A Nonclassical Mechanism of β-Lactam Resistance in Methicillin-Resistant *Staphylococcus aureus* and Its Effect on Virulence

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**ABSTRACT** Methicillin-resistant *Staphylococcus aureus* (MRSA) is a group of pathogenic bacteria that are infamously resistant to β-lactam antibiotics, a property attributed to the *mecA* gene. Recent studies have reported that mutations associated with the promoter region of *pbp4* demonstrated high levels of β-lactam resistance, suggesting the role of PBP4 as an important non-*mecA* mediator of β-lactam resistance. The *pbp4*-promoter-associated mutations have been detected in strains with or without *mecA*. Our previous studies that were carried out in strains devoid of *mecA* described that *pbp4*-promoter-associated mutations lead to PBP4 overexpression and β-lactam resistance. In this study, by introducing various *pbp4*-promoter-associated mutations in the genome of a MRSA strain, we demonstrate that PBP4 overexpression can supplement *mecA*-associated resistance in *S. aureus* and can lead to increased β-lactam resistance. The promoter and regulatory region of *pbp4* is shared with a divergently transcribed gene, *abcA*, which encodes a multidrug exporter. We demonstrate that the promoter mutations caused an upregulation of *pbp4* and downregulation of *abcA*, confirming that the resistant phenotype is associated with PBP4 overexpression. PBP4 has also been associated with staphylococcal pathogenesis, however, its exact role remains unclear. Using a *Caenorhabditis elegans* model, we demonstrate that strains having increased PBP4 expression are less virulent than wild-type strains, suggesting that β-lactam resistance mediated via PBP4 likely comes at the cost of virulence.

**IMPORTANCE** Our study demonstrates the ability of PBP4 to be an important mediator of β-lactam resistance in not only methicillin-susceptible *Staphylococcus aureus* (MSSA) background strains as previously demonstrated but also in MRSA strains. When present together, PBP2a and PBP4 overexpression can produce increased levels of β-lactam resistance, causing complications in treatment. Thus, this study suggests the importance of monitoring PBP4-associated resistance in clinical settings, as well as understanding the mechanistic basis of associated resistance, so that treatments targeting PBP4 may be developed. This study also demonstrates that *S. aureus* strains with increased PBP4 expression are less pathogenic, providing important hints about the role of PBP4 in *S. aureus* resistance and pathogenesis.

**KEYWORDS** PBP4, *Staphylococcus aureus*, beta-lactam resistance, virulence

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$t*aphylococcus aureus$ is a Gram-positive pathogen that can cause skin and soft tissue infections (SSTIs), bacteremia, osteomyelitis, and sepsis in humans (1). Along with being equipped with a wide array of virulence factors, *S. aureus* is also resistant to a wide range of antibiotics, making infections difficult to treat (2). In particular, methicillin-resistant *Staphylococcus aureus* (MRSA) is infamous for being resistant to β-lactams, resulting in over 120,000 deaths globally in 2019 (3).

β-Lactams are a class of antibiotics known for their safety, efficacy, and tissue distribution which makes them the most commonly prescribed antibiotics (4). β-Lactams...
bind to penicillin binding proteins (PBPs), which are integral proteins involved in the final stages of cell wall synthesis. The binding of β-lactams to PBPs causes their inactivation, leading to weakening of the cell wall and, subsequently, cell death (5). MRSA contains the gene mecA, which encodes PBP2a, a PBP that has decreased affinity toward β-lactams, allowing cells to survive even in high concentrations of β-lactams (2).

Historically, resistance mechanisms in S. aureus have been acquired in waves. With the introduction of every new generation of β-lactams, S. aureus has been able to develop new resistance mechanisms (2). This ability of S. aureus to constantly develop resistance mechanisms makes it important to focus on other, novel mechanisms of resistance. Keeping this in mind, our previous studies involved serial passaging of strains in increasing amounts of β-lactams with the aim of identifying non-mecA mechanisms of resistance (6). We determined that mutations associated with the promoter region of PBP4 were largely prevalent in resistant strains (6, 7). Various studies by other groups also identified pbp4-associated mutations in laboratory-generated (8, 9) as well as in clinically isolated (10, 11) resistant strains of both MRSA and MSSA backgrounds, suggesting the clinical relevance of these mutations. PBP4, a nonessential, low-molecular-weight (LMW) PBP in S. aureus, is produced in small amounts, and its role in resistance or pathogenesis is not very well described (12). We previously demonstrated that pbp4-promoter-associated mutations led to significant β-lactam resistance in strains devoid of mecA by causing increased PBP4 expression and enhanced cell wall cross-linking (7). However, the effect of these mutations in strains containing mecA is unknown. Thus, in this study, we inspected whether PBP4, through the means of overexpression, supplemented mecA-mediated β-lactam resistance. We used the strain SF8300, a derivative of USA300, which is one of the most prominent community-associated MRSA (CA-MRSA) strains detected in the United States (2). pbp4-promoter-associated mutations that were detected in a laboratory-passaged resistant strain that was devoid of mecA (namely, CRB) and in a resistant strain containing mecA (namely, CRT) were introduced in SF8300 (6). Furthermore, a pbp4-promoter-associated mutation detected in a β-lactam-resistant clinical isolate (namely, Strain 1) was also used in this study (13). Using these promoter mutants, we demonstrated that pbp4-promoter-associated mutations lead to PBP4 overexpression and increased β-lactam resistance in MRSA strains in a mechanism similar to what was previously shown in MSSA strains (7, 13).

pbp4 shares its promoter and regulatory region with abcA, a gene that encodes an ATP-binding cassette transporter protein. ABC transporters are notorious for enabling resistance to chemotherapeutic agents in both prokaryotes and eukaryotes via export activity. Since AbcA has previously been associated with antibiotic resistance (14), we were interested in understanding whether the promoter-associated mutations altered abcA expression that potentially led to AbcA-mediated β-lactam resistance. However, our results indicated that while the promoter-associated mutations led to increased pbp4 expression, they resulted in downregulation of abcA, indicating that AbcA did not have any role in β-lactam resistance and that the resistance phenotype observed in the promoter mutants was attributed to PBP4.

Along with β-lactam resistance, PBP4 has recently been associated with virulence in previous studies. However, the exact role of PBP4 in virulence remains unclear, as these studies had contrasting reports (15, 16). In order to get a better understanding of the role of PBP4 in virulence, we used a Caenorhabditis elegans infection model and demonstrated that MRSA strains containing pbp4-promoter-associated mutations had decreased virulence compared with the wild-type (WT) strain. We also generated abcA mutant strains to demonstrate that abcA downregulation due to the promoter mutations did not result in the observed resistance and virulence phenotypes.

Our findings suggest the importance of monitoring PBP4-associated resistance in both MRSA and MSSA strains and indicate that treatment options would potentially have to consider targeting both PBP2a and PBP4, as when present, both the proteins independently mediate β-lactam resistance via distinct mechanisms leading to increased resistance. Our results also confirm that promoter-associated mutations only allow for
PBP4 overexpression and do not facilitate AbcA-mediated resistance, indicating that PBP4-associated resistance has the potential of being a prominent resistance mechanism in the future. Finally, our results also provide important clues associated with the role of PBP4 in virulence and suggest that strains with increased PBP4 may have decreased virulence, a phenomenon that needs to be studied further in detail in the future.

RESULTS

*pbp4*-promoter-associated mutations led to increased PBP4 expression and \( \beta \)-lactam resistance in MRSA strains. Our previous studies demonstrated that *pbp4*-promoter-associated mutations led to PBP4 overexpression that subsequently resulted in \( \beta \)-lactam resistance (7, 13). These studies were performed by introducing promoter-associated mutations into strains devoid of *mecA*. Using allelic replacement, we created isogenic mutants by introducing three different, previously detected mutations in the *pbp4* promoter region of SF8300 (13, 17). Of these mutants, two mutations were detected in laboratory-generated resistant strains as a result of a passaging experiment. The first mutation was detected in the strain CRB and was a 36-bp duplication 290 bp upstream of the *pbp4* start codon. This mutation was introduced into the *pbp4*-promoter region of SF8300, giving rise to the strain SF8300 *Ppbp4* (CRB). Similarly, insertion of mutations detected in the strain CRT (a thymine insertion 377 bp upstream the start codon and a 90-bp deletion 275 bp upstream the start codon) into the *pbp4*-promoter region of SF8300 produced SF8300 *Ppbp4* (CRT). The third mutation was one detected in a clinically isolated strain (10, 13), namely, Strain 1, and consisted of a T-to-A substitution 266 bp upstream of the start codon. The introduction of this mutation in SF8300 gave rise to SF8300 *Ppbp4* (Strain 1) (Fig. 1a).

In order to determine if the introduction of these mutations affected PBP4 expression in the selected isogenic strains, immunoblotting was performed using an antibody specific to PBP4. Compared with the wild-type (WT) strain SF8300, strains containing promoter-associated mutations had a significantly increased expression of PBP4 (Fig. 1b; see Fig. S1 in the supplemental material). SF8300 *Ppbp4* (CRB) had the largest amount of expressed protein, followed by SF8300 *Ppbp4* (CRT) and SF8300 *Ppbp4* (Strain 1). ∆*pbp4* was used as a control, for which there was no PBP4 band detected (Fig. 1c). These results indicated that PBP4 overexpression occurred as a result of promoter-associated mutations in MRSA strains, in a manner similar to what was previously detected in MSSA strains (7, 13).

Following confirmation of PBP4 overexpression, we performed a growth assay in the presence of \( \beta \)-lactams, such as nafcillin and oxacillin, to examine the effect of PBP4 overexpression on \( \beta \)-lactam resistance. When exposed to \( \beta \)-lactams, strains with promoter-associated mutations were able to survive significantly better in the presence of either of the antibiotics than the WT and ∆*pbp4* strains for 4 \( \mu \)g/mL nafcillin (Fig. 1d) and 8 \( \mu \)g/mL oxacillin (Fig. 1e). The wild-type strain as well as the strains with promoter-associated mutations all had similar growth patterns in the absence of \( \beta \)-lactams (Fig. 1f). Taken together, these results suggested that promoter-associated mutations led to PBP4 overexpression and \( \beta \)-lactam resistance in the MRSA background, similar to what was previously detected in MSSA strains (7, 13).

*pbp4*-promoter-associated mutations led to increased expression of *pbp4* and decreased expression of *abcA*. The *pbp4* gene shares its 420-bp promoter and regulatory region with a neighboring, divergently transcribed gene, namely, *abcA* (18). AbcA is an ATP binding cassette-like transporter protein that has been reported to export various chemicals, dyes, and antibiotics, thus contributing to antibiotic resistance in *S. aureus* (14, 19). It also has the ability to export phenol-soluble modulins (PSMs) that are cytolytic toxins and, thus, also plays a role in *S. aureus* virulence (20). Since the mutations detected upstream of the *pbp4* start codon also lie upstream of the *abcA* start codon (Fig. 1a), we were interested in whether they caused any alterations in the expression of *abcA* that subsequently contributed to \( \beta \)-lactam resistance in *S. aureus*. We thus performed reverse transcription-quantitative PCR (qRT-PCR) to examine the expression pattern of *pbp4* and *abcA* in the presence of promoter-associated
pbp4-promoter-associated mutations caused PBP4 overexpression and increased β-lactam resistance in MRSA strains. (a) Schematic diagram of the pbp4-Ppbp4-abcA region. The pbp4 and abcA transcriptional start sites are separated by 420 bp of the promoter region. Along with the wild-type promoter (Ppbp4 WT), promoter mutations seen in the strain CRB (36-bp duplication at 290 bp upstream the start codon), CRT (T insertion at 377 bp and a 90-bp deletion at 275 bp upstream the start codon), and Strain 1 (T-to-A substitution 266 bp upstream the start codon) are represented. (b) Immunoblotting of PBP4 expression levels among selected strains. Proteins from the membrane fraction of the WT strain (SF8300) and strains containing promoter-associated mutations (SF8300 Ppbp4* [CRB], SF8300 Ppbp4* [CRT], and SF8300 Ppbp4* [Strain 1]) were probed with an antibody specific to PBP4 and were visualized for protein expression. (c) Densitometry analysis of PBP4 immunoblotting. Compared with the WT, the strains with promoter mutations had increased levels of PBP4 (48 kDa). pbp4 was used as a control, where there was no PBP4 band detected. (SF8300 WT versus SF8300 Δpbp4, P = 0.0003; SF8300 WT versus SF8300 Ppbp4* [CRB], P = 0.0030 and SF8300 WT versus SF8300 Ppbp4* [Strain 1], P = 0.0141). Band intensity is represented as arbitrary units (AU). (d) Growth assay of SF8300 WT and mutant strains in the presence of 4 μg/mL nafcillin. Strains containing promoter-associated (Continued on next page)
mutations. At 4 h, pbp4 transcripts were expressed in very small amounts in the SF8300 WT and were significantly increased in strains containing promoter-associated mutations (Fig. 2a) (SF8300 Ppbp4 WT versus SF8300 Ppbp4* [CRB], *P < 0.0001; SF8300 WT versus SF8300 Ppbp4* [CRT], *P < 0.0001; SF8300 WT versus SF8300 Ppbp4* [Strain 1], *P = 0.0004). On the other hand, abcA transcripts had increased expression in SF8300 WT but had significantly decreased expression in strains containing promoter-associated mutations (Fig. 2b) (SF8300 WT versus SF8300 Ppbp4* [CRB], *P = 0.0161; SF8300 WT versus SF8300 Ppbp4* [CRT], *P = 0.0016; SF8300 WT versus SF8300 Ppbp4* [Strain 1], *P = 0.0203). These results indicate that the promoter-associated mutations caused overexpression of ppb4 but led to a downregulation of abcA. Thus, the increased β-lactam resistance seen due to the promoter-associated mutations was likely to be solely attributed to PBP4 overexpression and not AbcA activity. The results of qRT-PCR also corresponded with the results seen with PBP4 immunoblotting in terms of different levels of PBP4 overexpression, which indicated that SF8300 Ppbp4* (CRB) had the largest amount of PBP4 overexpression, followed by SF8300 Ppbp4* (CRT) and SF8300 Ppbp4* (Strain 1) (Fig. 1c, Fig. 2a).

In order to determine whether the promoter mutations affected the expression of genes downstream of abcA or ppb4, we also performed qRT-PCR analysis of genes flanking abcA (nupC2) and ppb4 (tagD) (see Fig. S2a in the supplemental material). The results indicated that there was no significant difference between the expression of nupC2 (Fig. S2b) or tagD (Fig. S2c) among the WT strain and the promoter mutants, suggesting that the promoter mutants caused changes only in expression of ppb4 and abcA (SF8300 WT versus SF8300 Ppbp4* [CRB], SF8300 WT versus SF8300 Ppbp4* [CRT], SF8300 WT versus SF8300 Ppbp4* [Strain 1], *P = not significant [ns]).

One of the important functions performed by AbcA is the export of cytolytic toxins, such as phenol-soluble modulins (PSMs) that can lyse erythrocytes and neutrophils (20). In order to determine the effect of AbcA downregulation and its subsequent effect on its activity, we measured the amount of PSMs exported by WT cells and cells that contained the promoter-associated mutations. Using 1-butanol, PSMs were extracted from culture supernatants and were resuspended in urea, following which their ability to lyse sheep erythrocytes was measured by performing hemolysis assays as previously shown (21). It was seen that none of the strains containing promoter-associated mutations differed in their hemolytic capabilities compared with the WT strain, as they all showed equal amounts of hemolysis at the different dilutions used (Fig. 2c). A lagA strain was used as a negative control, which showed no hemolysis at all. Urea was used as a control, which by itself did not have any hemolytic abilities, whereas 0.5% Triton X-100 was used as a positive control that showed maximum hemolysis (Fig. 2d). Along with using sheep blood for the hemolysis assay, 10× and 100× dilutions of the extracted PSMs were spotted onto blood-tryptic soy agar (TSA) plates to analyze differences in hemolysis levels (Fig. 2e to g). The pattern of hemolysis seen on the plates also indicated that there was no difference in the hemolytic abilities between the WT and the mutant strains. The lagA, urea, and Triton X-100 controls were used here, too. Together, these results confirmed that the β-lactam-resistant phenotypes seen with promoter-associated mutations were due to PBP4 overexpression and that AbcA had no role in it.

To further confirm that abcA downregulation did not cause the resistance phenotypes (Fig. 1d and e), we generated abcA mutants in the mecA- and blaZ-devoid derivatives of SF8300 called SF8300ex. SF8300 contains the blaZ plasmid, which also contains...
Promoter mutations cause an upregulation of \(pbp4\) and downregulation of \(abcA\).

(a) qRT-PCR analysis of \(pbp4\). \(pbp4\) transcriptional expression was significantly increased in strains containing promoter-associated mutations compared with the WT strain (SF8300 WT versus SF8300 \(P_{pbp4}^*\) [CRB], \(P = 0.0001\); SF8300 WT versus SF8300 \(P_{pbp4}^*\) [CRT], \(P = 0.0001\); SF8300 WT versus SF8300 \(P_{pbp4}^*\) [Strain 1], \(P = 0.0004\)).

(b) qRT-PCR analysis of \(abcA\). \(abcA\) transcriptional expression was significantly decreased in strains containing promoter-associated mutations compared with the WT strain. (SF8300 WT versus SF8300 \(P_{pbp4}^*\) [CRB], \(P = 0.0161\); SF8300 WT versus SF8300 \(P_{pbp4}^*\) [CRT], \(P = 0.0016\); SF8300 WT versus SF8300 \(P_{pbp4}^*\) [Strain 1], \(P = 0.0203\)).

(c) Hemolysis assay. Analysis of the ability of butanol-extracted phenol-soluble modulins (PSMs) to lyse sheep erythrocytes was carried out using a hemolysis assay. There was no difference detected in the hemolytic abilities among the WT strain and strains containing promoter-associated mutations. \(\DeltaagrA\) was used as a control, which showed no hemolytic activity.

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ermB, an erythromycin resistant gene (22). In order to accommodate for the selection of transposon insertion mutants based on erythromycin resistance, we had to use a strain devoid of the blaZ plasmid. Eviction of this plasmid results in the constitutive expression of mecA, as the regulatory system associated with blaZ expression (namely, the BlaR1-BlaI system) also regulates mecA in USA300 (23). In order to avoid the effect of constitutive expression of mecA, we used SF8300ex, a strain devoid of both blaZ and mecA. An abcA insertional transposon was introduced in the wild-type strain (SF8300ex or WTex) to generate SF8300ex abcA::Tn and in a representative promoter mutant to generate SF8300ex Ppbp4* (CRB) abcA::Tn. Phenotypic verification of these strains was performed by qRT-PCR analysis for pbp4 and abcA (see Fig. S3a and S3b in the supplemental material) and Bocillin assay (Fig. S3c and S3d).

Growth assays for these strains with 0.25 μg/mL nafcillin or 0.5 μg/mL oxacillin indicated that the abcA mutant did not affect the phenotypes of the strains, as both the strains containing promoter mutants grew significantly better than strains with wild-type promoters (see Fig. S4 in the supplemental material). For 0.25 μg/mL nafcillin, the SF8300ex abcA::Tn and SF8300ex Ppbp4*(CRB) abcA::Tn P value was <0.0001. For 0.5 μg/mL oxacillin, the SF8300ex abcA::Tn and SF8300ex Ppbp4*(CRB) abcA::Tn P value was 0.0075.

S. aureus strains with pbp4-promoter-associated mutations were less virulent to C. elegans than the wild-type strain. Along with a role in β-lactam resistance, PBP4 has also been associated with pathogenesis. However, its exact role, if any, remains undetermined, as there have been contrasting reports regarding the role of PBP4 in pathogenesis (15, 16). We attempted to understand the role of PBP4 in pathogenesis under wild-type and overexpressed conditions using a C. elegans infection model. We selected one representative strain containing promoter-associated mutations, namely, SF8300 Ppbp4* (CRB) and used it to perform infection studies with C. elegans. Age-synchronized worms were infected with 1.5 × 10^5 bacteria and were incubated for 3 days, following which they were assessed for worm survival. Worms that responded to mechanical stimulus were considered live, whereas worms that did not respond were counted as dead. Compared with worms infected with WT cells, where the survival rates for the worms were approximately 30%, worms infected with Ppbp4* (CRB) had significantly higher survival rates (55%) (SF8300 WT versus SF8300 Ppbp4* (CRB), P = 0.0021) (Fig. 3a), indicating that the presence of promoter-associated mutations led to the decreased killing and thus decreased virulence in C. elegans. Infection with SF8300 Δpbp4 also resulted in decreased worm survival, similar to the results obtained by infection with WT cells (SF8300 Ppbp4* [CRB] versus SF8300 Δpbp4, P = 0.052). The Escherichia coli strain OP50 was used as a control, where worms displayed 100% survival, indicating that the killing detected was due to S. aureus virulence. Killing assays performed with SF8300ex background strains had similar survival patterns. The inclusion of abcA mutants did not affect the C. elegans killing pattern, as both strains with promoter mutations, i.e., SF8300ex Ppbp4* (CRB) and SF8300ex Ppbp4* (CRB) abcA::Tn, had increased worm survival compared with strains with wild-type promoters (SF8300ex and SF8300ex Ppbp4* [CRB], P = 0.0045; SF8300ex abcA::Tn and SF8300ex Ppbp4* [CRB] abcA::Tn, P = 0.0081) (see Fig. S5 in the supplemental material).

We carried out further experiments with C. elegans infection to determine why the Ppbp4* (CRB) strain showed increased survival. SF8300 WT and SF8300 Ppbp4* (CRB) strains were introduced with constitutively expressing GFP and RFP, respectively, via the constitutively expressing plasmid pTX, thus generating strains SF8300 WT + GFP and SF8300 Ppbp4* (CRB) + RFP. A competition-killing assay was performed, where an equal number of SF8300 WT and SF8300 Ppbp4* (CRB) cells were used to infect worms.

**FIG 2** Legend (Continued)

(d) Controls used for a hemolysis assay of sheep erythrocytes. A hemolysis assay of decreasing concentrations of 8 M urea indicated that urea in itself did not have any hemolytic ability. A total of 0.5% Triton X-100 was used as a positive control, which showed maximum hemolysis. An analysis of the hemolytic ability of butanol-extracted phenol-soluble modulins (PSMs) by spotting them onto blood agar-TSA plates. There was no difference in hemolysis among WT and promoter mutant strains when PSMs were diluted 10 times (e) or diluted 100 times (f). See Figure 2g for strain legend. (g) Diameter of the hemolytic zone detected on the blood agar-TSA plates for PSMs from the selected strains.
FIG 3 Cells with overexpressed PBP4 have decreased virulence. (a) *C. elegans* infection assay. Killing assay with isogenic strains demonstrated that SF8300 WT and SF8300 Δpbp4 strains had decreased worm survival (30%) compared with SF8300 Ppbp4* (CRB), which had increased worm survival (55%) indicating decreased *S. aureus* virulence (SF8300 WT versus SF8300 Ppbp4* [CRB], *P* = 0.0021; SF8300 Ppbp4* [CRB] versus SF8300 Δpbp4, *P* = 0.052; SF8300 WT versus SF8300 Δpbp4, *P* = ns). OP50 is an *E. coli* control strain, which is used as a food source to culture and maintain *C. elegans*. Fluorescence microscopy. Microscopy for *C. elegans* infected with GFP- or RFP-expressing isogenic strains demonstrated that SF8300 Ppbp4* (CRB) had a significantly decreased ability to colonize *C. elegans* compared with WT, after 3 days of infection using a 20× lens objective. Scale bar = 20 μm. (b) Infection of *C. elegans* with SF8300 WT + GFP. (c) Infection of *C. elegans* with SF8300 Ppbp4* (CRB) + RFP. (d) Merged image of *C. elegans* infected with SF8300 WT + GFP and SF8300 Ppbp4* (CRB) + RFP. (e) Infection of *C. elegans* with SF8300 WT + RFP. (f) Infection of *C. elegans* with SF8300 Ppbp4* (CRB) + GFP. (g) Merged image of *C. elegans* infected with SF8300 WT + RFP and SF8300 Ppbp4* (CRB) + GFP. (h) Analysis of RFP and GFP signals following fluorescence microscopy of *C. elegans* infected with an equal number of SF8300 WT + GFP and (Continued on next page)
After 3 days of infection, fluorescence microscopy was carried out where the GFP and RFP signals from within the gut of each worm were measured. On a subsequent analysis of the fluorescent signals, it was observed that there was a significantly increased GFP signal detected compared with RFP (Fig. 3b to d and h). This finding indicated that there was a higher proportion of SF8300 WT cells colonized within the gut of *C. elegans* than SF8300 Ppbp4* (CRB) cells (SF8300 WT + GFP versus SF8300 Ppbp4* [CRB] + RFP, \( P < 0.0001 \)). The experiment was repeated by interchanging the plasmids containing fluorescent proteins, i.e., with the strains SF8300 WT + RFP and SF8300 Ppbp4* (CRB) + GFP (Fig. 3e to g and i). Here, an increased RFP signal compared with GFP signal within the gut of *C. elegans* was detected, ensuring that it was due to the increased colonization of SF8300 WT and not due to a result of a potential anomaly of using fluorescent proteins (SF8300 WT + RFP versus SF8300 Ppbp4* [CRB] + GFP, \( P = 0.0026 \)).

Before *C. elegans* were infected with bacteria containing fluorescent proteins, the initial inoculum was plated onto tetracycline-containing TSA plates (Fig. 3j). After it was determined that the initial inoculum contained a similar number of each of the bacterial strains (SF8300 WT + GFP versus SF8300 Ppbp4* [CRB] + RFP, \( P = \text{ns} \)), *C. elegans* were subjected to lysis after 3 days of infection following which the bacteria accumulated within the gut of the worms were enumerated by performing serial dilutions of the lysate and plating them. There was a significantly higher number of GFP-expressing colonies (representing SF8300 WT) on the plate than that of RFP-expressing colonies (representing SF8300 Ppbp4* [CRB]) (Fig. 3k) (SF8300 WT + GFP versus SF8300 Ppbp4* [CRB] + RFP, \( P < 0.0001 \)). When plasmids were interchanged, there were increased RFP-expressing colonies (representing SF8300 WT) and decreased GFP-expressing colonies (representing SF8300 Ppbp4* [CRB]) (Fig. 3l and m). Together, the *C. elegans* experiments indicated that the SF8300 Ppbp4* (CRB) was unable to colonize the *C. elegans* gut as well as SF8300 WT, leading to decreased virulence.

**DISCUSSION**

MRSA is one of the most prominent agents contributing to the significant global antimicrobial resistance burden today (3). However, in recent years, the presence of \( \beta \)-lactam resistance in *S. aureus* strains without *mecA* has been reported (10, 24, 25). In previous studies, we have demonstrated the ability of PBP4 to mediate high-level \( \beta \)-lactam resistance via protein overexpression due to promoter-associated mutations in non-*mecA* strains (7, 17). In this study, we saw that PBP4 could mediate \( \beta \)-lactam resistance independent of PBP2a. As seen by the growth assays, both PBP2a and PBP4 contributed toward \( \beta \)-lactam resistance, as cells containing the promoter-associated mutations were able to survive the \( \beta \)-lactam challenge more significantly than strains that contained only PBP2a, i.e., the WT strains. This result indicated that, when present, PBP4 could supplement the action of PBP2a, causing a further increase in resistance and potentially leads to complications in treatment. Current clinical diagnostic and
therapeutic protocols are based on the categorization of the infecting strains as MRSA or MSSA, as the treatment for MRSA is more aggressive than that of MSSA (26). However, due to the rise in pbp4-associated resistance, it is likely that targeting only PBP2a for diagnosis and treatment protocols may not suffice.

PBP4 is a low-molecular-weight (LMW) PBP in S. aureus (27). LMW PBPs in other bacteria, such as E. coli (28), Bacillus subtilis (29), Listeria monocytogenes (30), and Streptococcus pneumoniae (31), have been described to possess carboxypeptidase activity, allowing them to maintain the degree of cell wall cross-linking. PBP4 in S. aureus has transpeptidase activity along with carboxypeptidase activity, giving it the ability to perform increased, secondary cell wall cross-linking compared with other bacteria (32). Increased PBP4 expression due to promoter-associated mutations leads to a further increase in cross-linking (17, 33), indicating PBP4’s propensity toward transpeptidase activity over carboxypeptidase activity. This information demonstrates the potential of PBP4 from S. aureus to be a powerful player in β-lactam resistance. The importance of PBP4 in β-lactam resistance was further reiterated when we saw that the promoter-associated mutations studied here led only to pbp4 upregulation and not abcA. AbcA can export various antimicrobial agents, making it an important mediator of antibiotic resistance (14, 19). However, based on the qRT-PCR analysis and hemolysis assay, it was clear that AbcA did not play any role in the resistant phenotype associated with the promoter mutations and that it was all attributed to PBP4 overexpression. It is likely that the presence of mutations possibly led to altered binding of transcriptional factors to the regulatory region, leading to overexpression of PBP4. Reports have suggested that pbp4 and abcA regulation are independent of each other (14); however, since the studied mutations also caused the downregulation of abcA, it appears that the regulation of both of these genes are at least partially interdependent, and they potentially share some of their regulatory sequences as well as some regulatory factors. Along with AbcA, S. aureus also has another known exporter of PSMs, namely, the Pmt system (21). It is likely that Pmt compensated for the export of PSMs during abcA downregulation, allowing for similar levels of hemolytic activities between various strains. abcA mutants did not affect the growth phenotypes in the presence of β-lactams, which indicated that downregulation of abcA also did not contribute to resistance (Fig. S3).

In S. aureus, PBP4 maintains the level of peptidoglycan cross-linking by its transpeptidase and carboxypeptidase properties, thus striking a balance between appropriate cross-linking of peptidoglycan monomers and allowing for anchoring of surface proteins that are necessary for S. aureus virulence (34). Our previous studies demonstrated that this well-balanced cell wall dynamic was disrupted in the form of increased cell wall cross-linking due to the overexpression of PBP4 (7, 33). However, the effect of this disruption on the abundance of surface-anchored proteins is unknown. As these surface proteins play vital roles in host attachment, colonization, and infection, we examined the virulence of a strain containing promoter-associated mutations using C. elegans, as this strain exhibited increased PBP4 expression. C. elegans has proven to be a useful infection model system to use to study host-pathogen interactions and has given multiple clues about host response to pathogenic bacteria (35). S. aureus cells mediate C. elegans killing by accumulating within the gut of the worm, leading to intestinal distension and subsequent worm death (36). Based on our results, strains with overexpressed PBP4 were unable to colonize within the gut of the worms to a sufficient enough degree to cause high levels of accumulation and worm death compared with the WT cells. The abcA mutants did not change the survival pattern of C. elegans on infection, suggesting that AbcA likely did not have a role in the virulence of C. elegans (Fig. S4). Gut epithelial cells of C. elegans have been described to have similarities with human intestinal epithelial cells, suggesting the possibility that strains containing increased PBP4 expression may have a decreased ability to colonize humans, as S. aureus has been shown to also colonize and infect human intestinal cells, making this study biologically relevant (37). It is likely that the inability of Ppbp4* (CRB) cells to colonize was due to a decrease in surface-anchored proteins due to increased PBP4-mediated cross-linking of the peptidoglycan.
In S. aureus, repeating monomers of the peptidoglycan consisting of penta-peptide stems are cross-linked via a penta-glycine bridge (38). The terminal glycine of this penta-glycine bridge forms a peptide bond with the α-alanine of the penta-peptide stem, via the transpeptidase activity of PBP4 (39). However, this glycine is also the site at which sortase A-mediated anchoring of cell surface proteins takes place (40). Due to the PBP4-mediated increase of cell wall cross-linking, we hypothesize that there are not sufficient free glycine sites available for the sortase A-mediated attachment of surface proteins, thus leading to decreased virulence (see Fig. S6 in the supplemental material). Further studies are required to understand the biology that leads to the decreased virulence phenotype of S. aureus strains having pbp4-promoter associated mutations, which can potentially help in exploiting this phenomenon for treatment purposes.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** S. aureus strains were all cultured at 37°C in tryptic soy broth (TSB), with agitation at 180 rpm. Promoter-associated mutations in S. aureus were introduced via splice-overlap PCR and allelic replacement as described previously (41), using the plasmid pJB38 (42). The GFP and RFP encoding regions were amplified from the integration plasmids pGFP-F and pRFP-F, respectively (42), and were cloned into the constitutively expressing plasmid pTXα, as described previously (17). The plasmid was introduced into RN4420 by electroporation, following which they were introduced in SF8300 or SF8300 devoid of meca gene and blaZ plasmid (BEI Resources).

**Growth curve assays.** Growth assays were performed using the automated microbiology growth curve analysis system Bioscreen C (Growth Curves USA). Overnight cultures of bacteria were diluted to an optical density (OD) of 0.1 in TSB with or without antibiotics, and 200 µL was added to each well of a honeycomb Bioscreen C plate in triplicates. The assay was carried out for 20 h with continuous orbital shaking at 37°C. Each condition was tested in triplicate, and the experiment was performed twice to ensure reproducibility.

**Immunoblotting.** Overnight cultures of bacteria were subcultured into 50-mL flasks containing TSB such that the initial OD at 600 nm (OD000) of the flask was 0.1. The cells were cultured to an OD000 of 1, following which cells were collected and resuspended in phosphate-buffered saline (PBS) containing CompleteMini protease inhibitor cocktail (Roche). The cells were mechanically lysed using the FastPrep (MP Biochemicals) instrument, and whole-cell lysates were obtained. The cell membrane fraction was isolated from the lysates by performing ultracentrifugation at 66,000 × g for 1 h (Sorval WX Ultra 80 centrifuge, Thermo Fisher Scientific). After the obtained pellet was resuspended with PBS, protein estimation was carried out using the Pierce BCA protein assay kit (Thermo Fisher). The samples were separated by performing SDS-PAGE on a 10% gel, following which they were transferred onto a low-fluorescence polyvinylidene difluoride (PVD) membrane (Millipore). Blocking was performed for 1 h (5% skimmed milk in Tris-buffered saline containing 0.5% Tween), primary antibody staining was carried out overnight at 4°C (polyclonal anti-PBP4, custom antibody from Thermo Fisher, 1:1,000), and secondary antibody staining was performed using an anti-rabbit antibody (Azure anti-rabbit NIR700, 1:20,000 dilution). The blots were imaged using the Azure C600 imager, and analysis was performed using ImageJ.
### TABLE 2 Primers used in this study

| Primer          | Sequence (5’–3’)                                      | Use                                      |
|-----------------|-------------------------------------------------------|------------------------------------------|
| PBP4-P1         | GGGGACAAGTTTGTAACAAAAAGCGACTGTTGCATTCAATTAGTTGTACCTTGCGATATCGATCTTTGCAATATACGACC | pbp4 deletion using pJB38                |
| PBP4-P2         | AAAGCGTAACTCTCCCTTTTCCATAATTCAAATTCTCCAAAGGC         | pbp4 deletion using pJB38                |
| PBP4-P3         | AAAAGGGAAGATACAGCTTCACTATACAAAACGCCGCAAGTTTGCAATTATTATGAACTTGCGAACTTTGTCCGG | pbp4 deletion using pJB38                |
| PBP4-P4         | GGGGACAAGTTTGTAACAAAAAGCGACTGTTGCATTCAATTAGTTGTACCTTGCGATATCGATCTTTGCAATATACGACC | pbp4 deletion using pJB38                |
| Pbp4*-plmay-NotI-for | AGTTTGCGCGCGCACATTGTACCTGCGATATCTTTTG     | Cloning of Pbp4 into the plasmid plmay    |
| Pbp4*-pImay-kpn1-rev | TCCTGGATCTTGGTGCGAATGATCGTAATCTTG  | Cloning of Pbp4 into the plasmid plmay    |
| Pbp4*- (CRB)-rev | ACAAAAAATCAGATAATATCTCATATACAAATGTGAGCGGTATTATTG | Introduction of CRB mutations in Pbp4*   |
| Pbp4*- (CRB)-for | ATATTTCTATGCTATTCTTATTATATAGATATTCTATTG        | Introduction of CRB mutations in Pbp4*   |
| Pbp4*- (CRT)-kpn1-rev | ATTTGGATCTAGATCGTAATTTTGAGGAGG     | Introduction of CRT mutations in Pbp4*   |
| Pbp4*- (CRB)-rev | AGTTTGCGCGCGCACATTGTACCTGCGATATCTTTTG     | Introduction of CRT mutations in Pbp4*   |
| Pbp4*- (CRB)-for | ATATTTCTATGCTATTCTTATTATATAGATATTCTATTG        | Introduction of CRT mutations in Pbp4*   |
| Pbp4*- (CRT)-kpn1-rev | ATTTGGATCTAGATCGTAATTTTGAGG     | Introduction of CRT mutations in Pbp4*   |
| GFP_BamHI_FP    | AGGATCTTAAAAGTAAAGAGTTTGGAATATGTCAAAAAGGAGAAGAATTATTTACAG | Cloning GFP into pTxK |
| GFP_MluI_RP     | ATACGCGTTTCTATATTAATCTATCC                          | Cloning GFP into pTxK                    |
| RFP_BamHI_FP    | ATATGCTCATTGTAATACATTTATGAAAGG                  | Cloning GFP into pTxK                    |
| RFP_MluI_RP     | ATACGCGTTTATAAAAACATGACAGG                   | Cloning GFP into pTxK                    |
| NMC-Tn-upstream | CTGATTTCTATTAAAGGG                                         | Transposon insertion verification     |
| abcA-1          | TTAATCTGGATTATTTTGAATGACTAC                    | Transposon insertion verification     |
| pbp4-1          | GGTTTTAATGAAATACAAAGGCTAAAGC               | qRT-PCR for pbp4                        |
| pbp4-rev        | AGCTCTACTGTCAGCTGACAGATCGTAATCC               | qRT-PCR for pbp4                        |
| abcA-1          | ACCAATTACTGTAAGATCTGAGTACGTGACAG             | qRT-PCR for abcA                        |
| abcA-rev        | TGGAAGATGAGATAAAGGCCTAGATAAAT              | qRT-PCR for abcA                        |
| gyrB-1          | ATTGCTTAGTAAGCTCCTAGAATG                   | qRT-PCR for gyrB                        |
| gyrB-rev        | TAATCGTGCCTTATCAGATATATAC                 | qRT-PCR for gyrB                        |
TABLE 3 Plasmids used in this study

| Plasmid          | Notes                              | Reference |
|------------------|------------------------------------|-----------|
| plmay + Ppbp4 (CRB) | Insertion of Ppbp4 promoter mutation | This study |
| pJB38 + Ppbp4 (CRT) | Insertion of Ppbp4 promoter mutation | This study |
| pJB38 + Ppbp4 (Strain 1) | Insertion of Ppbp4 promoter mutation | This study |
| pJB38 + Δpbp4     | Deletion of pbp4                  | This study |
| pTX3 + GFP       | Constitutive expression of GFP     | This study |
| pTX3 + RFP       | Constitutive expression of RFP     | This study |

instrument following which RNA isolation was performed using the Qiagen RNeasy minikit. On confirmation of RNA quality, cDNA synthesis was performed using the SuperScript IV reverse transcriptase (RT) kit. qRT-PCR was performed using SYBR PCR Mastermix in an ABI 7500 system (Applied Biosystems) and primers for pbp4, abcA, and the housekeeping gene gyrB.

Butanol extraction of PSMs. PSMs were extracted from culture supernatants as described previously (21, 43). Briefly, overnight cultures of bacteria were subcultured into 50-mL flasks containing TSB such that the initial OD600 of the flasks was 0.1. After 24 h, the cells were collected and centrifuged for 30 min, and 30 mL of supernatant from each strain was collected to which 10 mL 1-butanol was added. The samples were mixed by shaking at 180 rpm for 2 h at 37°C. Samples were then centrifuged, and 7 mL of the upper layer from each sample was collected. The samples were dried using a vacuum centrifuge (Eppendorf), and the dried pellet was resuspended in 8 M urea. Samples were diluted 10-fold for hemolysis experiments.

Hemolysis assay. Two percent sheep blood was prepared using chilled PBS and was washed twice to get rid of lysed erythrocytes. After a washing step, 100 μL of the blood was added to a 96-well round-bottom plate, to which 100 μL of samples of PSMs extracted with butanol was added in decreasing concentrations (from 1/10th to 1/280th of the sample). The plates were incubated for 1 h at 37°C, following which they were centrifuged at 1,500 rpm for 10 min. A total of 100 μL of the supernatant was collected and transferred onto a flat-bottom 96-well plate. Absorbance was measured at 540 nm using a SpectraMax M5 (Molecular Devices) instrument. A total of 0.5% Triton was used as a control.

A total of 2 μL of butanol-extracted PSMs was spotted onto TSA-blood plates (5% sheep blood) for each sample. Urea was spotted as a negative control and 0.5% Triton was spotted as a positive control. After samples were dried, plates were incubated at 37°C overnight.

C. elegans-S. aureus infection assays. C. elegans worms were obtained from the Caenorhabditis Genetics Center (CGC). The liquid killing assay was performed as described previously (44), with certain modifications. The C. elegans strain used in this study was DH26 [rf-3(b26) II], a temperature-sensitive, spermatogenesis-defective strain. Briefly, adult hermaphrodites were age synchronized (45) using householder bleach and 5 N NaOH and allowed to grow on nematode growth medium (NGM) plates for 40 h at 26°C. Fifteen young adult worms were picked from this plate and added to 96-well plates containing tetracycline, and incubated at 37°C. The next day, colonies were counted based on the light they expressed. The experiment was performed twice, in triplicates to ensure reproducibility.

TABLE 3 Plasmids used in this study

| Plasmid          | Notes                              | Reference |
|------------------|------------------------------------|-----------|
| plmay + Ppbp4 (CRB) | Insertion of Ppbp4 promoter mutation | This study |
| pJB38 + Ppbp4 (CRT) | Insertion of Ppbp4 promoter mutation | This study |
| pJB38 + Ppbp4 (Strain 1) | Insertion of Ppbp4 promoter mutation | This study |
| pJB38 + Δpbp4     | Deletion of pbp4                  | This study |
| pTX3 + GFP       | Constitutive expression of GFP     | This study |
| pTX3 + RFP       | Constitutive expression of RFP     | This study |

For the competition assay, WT + GFP and Ppbp4* (CRB) + RFP cells were mixed in equal amounts before 1.5 × 10^6 cells were added to each well. The media also contained 12.5 μg/mL tetracycline, which is the selection antibiotic for the pTX3 plasmid.

Fluorescence microscopy and image analysis. Fluorescence microscopy and analysis were performed as described previously (46). After 3 days of infection with S. aureus, worms were transferred from the wells to 1.5-mL microcentrifuge tubes. The worms were washed with M9 buffer containing 10 mM sodium azide, following which they were treated with 100 μg/mL gentamicin for 30 min, twice. Following the gentamicin treatment, worms were washed thrice with M9 buffer containing 10 mM sodium azide. The worms were then placed onto an agar pad (2% agar) on glass slides, and microscopy was performed using the Keyence BZ-X800 all-in-one fluorescence microscope using the 20× objective. The exposure time and all other settings remained the same for all samples.

The BZX analyzer software was used to measure fluorescence intensities within a worm. After stitching images wherever necessary, the “hybrid cell count” function was used to select the area of the worm and measure fluorescence intensities for GFP and RFP. For each condition, at least 5 worms were imaged and analyzed.

Gut CFU determination. Gut bacterial enumeration was performed as described previously (47). After the gentamicin treatment and wash steps as described above, the worms were resuspended in 250 μL M9 buffer containing 10 mM sodium azide, mixed with 200 mg of 1-mm zirconia beads (Research Products International), and lysed by vortexing the tubes for 2 min. Before lysis, 20 μL of the supernatant was collected for plating. Following lysis, samples were diluted to 10^−4, plated onto TSA plates containing tetracycline, and incubated at 37°C. The next day, colonies were counted based on the color they expressed. The experiment was performed twice, in triplicates to ensure reproducibility. Each tube contained 10 worms, and the experiment was performed twice, in triplicates.

Sequencing. The fidelity of all the mutants and plasmid constructs was validated through Sanger sequencing (Eurofins Genomics, USA).
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