Stp1 Loss of Function Promotes β-Lactam Resistance in *Staphylococcus aureus* That Is Independent of Classical Genes

Aditi Chatterjee,[a] Raymond Poon,[a] Som S. Chatterjee[a]

aDepartment of Microbial Pathogenesis, School of Dentistry, University of Maryland and Institute of Marine and Environmental Technology, Baltimore, Maryland, USA

ABSTRACT β-Lactam resistance in *Staphylococcus aureus* limits treatment options. Stp1 and Stk1, a serine-threonine phosphatase and kinase, respectively, mediate serine-threonine kinase (STK) signaling. Loss-of-function point mutations in *stp1* were detected among laboratory-passaged β-lactam-resistant *S. aureus* strains lacking *mecA* and *blaZ*, the major determinants of β-lactam resistance in the bacteria. Loss of Stp1 function facilitates β-lactam resistance of the bacteria.

KEYWORDS Stp1, β-lactam resistance, *Staphylococcus aureus*

Over several decades, *Staphylococcus aureus* has successfully bypassed the action of antimicrobials through sophisticated resistance mechanisms (1). Its ability to develop resistance together with its vast array of virulence genes has made it a serious threat to global health (2). β-Lactam drugs have been a benchmark for treatment of *S. aureus* due to their superior efficacy and safety over other drugs. However, rampant β-lactam resistance among clinical strains of *S. aureus* makes it difficult to treat infections caused by the bacteria.

The two major factors that traditionally drive β-lactam resistance in *S. aureus* are penicillin binding protein 2a (PBP2a) and β-lactamase (3), encoded by *mecA* and *blaZ*, respectively. We previously reported that *S. aureus* strains (COLnex and SF8300ex; derivatives of COL and SF8300 strains, respectively, lacking both *mecA* and *blaZ*) can also produce high-level β-lactam resistance when passaged in the laboratory (4, 5). These results indicated that unknown factors that are independent of *mecA* and *blaZ* were responsible for resistance in these strains. Whole-genome sequence analysis of the resistant passaged strains (i.e., COLnex and SF8300ex strains passaged in ceftobiprole, ceftaroline, and nafcillin; i.e., six strains total) revealed mutations in several interesting genes, which suggested that they might play a role in this uncanonical mode of β-lactam resistance (5). One of these candidate genes, which codes for a serine-threonine phosphatase (*stp1*), had point mutations G169S and Q31X in the SF8300ex strain that was passaged in ceftobiprole and ceftaroline, respectively. Interestingly, none of the COLnex background strains showed any mutations in *stp1* (5).

Although both *stk1* and *stp1* are conserved in COLnex and SF8300ex strains, the former possesses a truncated Stk1 due to a natural point mutation that has been reported in COL (the parent of COLnex) (6). Thus, an absence of *stp1* point mutation may be due to an inherent defect in STK signaling among COLnex passaged strains.

STK signaling is predominantly present in eukaryotes, and it was recently shown to be present in bacteria. Since the discovery of the first serine-threonine kinase in *Myxococcus xanthus* isolates, several studies have elucidated the roles of STK signaling in bacteria (7). In *S. aureus*, STK signaling is mediated by a serine-threonine kinase and phosphatase, encoded by *stk1* and *stp1*, respectively (8). Stk1 and Stp1, through their opposing functions, maintain the balance of STK signaling.

Recent studies have shown that STK signaling plays important roles in *S. aureus* pathogenesis (9). An *stp1* deletion mutant strain displayed attenuated hemolysis due to
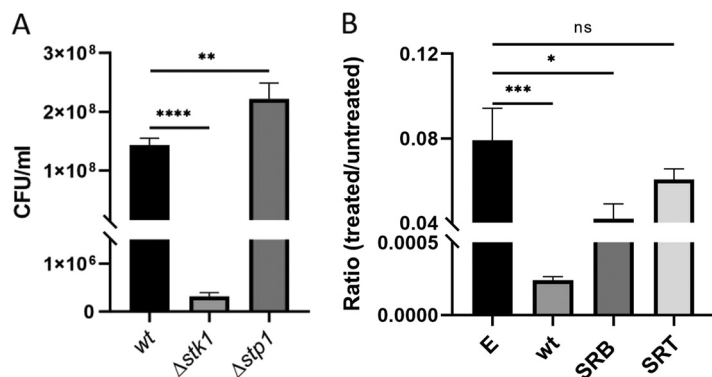
FIG 1 Deletion of *stp1* facilitates resistance in SF8300ex. (A) CFU/ml of knockout strains (Δ*stk1* and Δ*stp1*) and wild-type SF8300ex after 4 h of treatment with 0.25 μg/ml nafcillin. (B) Ratio of survival of Δ*stp1*-complemented strains after 4 h of treatment with 0.25 μg/ml nafcillin. Δ*stp1* complemented with empty-vector pTX_{Δ} (E), wild-type *stp1* (wt), mutant G169S *stp1* (SRB), and mutant Q31X *stp1* (SRT). *, $P < 0.01$; **, $P < 0.009$; ***, $P < 0.0008$; ****, $P < 0.0001$. Student's *t* test was used to analyze the P values using GraphPad Prism software. All experiments were repeated at least twice. ns, not significant.

low-level production of alpha-toxin compared with its isogenic wild-type (wt) strain (10). Stp1 has also been shown to play a role in resistance of cell wall-reactive antibiotics in *S. aureus* isolates (11, 12). On the other hand, an *stk1* deletion mutant was found to be susceptible to many cell wall-active antibiotics (6, 9, 13). Although much of the mechanistic detail on how STK signaling mediates these processes is currently lacking, it was recently shown that Stk1 controls the activity of β-lactamase in *S. aureus* through phosphorylation of its sensory inducer, BlaR1 (14). These results indicated that STK signaling mediates β-lactam resistance in *S. aureus*, at least in part, via the known mediators of resistance. Our results, i.e., detection of Stp1 mutations among resistant passaged strains lacking *mecA* and *blaZ*, indicated that STK signaling may also mediate β-lactam resistance independent of the known mediators of resistance. Thus, in this study, we sought to determine the role of STK signaling in β-lactam resistance in strains that lacked *mecA* and *blaZ* and to evaluate the role of the Stp1 mutations (G169S and Q31X) detected among our resistant passaged strains.

We created *stk1* and *stp1* deletion mutants (Δ*stk1* and Δ*stp1*, respectively) in the SF8300ex strain and compared their susceptibilities to nafcillin, a β-lactam antibiotic commonly used for staphylococcal infection (Fig. 1A) (15). Creation of isogenic strains in this manner allowed us to determine the roles of *stk1* and *stp1* in β-lactam resistance and to compare them with their isogenic parental strain (SF8300ex). For this assay, bacteria were grown in the presence of nafcillin 0.25 μg/ml (half the MIC of SF8300ex) for 4 h and then plated to determine bacterial CFU counts (15). Our results indicated that the Δ*stk1* mutant strain was highly susceptible to nafcillin treatment, whereas Δ*stp1* survived the nafcillin challenge better than the SF8300ex strain. Comparison of the growth profiles of these strains in the absence of nafcillin ensured that the difference in survival of these strains on nafcillin challenge was not due to any inherent growth defect (see Fig. S1 in the supplemental material). The resistance phenotype was confirmed with another β-lactam drug, cefoxitin, at a dose that represented half its MIC (15) (see Fig. S3 in the supplemental material). Thus, STK signaling appeared to be important in mediating survival against a β-lactam challenge in strains that lacked both *mecA* and *blaZ*. Furthermore, our results suggested that deletion of *stk1* and *stp1* had opposite effects on β-lactam resistance in the SF8300ex strain.

To determine the role of *stp1* point mutations G169S and Q31X detected among our resistant passaged strains SRB and SRT, respectively, we cloned *stp1* genes from the wt SF8300ex strain and the mutants in a constitutively expressing vector (pTX_{Δ}) (15). The resultant vectors along with the empty-vector control were transformed to the Δ*stp1* strain to generate Δ*stp1*-complemented strains. Growth assay of the resultant strains in rich medium showed attenuated growth of the strains that were complemented with
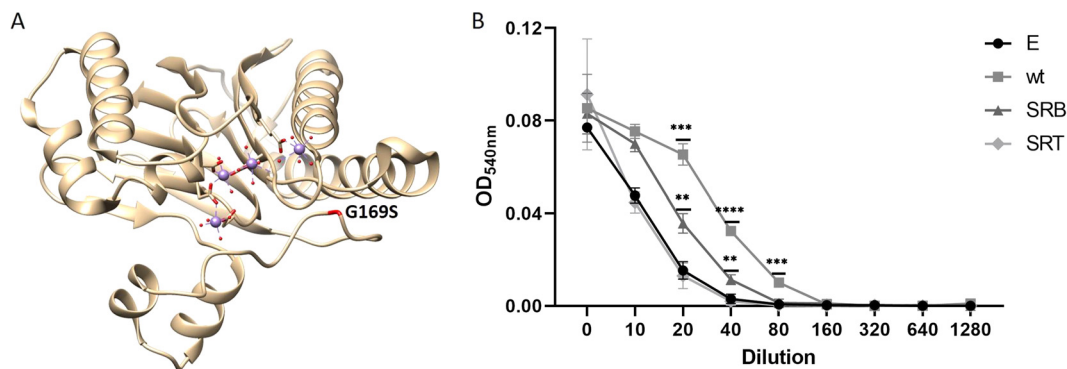
FIG 2 Loss of function of Stp1 reduces hemolytic activity of SF8300ex. (A) G169S mutation annotated in the crystal structure of Stp1 bound to metals (purple spheres). The figure was generated using UCSF Chimera and PDB entry 5F1M. (B) Decrease in hemolysis in Δ*stp1* strains expressing mutant Stp1s. Δ*stp1* complemented with empty-vector pTX_Δ (E), wild-type *stp1* (wt), and mutant *stp1* strains SRB (G169S) and SRT (Q31X). *, $P < 0.01$; **, $P < 0.009$; ***, $P < 0.0008$; ****, $P < 0.0001$. Student's *t* test used to analyze the *P* values using GraphPad Prism software. Experiments were repeated at least twice.

the wt and SRB *stp1* compared with those complemented with the empty-vector or the SRT *stp1* (see Fig. S2 in the supplemental material). Because the SRT mutation (Q31X) produced a truncated Stp1 after 31 amino acids, we hypothesized that the resultant growth defects of the above-mentioned strains were likely due to the overexpression of full-length Stp1 (wt and SRB). Because of this growth difference, we challenged the complemented strains with or without nafcillin, and bacterial survival was presented as the ratio of bacterial CFUs between these treatment conditions. Complementation of wt *stp1* reduced bacterial survival compared with the empty-vector control (Fig. 1B), supporting an important role for Stp1 in modulating a β-lactam challenge. The survival of the strain with SRB *stp1* was detected to be significantly less than that of the empty-vector complemented strain, whereas the strain with SRT *stp1* showed bacterial survival that was comparable to that of the strain that received the empty vector. These results suggested that G169S *stp1* likely had a loss of Stp1 function mutation, and the Q31X *stp1* mutant did not have any Stp1 function, as expected due to its premature truncation caused by a nonsense mutation. Stp1 belongs to the protein phosphatase 2C (PP2C) family, which is known to use metal ions for activity. On mapping the G169S mutation onto the *stp1* crystal structure, we found that the point mutation is near the metal binding site of the protein, which may affect its catalytic activity (Fig. 2A) (16, 17).

Stp1 is known to modulate *S. aureus* hemolysis. As shown previously, a Δ*stp1* strain causes attenuated hemolysis of sheep blood compared with its wt strain (10). To examine whether the Stp1 mutants had a loss of function mutation through an alternative way, we checked the hemolytic activity of the Δ*stp1*-complemented strains. Culture filtrates from 12-h-grown bacterial cultures were used in various dilutions to check their hemolytic potential. The 0.5% (vol/vol) washed sheep erythrocytes were incubated with culture filtrates at 37°C for 1 h, as described previously (6). Both SRB and SRT complements had attenuated hemolytic activity compared with wt Stp1 (Fig. 2B). These results further indicated that the mutated Stp1s (G169S and Q31X) among our resistant passaged strains were loss-of-function mutations.

Our results indicate that loss of Stp1 mutation facilitates β-lactam resistance in *S. aureus* isolates that lack *mecA* and *blaZ*. Previously, we showed that *pbp4* played a pivotal role in β-lactam resistance of SRB and SRT (15). To determine whether the Stk1/Stp1 pathway modulates *pbp4* expression, we performed reporter assays that suggested that deletion of *stk1* or *stp1* did not play any role in *pbp4* regulation (see Fig. S4 in the supplemental material). Thus, the mechanistic basis of how the Stk1/Stp1 pathway mediates β-lactam resistance that lacks the classical mediators (*mecA* and *blaZ*) remains to be elucidated. In a recent study, Stp1 was shown to regulate the SarA/MgrA family of transcription regulators, which in turn affect staphylococcal viru-

lence and resistance (18–21). Thus, it is possible that Stp1-mediated β-lactam resistance is carried out via the SarA/MgrA family of transcriptional factors. The proteins that mediate this mode of β-lactam resistance are currently unknown.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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