animals challenged with an equivalent amount of SA75ΔcapB. Likewise, significant differences between the SA75ΔcapB and the
SA75ΔcapB-C strains were observed from the second day to the ninth day of infection. These data demonstrate that the presence of capB2 in *S. aureus* promotes bacterial virulence.

**Electron Microscopy Study**

To analyze the influence of the deletion of capB2 gene in *S. aureus* SA75, we used the transmission electron microscopy to detect the difference between *S. aureus* SA75, SA75ΔcapB and SA75ΔcapB-C strains. As shown in Figure 4, the related components of *S. aureus* SA75 cell wall in SA75ΔcapB was thinner than SA75 and SA75ΔcapB-C.

**Effect of sarA on capB2 Expression**

In our previous work, we constructed a sarA mutant strain. To eliminate the possibility of a second mutant, we constructed a sarA-complemented strain containing a plasmid bearing the sarA gene. Since the transcriptional expression of capB2 was
highest at the 6 h time point, we selected that time point to assess expression differences among the three strains. The effect of sarA on expression of capB2 was assessed by qRT-PCR. As shown in Figure 5, deletion of sarA had a dramatic effect on capB2 expression, with a 326-fold increase at 6 h of growth compared with that of the wild type strain. The difference in capB2 expression was thus reversed by complementation of sarA. These results suggest that sarA represses the expression of capB2.

**DISCUSSION**

Capsular polysaccharide produced by *S. aureus* has been acknowledged as an important virulence factor for bacterial infection. Methods for *S. aureus* capsule serotyping include enzyme-linked immune sorbent assay (ELISA) inhibition, colony immune blotting, and double immune diffusion assay (the gold standard). However, all of them are based on the availability of specialized antibodies, and are not suitable for rapid capsule serotyping. Grunert et al. (2013) have developed a high-throughput method to identify capsular serotypes 5, 8 and NT (non-typable). DNA sequence analysis of *S. aureus* has determined that the cap5HIJK and cap8HIJK genes are the primary capsular type-specific genes. This information allowed us to identify the capsular genotype of SA75 using type-specific oligonucleotide primers (Verdier et al., 2007) designed against the cap5K and cap8I genes, with the results demonstrating that SA75 belongs to capsular type CP8.

Since the skin infection model was shown to be more sensitive, the role of CapB2 in *S. aureus* pathogenicity was evaluated using the skin infection model. Notably, the capB2 deletion decreased virulence in the mouse model of *S. aureus* skin infection, but full virulence was restored by transformation with the complementation plasmid containing the capB2 gene. Neither the capB2 mutant strain nor the complementation strain showed any difference in bacterial growth compared with the wild type strain when grown in TSB medium. Of important, the mutant strain when grown in TSB medium. Of important, the mutant strain, deletion of capB2 in *S. aureus* strain resulted in difference in the related components of cell wall. The role of CP in the pathogenesis of *S. aureus* infection has been reported by a number of studies (Baddour et al., 1992; Luong and Lee, 2002; O’Riordan and Lee, 2004; Nanra et al., 2013), which found that expression of CP5 or CP8 can enhance bacterial virulence both in vitro and in vivo. Taken together, we conclude that the decrease in virulence of the capB2 mutant strain is likely due to decreased synthesis of CP rather than the effect on overall growth.

Similar to other virulence proteins, CP and CapB2 are also regulated by a complex regulatory network. Luong T. et al. (2002) investigated the regulation of *S. aureus* CP8 expression by the global regulators agr and sarA, and found that the CP8 expression was positively regulated by agr and sarA, with agr being the major activator. However, our results demonstrate that capB2 expression was dramatically repressed by sarA, suggesting that SarA is a negative regulator of capB2 expression. The difference in regulation may be due to the complex regulatory network present in *S. aureus*, with the sarA interface within the regulatory network controlling the expression of virulence-associated proteins (Arvidson and Tegmark, 2001). In addition, the expression of CP is also influenced by several regulatory proteins. The regulation of CP by the two-component system KdpDE has been reported by Zhao et al. (2010), who found that CP expression was reduced in the kdpDE mutant strain, and that positive regulation occurred via the phosphorylation of KdpE. Furthermore, Lei and Lee (2015) found that RbsR (a Lac family of repressor) positively regulates cap gene expression by directly binding to the cap operon promoter region, as evidenced by electrophoretic mobility shift assays (EMSA). In brief, the complexity of virulence regulatory networks in *S. aureus* is such that a single virulence protein may be controlled by a remarkable number of regulators.

In summary, CapB2 is a probable virulence gene in *S. aureus* infection, with deletion of capB2 resulting in decreased virulence in a mouse model of skin infection. SarA, on the other hand, is a negative regulator of capB2 expression, but further in-depth studies are required to determine if it directly regulates capB2.

**ETHICS STATEMENT**

This study was reviewed by the Ethics Committee of the first Affiliated Hospital of Wenzhou Medical University.

**AUTHOR CONTRIBUTIONS**

DL performed the experiments, wrote the main manuscript text and analyzed the data. YG, SW, ZW, JL, and XQ helped to conduct and perform the experiments. ZC, LH, and XZ contributed to preparing the reagents/materials and supervised the project. LW and FY conceived the experiments and helped with the discussion of results. All authors reviewed the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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