

PBP 4 Mediates High-Level Resistance to New-Generation Cephalosporins in *Staphylococcus aureus*

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Staphylococcus aureus is an important cause of both hospital- and community-associated methicillin-resistant *S. aureus* (MRSA) infections worldwide. β -Lactam antibiotics are the drugs of choice to treat *S. aureus* infections, but resistance to these and other antibiotics make treatment problematic. High-level β -lactam resistance of *S. aureus* has always been attributed to the horizontally acquired penicillin binding protein 2a (PBP 2a) encoded by the *mecA* gene. Here, we show that *S. aureus* can also express high-level resistance to β -lactams, including new-generation broad-spectrum cephalosporins that are active against methicillin-resistant strains, through an uncanonical core genome-encoded penicillin binding protein, PBP 4, a nonessential enzyme previously considered not to be important for staphylococcal β -lactam resistance. Our results show that PBP 4 can mediate high-level resistance to β -lactams.

Antibiotic resistance presents an important therapeutic challenge and constitutes a considerable burden medically and economically (1–3). *Staphylococcus aureus* is an important bacterial pathogen that causes a wide variety of community- and health care-related infections, and widespread β -lactam resistance in *S. aureus* has been a growing problem worldwide (1, 4, 5).

The antibacterial activity of β -lactams is mediated by covalent binding to penicillin binding proteins (PBPs), thereby inhibiting the transpeptidase (TPase) activity of PBP-mediated bacterial cell wall synthesis (6–9). In *S. aureus*, class resistance to β -lactams, termed methicillin resistance, is widespread. Class resistance to β -lactams in methicillin-resistant strains of *S. aureus* (MRSA) is due to expression of PBP 2a or PBP 2a', which are encoded by horizontally acquired genes (1, 10), *mecA* and *mecC* (11), respectively. PBP 2a confers class resistance to β -lactam antibiotics due to its low-affinity binding to β -lactams (8, 12). In addition to PBP 2a, MRSA has four other PBPs (PBPs 1 through 4), which are encoded in the bacterial core genome. PBPs 1 to 4 have not been thought to play a role in high-level β -lactam resistance (7, 9, 13), although PBP 2a-mediated resistance depends on PBP 2 (14).

Ceftaroline and ceftobiprole are broad-spectrum β -lactam antibiotics that are active against MRSA strains, and ceftaroline is FDA approved for treatment of skin and skin structure MRSA infections (15–18) and is clinically effective for other infections, including pneumonia and bacteremia (19). Ceftaroline and ceftobiprole are active against MRSA strains because they effectively bind to and inhibit PBP 2a. Ceftaroline inhibits PBP 2a allosterically by binding to the protein and causing a conformational change, which makes PBP 2a vulnerable to the action of a second molecule of ceftaroline or another β -lactam (20). Ceftobiprole bypasses the resistance mechanism of PBP 2a by having a vinylpyrrolidinone moiety (R2 group) that allows access to the PBP 2a active site (20, 21). MRSA strains that are resistant to ceftaroline and ceftobiprole have already been reported (13, 22–25). High-level resistance to both drugs has been attributed to point mutations in two amino acid residues (Y446 and E447) in the *mecA* gene (13, 26, 27). Both amino acid residues are located near the transpeptidase active site of PBP 2a, and mutations in them are

predicted to interfere with drug binding (20). Moreover, Y466N and E447K mutations have been shown to cause ineffective binding of ceftaroline to PBP 2a (27).

In previous studies, we have shown that a *mecA*-negative mutant of the archaic *S. aureus* COL strain, which expresses homogeneous resistance to methicillin and other β -lactams, can express high-level ceftobiprole resistance independently of PBP 2a, indicating alternative mediators of its resistance (26). Genome sequencing of the *mecA*-negative ceftobiprole-resistant mutant strain (CRB) revealed mutations in three genes: *pbp4*, *gdpP*, and *acrB* (28). The roles of GdpP, a cyclic-di-AMP phosphodiesterase, and AcrB, an efflux pump, in β -lactam resistance are not well delineated. PBP 4 is a low-molecular-weight, core genome-encoded, nonessential PBP implicated in low- to moderate-level β -lactam resistance (29, 30). In this study, we sought to address (i) if passage in another lineage generates mutants that are similar to those found in CRB, (ii) if ceftaroline selects for mutations similar to those selected for by ceftobiprole, and (iii) what is the contribution of PBP 4, as this has not been addressed in previous work. We show that a representative strain of the USA300 community MRSA genotype, which expresses heterogeneous resistance to β -lactams and which is responsible for the majority of MRSA infections in the United States (31), can also exhibit high-level

Received 14 February 2016 Returned for modification 14 March 2016

Accepted 8 April 2016

Accepted manuscript posted online 11 April 2016

Citation Chan LC, Gilbert A, Basuino L, da Costa TM, Hamilton SM, dos Santos KR, Chambers HF, Chatterjee SS. 2016. PBP 4 mediates high-level resistance to new-generation cephalosporins in *Staphylococcus aureus*. Antimicrob Agents Chemother 60:3934–3941. doi:10.1128/AAC.00358-16.

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.00358-16>.

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TABLE 1 Strains used in the study

Strain	MecA	Passage in:	Notes	Reference
<i>E. coli</i>				Invitrogen
RN4220	–		Laboratory <i>S. aureus</i> strain	BEI Resources
COLnex	–			13
COLnex Δ <i>pbp4</i>	–		<i>pbp4</i> deletion mutant in COLnex	This study
SF8300	+		USA300 MRSA clinical isolate	29
SF8300ex	–		SF8300 strain with <i>mecA</i> excised	29
SF8300ex Δ <i>pbp4</i>	–		<i>pbp4</i> deletion mutant in SF8300ex	This study
SRT	–	Ceftaroline		This study
SRB	–	Ceftobiprole		This study
Sgap	–		SF8300ex strain in which <i>pbp4</i> (E183A, F241R), <i>gdpP</i> (N182K), and <i>acrB</i> (I960V) mutations analogous to those in the CRB strain (28) were introduced	This study
SgapT	–	Ceftaroline		This study
Sp	–		SF8300ex strain in which <i>pbp4</i> (E183A, F241R) mutations analogous to those in the CRB strain (28) were introduced	This study
SpT	–	Ceftaroline		This study
SRB Δ <i>pbp4</i>	–		<i>pbp4</i> deletion mutant in SRB	This study
SRT Δ <i>pbp4</i>	–		<i>pbp4</i> deletion mutant in SRT	This study
SgapT Δ <i>pbp4</i>	–		<i>pbp4</i> deletion mutant in SgapT	This study
SpT Δ <i>pbp4</i>	–		<i>pbp4</i> deletion mutant in SpT	This study
SRB Δ <i>pbp4</i> [empty plasmid]	–		SRB Δ <i>pbp4</i> with empty plasmid pTX Δ	This study
SRB Δ <i>pbp4</i> [<i>pbp4</i> (SF8300)]	–		SRB Δ <i>pbp4</i> complemented with pTX Δ containing <i>pbp4</i> from SF8300	This study
SRB Δ <i>pbp4</i> [<i>pbp4</i> (SRB)]	–		SRB Δ <i>pbp4</i> complemented with pTX Δ containing <i>pbp4</i> from SRB	This study
SRT Δ <i>pbp4</i> [empty plasmid]	–		SRT Δ <i>pbp4</i> with empty plasmid pTX Δ	This study
SRT Δ <i>pbp4</i> [<i>pbp4</i> (SF8300)]	–		SRT Δ <i>pbp4</i> complemented with pTX Δ containing <i>pbp4</i> from SF8300	This study
SRT Δ <i>pbp4</i> [<i>pbp4</i> (SRT)]	–		SRT Δ <i>pbp4</i> complemented with pTX Δ containing <i>pbp4</i> from SRT	This study

^a +, present; –, absent.

resistance to ceftobiprole, ceftaroline, and other β -lactams in the absence of *mecA*. Both ceftobiprole- and ceftaroline-resistant strains displayed mutations in *pbp4* and *gdpP*. Finally, we show PBP 4 to be the driving factor responsible for this high-level resistance. These results underscore the potential role of PBP 4 in β -lactam resistance in *S. aureus* (32).

MATERIALS AND METHODS

Bacterial strains and plasmids. All the strains were grown in Trypticase soy broth (TSB) with aeration, on Trypticase soy agar (TSA), or on blood agar (Remel) at 37°C. The plasmid pKOR1 was selected in bacterial medium containing ampicillin (100 μ g ml⁻¹) or chloramphenicol (10 μ g ml⁻¹) for *Escherichia coli* and *S. aureus*, respectively. Bacterial medium supplemented with tetracycline (12.5 μ g ml⁻¹) was used to select for pTX Δ . The strains used in this study are listed in Table 1. The plasmids and primers used in the study are listed in Table S1 in the supplemental material.

Antibiotics. Ceftobiprole solution was prepared fresh daily from ceftobiprole powder (provided by Johnson and Johnson Pharmaceutical Research and Development) at a concentration of 2 mg ml⁻¹. Ceftaroline solution was prepared from ceftaroline active powder (provided by Forest Laboratories) at a concentration of 1 mg ml⁻¹. All other antibiotics were purchased from Sigma-Aldrich.

Multipassage selection. SF8300ex was serially passaged in ceftobiprole as previously described (26) to obtain strain SRB. Briefly, 10-ml preparations of TSB containing various concentrations of ceftobiprole were inoculated at a 1:100 dilution with overnight cultures containing 10⁹ CFU ml⁻¹. The ceftobiprole concentration was doubled at each passage, as tolerated. The strains SF8300ex, Sp, and Sgap were serially passaged in ceftaroline in a similar manner to obtain strains SRT, SpT, and SgapT, respectively (Table 1). The strains mentioned above were passaged until bacterial growth was observed in at least 128 μ g ml⁻¹ of the drug specified (Fig. 1). Two to four single colonies were isolated from each passaging study. MIC tests revealed that each of the colonies was similarly resistant

to the respective antibiotic that was used in the passaging study (data not shown). A single colony was selected from each passaged strains and used throughout the study.

Serial passaging of COLnex, SF8300ex, and their isogenic Δ *pbp4* strains was carried out in nafcillin as described above, except that they were grown in 50 ml TSB medium. The passaging experiment for COLnex and SF8300ex was terminated when bacterial growth was observed at 256 μ g ml⁻¹ of nafcillin (see Fig. 5).

Allelic exchange and complementation. The plasmid pKOR1 was used to conduct allelic exchanges as previously described (33). Briefly, constructs for allelic exchange were created via splice overlap extension (SOE) PCR. The PCR fragments generated with the primers listed in Table S1 in the supplemental material were cloned into the plasmid pKOR1 using BP Clonase II. The constructs were transformed into *E. coli* Top10 cells. Purified plasmids from *E. coli* Top10 cells were electroporated into competent *S. aureus* RN4220. The plasmids were either transformed or transduced with phage ϕ 11 from RN4220 to host *S. aureus* strains of choice. The strains were grown at 30°C and 42°C for allelic exchange. Cultures were plated onto 1 μ g ml⁻¹ anhydrotetracycline plates for counterselection.

Complementation was carried out with the constitutively expressed plasmid pTX Δ (34). The *pbp4* gene was amplified with the primers specified in Table S1 in the supplemental material by standard PCR, digested with the restriction enzymes BamHI and MluI, and ligated to the empty pTX Δ plasmid digested with the same enzymes prior to the ligation step. The resultant plasmids were first selected in strain RN4220, followed by transformation or transduction to the strain of choice as stated.

Antibiotic resistance measurement. MICs were determined by broth microdilution. Briefly, 1 \times 10⁵ CFU were incubated for 24 or 48 h at 37°C in 0.2 ml cation-adjusted Mueller-Hinton broth (CAMHB) containing increasing concentrations of antibiotic. MIC levels were recorded as the lowest concentration without growth.

Population analyses were done by the agar method, as previously described (26). Tetracycline (12.5 μ g ml⁻¹) was added to the agar plates to

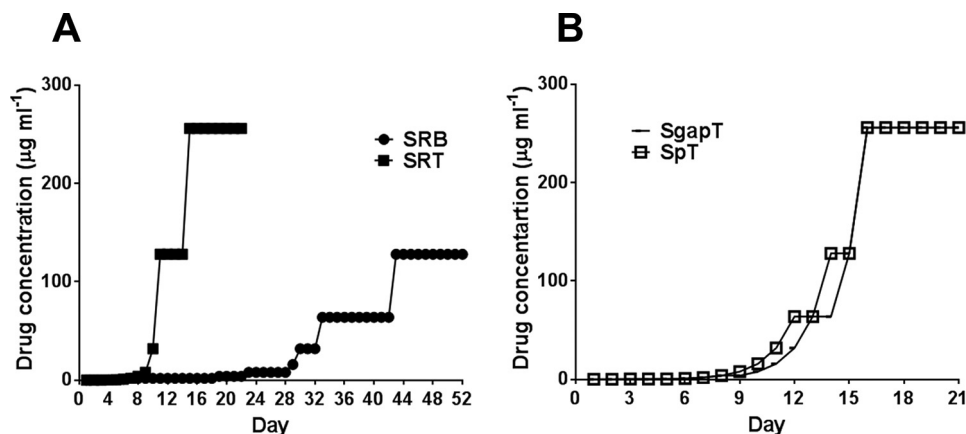


FIG 1 Serial passing of bacteria in ceftobiprole and ceftaroline. Bacteria were passaged every day in ceftobiprole or ceftaroline as described in Materials and Methods. (A) Strain SF8300ex passage in ceftobiprole (SRB) and ceftaroline (SRT). (B) Strain Sgap and Sp passage in ceftaroline.

select for the plasmid pTX_Δ whenever stated. A 10- μ l volume of serially diluted culture was spotted onto agar plates containing various concentrations of antibiotic. The plates were incubated at 37°C for 72 h. The plates were read, and the results were expressed as CFU per milliliter. Both MIC and population analyses were repeated at least twice, and the data from one experiment are presented.

Sanger sequencing. Mutations (see Table 3), including point mutations introduced in strains Sgap and Sp, were verified by Sanger sequencing. *pbp4* knockout strains and complemented constructs were verified by Sanger sequencing.

Bioinformatic and statistical analyses. Statistical analyses were performed using GraphPad Prism 5.04. Comparisons between groups were analyzed using two-way analysis of variance (ANOVA) whenever stated. DNA sequence analysis was performed using DNASTar. *In silico* analysis of the mutations on the cefotaxime-bound PBP 4 crystal structure (Protein Data Bank [PDB] 3HUM) was carried out with UCSF Chimera (35).

RESULTS

A USA300 strain of *S. aureus* can attain high-level resistance to ceftobiprole and ceftaroline in the absence of *mecA*. A SF8300 strain with *mecA* excised (SF8300ex) (36) was highly susceptible to a wide range of β -lactams compared to the isogenic wild-type strain, SF8300 (Table 2), underscoring the importance of *mecA* in β -lactam resistance in an *S. aureus* USA300 background. In contrast, both strains SF8300 and SF8300ex showed susceptibility to ceftaroline and ceftobiprole, confirming the activity of these drugs

TABLE 2 MICs for antibiotic-treated strains^a

Strain	MIC (μ g ml ⁻¹) ^a						
	NAF	AMP	CTX	FOX	CFZ	BPR	CPT
SF8300	32	128	>256	32	256	1	0.5
SF8300ex	0.5	0.25	4	4	1	0.5	0.25
SRB	8	4	32	1	16	>64	4
SRT	64	>256	>256	8	>256	8	>64
Sgap	0.5	<0.25	4	4	0.5	1	<0.25
SgapT	>256	>256	256	8	256	>64	64
Sp	1	0.25	4	4	0.5	0.5	0.25
SpT	>256	>256	>256	8	>256	>64	>64

^a NAF, nafcillin; AMP, ampicillin; CTX, ceftriaxone; FOX, cefoxitin; CFZ, cefazolin; BPR, ceftobiprole; and CPT, ceftaroline. The CLSI breakpoint for resistance is ≥ 4 μ g ml⁻¹ (45).

against the *mecA*-positive *S. aureus* USA300 background strain (Table 2).

To investigate if a *mecA*-negative strain can attain resistance to ceftobiprole and ceftaroline, we serially passaged strain SF8300ex in increasing concentrations of each drug. Strain SF8300ex attained growth in 256 μ g ml⁻¹ of ceftaroline within 15 days; the mutant strain selected for study is designated SRT. Strain SF8300ex grew in 128 μ g ml⁻¹ of ceftobiprole after 43 days of passage; the mutant strain selected for study is designated SRB (Fig. 1A; see Fig. S1 in the supplemental material). These data show that a representative USA300 strain can adapt to attain high-level resistance to either ceftobiprole or ceftaroline in the absence of *mecA*.

As described previously, COLnex, an archaic MRSA isolate of non-USA300 lineage and lacking the *mecA* gene, when passaged in ceftobiprole resulted in a highly broad-spectrum β -lactam-resistant derivative, strain, CRB (26). Whole-genome sequencing of CRB revealed point mutations in 3 genes, *pbp4*, *gdpP*, and *acrB*, that led to amino acid substitutions (28). To determine whether mutations detected in CRB play a role in β -lactam resistance in *S. aureus* USA300 background strains, we introduced mutations (N182K in GdpP, I960V in AcrB, and E183A and F241R in PBP 4) in strain SF8300ex to create strain Sgap and E183A and F241R in PBP 4 to create strain Sp (Table 1). Strains Sgap and Sp showed no detectable resistance in MIC tests performed using β -lactam drugs, including ceftaroline and ceftobiprole (Table 2). Thus, mutations associated with resistance in strain CRB were not able to confer β -lactam resistance in the USA300 background strain, probably due to marked differences in background between strains USA300 and COL (the parent of CRB).

Next, we tested if the mutations in strains Sgap and Sp can confer earlier resistance to ceftaroline by passaging both strains in increasing concentrations of ceftaroline, creating strains SgapT and SpT, respectively. Both SgapT and SpT grew at 256 μ g ml⁻¹ of ceftaroline within 15 days at a pace similar to that of strain SRT (Fig. 1A and B; see Fig. S1 in the supplemental material), suggesting that the mutations that were introduced in Sgap and Sp did not facilitate earlier development of resistance to ceftaroline.

The new-generation broad-spectrum cephalosporin-resistant isolates show broad-spectrum β -lactam resistance. The

TABLE 3 Point mutations detected in *S. aureus* PBP3, GdpP, and AcrB

Strain	Mutation(s) ^a					
	PBP1	PBP2	PBP3	PBP4	GdpP	AcrB
SRB	H499R; E567K	Y437C; V445L; Q453R; M559I	W228X	E183V; F241R	T509A	
SRT				N138K; H270L	Y306X	
Sgap				E183A ^b ; F241R ^b	N182K ^b	I960V ^b
SgapT	H499R			N138I; E183A ^b ; R200L; F241R ^b	N182K ^b	I960V ^b
Sp				E183A ^b ; F241R ^b		
SpT		G581D		N138I; E183V ^{b,c} ; T201A; F241R ^b	N214del	

^a X, stop codon.

^b Mutation was introduced by allelic replacement.

^c E183A was spontaneously mutated to E183V during the construction of SpT.

strains passaged in ceftobiprole and ceftaroline showed high-level stable resistance against the drugs in which they were passaged. SRB (passaged in ceftobiprole) and SRT, SgapT, and SpT (passaged in ceftaroline) were highly resistant (MICs > 64 $\mu\text{g ml}^{-1}$) compared to their parental strain (MICs $\leq 1 \mu\text{g ml}^{-1}$) (Table 2). Strains SRB and SRT showed lower levels of resistance to ceftaroline (MIC = 4 $\mu\text{g ml}^{-1}$) and ceftobiprole (8 MIC = $\mu\text{g ml}^{-1}$), respectively, supporting different modes of action for these drugs, as described previously (Table 2) (20, 21). On the other hand, both strains SpT and SgapT (passaged in ceftaroline) showed high-level cross-resistance to ceftobiprole (MICs $\geq 64 \mu\text{g ml}^{-1}$), suggesting the mutations introduced prior to passaging (N182K in GdpP, I960V in AcrB, and E183A and F241R in Pbp4 in Sgap and E183A and F241R in PBP 4 in Sp) might have contributed to the cross-resistance postpassaging (Table 2).

In addition, all the passaged strains showed across-the-board moderate to very high-level β -lactam resistance (nafcillin, ≥ 8 ; ampicillin, ≥ 4 ; ceftriaxone, ≥ 32 ; and ceftazidime, $\geq 16 \mu\text{g ml}^{-1}$) compared to their parental strain, SF8300ex (Table 2).

The resistant isolates revealed multiple mutations in genes known to confer β -lactam resistance. To investigate the underlying cause of resistance to ceftobiprole and ceftaroline in the passaged isolates, we sequenced all the core genome-encoded penicillin binding proteins (encoded by *pbp1* through *pbp4*). In addition, we also sequenced the *gdpP* and *acrB* genes, in which mutations were detected in the ceftobiprole-resistant passaged strain CRB (28). Sanger sequencing of the above-mentioned candidate genes revealed that our passaged isolates primarily contained mutations in all *pbp* and *gdpP* genes (Table 3). None of the passaged isolates revealed any mutations in *acrB* except SgapT, in which I960V in *acrB* was introduced during the creation of strain Sgap (see Materials and Methods).

Among mutations found in PBP 1, H499R was detected in strains SRB and SgapT. PBP 1 is an essential PBP and primarily functions as a transpeptidase in *S. aureus* (37). Several mutations were detected in *pbp2* in strains SRB and SpT. PBP 2 is the only bifunctional PBP in *S. aureus*, displaying both transglycosylase and transpeptidase activities (14). Interestingly, all the mutations detected in *pbp2* were located in the transpeptidase domain. A nonsense mutation was detected in *pbp3* of strain SRB that led to a premature stop codon, abolishing the entire C-terminal transpeptidase domain of PBP 3 (38). All of our passaged strains showed at least two point mutations in PBP 4, making it a highly mutated candidate in our study (Table 3). PBP 4 is a supplementary PBP known to function as a transpeptidase and a carboxypeptidase (CPase) (14, 39). Thus far, PBP 4 has been associated only

with low to moderate β -lactam resistance in *S. aureus* (30, 32). The PBP 4 mutations detected in this study clustered near its active site (Fig. 2). Additionally, each mutation detected in PBP 4 brought a change to the chemical properties of the amino acids. N138K/I and T201A brought in an electrically charged or hydrophobic amino acid in place of a polar amino acid. The E183V, R200L, and H270L mutations changed an electrically charged amino acid to a hydrophobic amino acid. F241R caused a change of a hydrophobic amino acid to an electrically charged one. All these amino acid changes suggest a significant change in enzyme activity for the mutant PBP 4. Furthermore, accumulation of all the PBP mutations in the transpeptidase domain affected the β -lactam target site, suggesting a common feature among the detected mutations.

Our study also shows several point mutations in GdpP, an enzyme crucial for maintaining cyclic-di-AMP (CDA) balance in bacteria. CDA is a recently discovered second messenger known to influence several life processes in bacteria, including virulence, ion export, and biofilm formation. Recent studies have suggested a role for CDA in the β -lactam resistance of *S. aureus* (40, 41). Mutations in GdpP in strains SRT, SRB, and SpT (Table 3) (only SgapT had a *gdpP* mutation introduced by allelic exchange), particularly the Y306Stop and T509A mutations (in SRT and SRB, respectively), were found adjacent to the DHH domain, crucial for the phosphodiesterase activity of GdpP (40). These mutations may alter the function of GdpP and could help mediate bacterial



FIG 2 Point mutations mapped on the PBP 4-cefotaxime complex. PBP 4 point mutations (yellow) detected in this study are depicted near the active site of cefotaxime-bound PBP 4 (PDB 3HUM).

TABLE 4 MICs of passaged strains and their isogenic *pbp4* deletion knockout strains

Strain	MIC ($\mu\text{g ml}^{-1}$) ^a						
	NAF	AMP	CTX	FOX	CFZ	BPR	CPT
SRB	8	4	32	1	16	>64	4
SRB $\Delta pbp4$	0.25	0.25	0.25	0.5	0.25	4	0.125
SRT	64	>256	>256	8	>256	8	>64
SRT $\Delta pbp4$	0.25	0.25	2	4	0.25	0.5	0.125
SgapT	>256	>256	256	8	256	>64	64
SgapT $\Delta pbp4$	0.25	0.25	1	2	0.25	0.25	0.125
SpT	>256	>256	>256	8	>256	>64	>64
SpT $\Delta pbp4$	0.25	0.25	2	4	0.25	0.5	0.125

^a NAF, nafcillin; AMP, ampicillin; CTX, ceftriaxone; FOX, ceftioxin; CFZ, cefazolin; BPR, ceftobiprole; and CPT, ceftaroline. The CLSI breakpoint for resistance is $\geq 4 \mu\text{g ml}^{-1}$ (45).

resistance or survival in our passaged isolates. However, further studies are needed to support this hypothesis.

PBP 4 mediates high-level class resistance to β -lactams in the resistant isolates. To determine if *pbp4* is the driving factor for high-level β -lactam resistance, we deleted the *pbp4* gene in the passaged isolates (Table 1). MIC assays performed for a variety of β -lactam drugs, including ceftobiprole and ceftaroline, revealed that the strains were fully susceptible to the entire class of β -lactams tested. MIC tests with ceftioxin, a known semiselective inhibitor of PBP 4 (42), showed only moderate decreases (2- to 4-fold) in the MIC for the $\Delta pbp4$ knockout strains (Table 4). Population analysis of the passaged isolates and their *pbp4* knockout strains for nafcillin also showed complete susceptibility of the knockouts compared to their isogenic wild-type strains, indicating that the resistant phenotype of the passaged strain was dependent on *pbp4* (Fig. 3A to D).

Next, the SRB $\Delta pbp4$ and SRT $\Delta pbp4$ knockout strains were complemented in *trans* with *pbp4* that originated either from the wild-type strain SF8300 or from the passaged strain SRB or SRT.

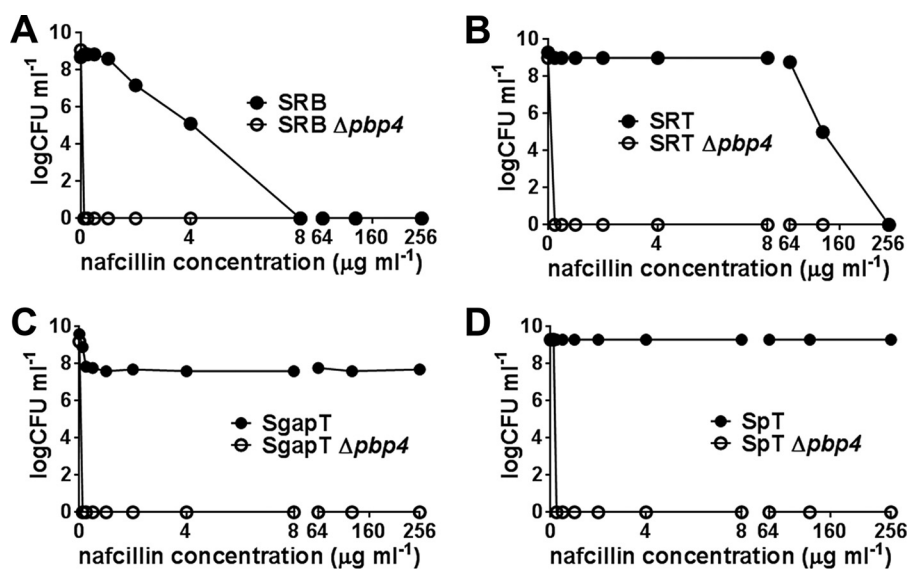


FIG 3 Population analysis of the passaged strains and their isogenic *pbp4* knockout strains in nafcillin. (A to D) Ten microliters of serially diluted bacterial overnight cultures was spotted onto agar plates containing various concentrations of nafcillin, as indicated. Bacterial colonies were enumerated (CFU ml^{-1}) after 72 h of incubation at 37°C.

Population analysis for nafcillin showed that PBP 4s originating either from SRB or from SRT provided significantly ($P < 0.0001$) higher resistance than the wild-type *pbp4*, clearly underscoring the importance of the mutations in PBP 4s in conferring elevated resistance (Fig. 4A and B). The wild-type *pbp4* from strain SF8300 provided significantly ($P < 0.0001$) higher resistance to SRB than the empty-vector control. In contrast, wild-type *pbp4* failed to confer resistance on SRT (Fig. 4A and B). These results suggest cooperation between PBP 4 and the other PBPs, all of which had the mutations in SRB and none of which had mutations in SRT. However, other, unknown candidate genes could also account for the resistant phenotype in the SRB strain.

PBP 4 is essential for conferring high-level β -lactam resistance on *S. aureus*. Cross-linking of mucopeptides during *S. aureus* cell wall synthesis is thought to be coordinated by multiple PBPs. It has been proposed that PBP 2, in particular, cooperates with PBP 4 to mediate proper cell wall synthesis (14). To determine if high-level β -lactam resistance can occur in the absence of *pbp4*, we serially passaged strains COLnex and SF8300ex, along with their isogenic $\Delta pbp4$ strains, in nafcillin. Nafcillin was used as a prototype β -lactam for this experiment due to its easy commercial availability. While strains COLnex and SF8300ex attained growth in 256 $\mu\text{g ml}^{-1}$ of nafcillin within 18 days, their isogenic $\Delta pbp4$ strains did not attain growth beyond 8 $\mu\text{g ml}^{-1}$ of nafcillin even after 60 days of our passaging experiment (Fig. 5). These results together further underline the critical role played by PBP 4 in the emergence of high-level β -lactam resistance.

DISCUSSION

Widespread antimicrobial resistance among bacterial pathogens, including *S. aureus*, has spurred renewed interest in searching for new and better drugs. The search will be facilitated by a detailed understanding of the mechanisms that lead to susceptibility and resistance to antibiotics (43; <http://www.cdc.gov/drugresistance/>). We show that *S. aureus* strain USA300 can attain high-level resistance to β -lactams, including those active against MRSA, in the absence

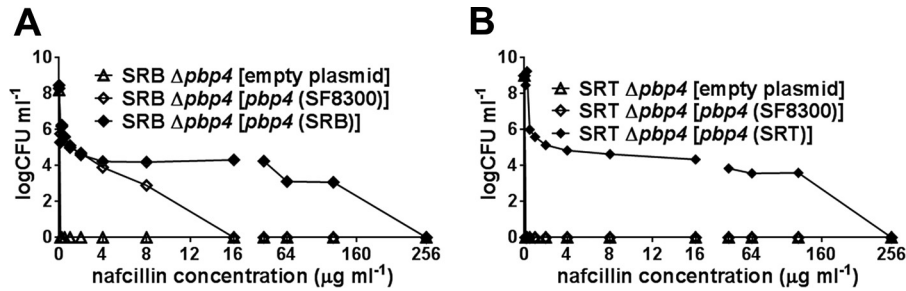


FIG 4 Population analysis of the complemented strains in nafcillin. (A and B) Ten microliters of serially diluted bacterial overnight cultures, as indicated, were spotted onto agar plates containing tetracycline (12.5 µg ml⁻¹) and various concentrations of nafcillin, as indicated. Bacterial colonies were enumerated (CFU ml⁻¹) after 72 h of incubation at 37°C. *pbp4* (SF8300), *pbp4* (SRB), and *pbp4* (SRT) represent the *pbp4* genes from strains SF8300, SRB, and SRT, respectively, that were cloned into pTX_Δ and introduced into the indicated strains. Two-way ANOVA of the data revealed significant differences ($P < 0.0001$) between the strains.

of *mecA*. Our observation supports previous work showing that a *mecA*-negative COLn strain was able to develop high-level β-lactam resistance (26). We have extended those results by demonstrating that an uncanonical member of the penicillin binding protein family, PBP 4, can mediate resistance in the community MRSA USA300 strain background (Fig. 3A to D and Table 4). In *S. aureus*, *pbp4* codes for a low-molecular-weight penicillin binding protein that is believed to function primarily as a transpeptidase and carboxypeptidase to perform the penultimate stages of bacterial cell wall synthesis (39). Although *pbp4* is conserved among all *S. aureus* strains, its function is generally considered supplementary and nonessential for both bacterial cell wall synthesis and high-level β-lactam resistance (30). Earlier work suggested that PBP 4 is responsible for forming higher cross-linked peptidoglycan chains and can be associated with moderate-level β-lactam resistance in CA-MRSA strains (14, 32). Our work supports these data and, in addition, indicates that PBP 4 can be associated with very high-level β-lactam resistance if needed.

Our study identified several point mutations in PBP 4 that were shown to provide elevated resistance (Fig. 4A and B). Mapping of these mutations to the recently crystalized PBP 4 identified all of them in close proximity to the active site of PBP 4 (Fig. 2). Interestingly, the E183A mutation that was introduced into strain Sp

was mutated to E183V postpassaging (Table 3). Although our study does not address the biochemical basis of the altered/superior function of the mutated PBP 4, we hypothesize the following scenarios: (i) the mutations may alter the binding affinity of PBP 4 for the β-lactams and/or (ii) the mutations may cause a gain of function, thereby turning the protein into a major TPase and CPase to allow bacterial cell wall synthesis. Experiments are ongoing to address these possibilities.

In addition to PBP 4, several point mutations were also detected in other PBPs (PBPs 1 to 3) in our study. Interestingly, all these mutations were present near the transpeptidase active site of the PBPs, the primary target of β-lactam drugs. The β-lactam susceptibility in our *pbp4* knockout strains to the β-lactam drugs tested here (Fig. 3A to D and Table 4), along with the inability of the *pbp4*-negative strains to display high-level resistance upon passaging in nafcillin (Fig. 5), confirm the key role of PBP 4 for the emergence and expression of resistance in our passaged isolates. It also seems likely that mutations in PBPs 1 to 3 have some supplementary role(s) in providing resistance to our isolates.

Our study also detected mutations in the *gdpP* gene in each of the passaged isolates examined. Recently, several points of evidence have associated GdpP with β-lactam resistance (44). Although a direct role of GdpP in *S. aureus* β-lactam resistance has yet to be identified, our study suggests that GdpP may provide some fitness to the bacteria to survive the β-lactam challenge. Finally, additional mutations that are not described in this study are likely to be present. Genome-sequencing studies are under way to provide further details of our finding on a genome-wide basis.

ACKNOWLEDGMENTS

We thank Carolina Roberte de Oliveira for technical help and Michael Otto for providing the plasmid pTX_Δ.

This work was funded in part by NIH grant R01-AI100291 (to H.F.C.). T.M.D.C. is sponsored by CNPq-Brazil.

FUNDING INFORMATION

This work, including the efforts of Henry F. Chambers, was funded by HHS | National Institutes of Health (NIH) (R01-AI100291).

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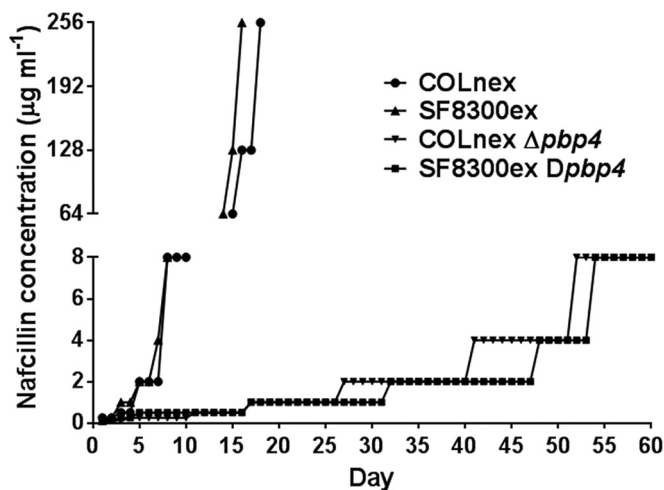


FIG 5 Serial passaging of bacteria in nafcillin. Bacteria were passaged every day in nafcillin as described in Materials and Methods. Each day, the drug concentration at which visible bacterial growth was observed was plotted.

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