Previously, we have shown that broad-spectrum 
resistance can also be mediated by penicillin-binding protein 4 (PBP4), 
encoded by the mecA gene of S. aureus, creating the CRB strain. This strain has two missense mutations in pbp4 and a mutation in the pbp4 promoter, both of which play an instrumental role in 
β-lactam resistance. To better understand PBP4’s role in resistance, here we have characterized its kinetics and structure with clinically relevant β-lactam antibiotics. We present the first crystallographic PBP4 structures of apo and acyl-enzyme intermediate forms complexed with three late-generation β-lactams, making them intractable to treatment. Although β-lactam resistance in MRSA has been ascribed to the acquisition and activity of penicillin-binding protein 2a (PBP2a), it has recently been observed that resistance can also be mediated by penicillin-binding protein 4 (PBP4).

Previously, we have shown that broad-spectrum β-lactam resistance can arise following serial passaging of a mecA-negative COL strain of S. aureus, creating the CRB strain. This strain has two missense mutations in pbp4 and a mutation in the pbp4 promoter, both of which play an instrumental role in β-lactam resistance. To better understand PBP4’s role in resistance, here we have characterized its kinetics and structure with clinically relevant β-lactam antibiotics. We present the first crystallographic PBP4 structures of apo and acyl-enzyme intermediate forms complexed with three late-generation β-lactam antibiotics: ceftobiprole, ceftaroline, and nafcillin. In parallel, we characterized the structural and kinetic effects of the PBP4 mutations present in the CRB strain. Localized within the transpeptidase active-site cleft, the two substitutions appear to have different effects depending on the drug. With ceftobiprole, the missense mutations impaired the Km value 150-fold, decreasing the proportion of inhibited PBP4. However, ceftaroline resistance appeared to be mediated by other factors, possibly including mutation of the pbp4 promoter. Our findings provide evidence that S. aureus CRB has at least two PBP4-mediated resistance mechanisms.

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This article contains Figs. S1–S9.

The atomic coordinates and structure factors (codes 6C39, 6C3X, 5TX1, 5TW8, 5TY7, 5TX9, 5TW4, and 5TY2) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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3 The abbreviations used are: MRSA, methicillin-resistant Staphylococcus aureus; Co, α-carbon; PG, peptidoglycan; PBP, penicillin-binding protein; PDB, Protein Data Bank; r.m.s.d., root mean square deviation; MIC, minimum inhibitory concentration.

Methicillin-resistant Staphylococcus aureus (MRSA)3 infections are a serious cause of both nosocomial and community-acquired infections, causing mortality and morbidity throughout the world. Indeed, the 2017 World Health Organization report, Priority Pathogens List for R&D of New Antibiotics, listed MRSA as a “high” priority pathogen for the development of new antimicrobials (1). Resistance to β-lactam antibiotics seen in MRSA is especially serious as they remain the most widely prescribed class globally, typically having a favorable safety profile and being relatively affordable and accessible. Although broad-spectrum MRSA resistance to β-lactam antibiotics has long been attributed to impaired acylation of the mecA gene product, penicillin-binding protein 2a (PBP2a) (2), recent evidence shows penicillin-binding protein 4 (PBP4), a low-molecular-weight monofunctional transpeptidase, can facilitate antibiotic resistance independently of PBP2a (3–9). It has been previously demonstrated that the only essential penicillin-binding proteins (PBPs) in S. aureus are the monofunctional high-molecular-weight transpeptidase, PBP1, and the bifunctional PBP2 (glycopeptidase/transpeptidase) (10). However, this strain expressing only the essential PBPs showed deficiencies in virulence and antibiotic resistance (10). Additionally, PBP4 has been shown to play an important role in mediating β-lactam resistance in some community-acquired MRSA strains (11).

PBPs ensure the integrity of the peptidoglycan (PG) sacculus by catalyzing peptide linkages between polymerized PG glycan chains, the hallmark transpeptidation reaction that β-lactam antibiotics such as penicillin and cephalosporins inhibit (12, 13). PBP4 is composed of two domains, one more N-terminal composed of a classic α-β-α sandwich transpeptidase domain and a second C-terminal domain of unknown function and composed of seven β-strands (14). Near the end of the C-terminal domain there is a transmembrane segment, anchoring PBP4 to the cell membrane.

The transpeptidase reaction is facilitated by three highly conserved signature motifs in PBPs: SXXK, (S/Y)(N/C), and (K/H)(S/T)G (15). In S. aureus PBP4, these motifs are S75MTK, S139SN, and K259TG (Fig. 1). The γ-O of Ser-75 is thought to...
be activated by the abstraction of the proton with Lys-78 serving in the general base role. The activated Ser-75 nucleophile then attacks the carbonyl carbon of the D-Ala–D-Ala bond in the donor PG stem peptide (Fig. 2), forming a tetrahedral oxyanion transition state before collapsing to an acyl-enzyme intermediate and releasing the leaving group terminal D-Ala from the stem peptide in the process (15). Deacylation follows when the N-terminal moiety of the acceptor strand pentaglycine bridge attacks, creating a stem peptide linkage between the two glycan chains and regenerating the resting state of the enzyme (15). The SXXK motif has been proposed to have roles in enzyme acylation and deacylation in the transpeptidase reaction mechanism (16). It is thought the serine in the SXXK motif may play a role in mediating the transfer of a proton from the lysine side chain N-ζ in the SXXK motif to the D-Ala leaving group nitrogen during acylation (16). The lysine of the KTG motif has been proposed to activate the serine of the SXN motif, which then abstracts a proton from the attacking amino nitrogen of the PG acceptor strand preparing it for nucleophilic attack of the α-carbon of the acyl-enzyme ester bond (16). The KTG residues also appear to play a role in orientating β-lactam antibiotics in the active site through hydrogen bonding with the carboxylate bound to the thiazolidine and dihydrothiazine ring in penicillin and cephalosporin antibiotics, respectively (16).

In an effort to better understand S. aureus β-lactam resistance, Chambers and colleagues (18) took S. aureus COL, a strain initially isolated from a hospital in Colindale, England, in the early 1960s (17), and excised the SCCmec cassette from a tetracycline-sensitive isolate creating the mecA (PBP2a)-negative S. aureus COLnex strain. Following the creation of the S. aureus COLnex strain, researchers explored its ability to...
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**Figure 2. *S. aureus* peptidoglycan monomer structure.** The PG glycan chain is characterized by repeating units of GlcNAc and N-acetyl-muramic acid. The stem peptide allows the glycan strands to be cross-linked, creating an essential protective mesh around the bacterium. There is some variation in the *S. aureus* stem peptide between strains with the d-iso-Gln carboxyl group being converted to an amide group in some strains.

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We have recently shown that PCRB high-level resistance within 18 days. By contrast, the isogenic strains SF8300 strains were passaged in nafcillin, they attained high-level resistance even after 60 days of passaging (4, 5). Additionally, changes in peptidoglycan composition and thickening of the cell wall have been observed in the CRB strain, suggesting increased peptidoglycan cross-linking is occurring (4, 5). We set out to understand therefore how PBP4 could be facilitating high-level β-lactam resistance in notoriously drug-resistant *S. aureus* infections.

To probe the structural and mechanistic contributions of the enzyme to β-lactam resistance, we have determined and compared the apo and acyl-enzyme intermediate X-ray crystallographic structures of *S. aureus* PBP4 with clinically relevant β-lactam antibiotics. Specifically, the atomic structures of PBP4 from both the COLnex strain (identical sequence to PBP4 from the parent COL strain) and PBP4CRB from the CRB strain (substitutions E183A and F241A) were solved in complex with the late-generation cephalosporins ceftaroline and ceftobiprole (designed to specifically inhibit PBP2a and approved to treat MRSA) (21–23), as well as nafcillin (a penicillin used to treat methicillin-sensitive *S. aureus* infections) (24). We also show PBP4 and PBP4CRB have differing kinetic behavior, with PBP4 having higher kinetic efficiency with several β-lactam antibiotics compared with the PBP4CRB.

**Results**

**X-ray crystallographic analysis of apo-PBP4 and PBP4** as well as acyl-enzyme intermediate structures with ceftaroline, ceftobiprole, and nafcillin

To investigate the structures of PBP4 and PBP4CRB with and without β-lactam antibiotics we grew apo-crystals of both recombinantly produced variants and subsequently soaked in ceftobiprole, ceftaroline, and nafcillin to allow characterization of the generated covalent adducts. The eight resulting crystal structures (Figs. 1, 3, and 4), represent the highest resolution *S. aureus* PBP4 structures published to date and the first characterizations of the apo and acyl-enzyme intermediate forms. All structures were generated from isomorphous crystals in the space group C121, giving us confidence that any differences observed between the mutant and WT structures are due to mutations in PBP4 and conformational effects of the bound ligands rather than differences in crystal packing. Additionally, comparison of existing PBP4 structures, all of which have been crystallized in space group P 21 21 21, show they closely align with the PBP4 apo structure solved here providing further support that the differences observed are not crystallization artifacts. The crystallographic data collection and refinement statistics for all structures described here can be found in Tables 1 and 2. The crystals have two highly similar monomers in the asymmetric unit (Car r.m.s. deviation for chains A and B for the various structures are presented in Table 3) with no obvious physiological dimeric interface (interfacial buried surface of $\sim$580 Å² in apo-PBP4 as calculated by PISA (25)). The uniformly excellent quality of crystals and data allowed us to obtain high-resolution structures at 1.7–1.6, 1.7, 1.9–1.6, 1.6–1.7, and 1.7 Å resolution for apo-PBP4, PBP4-ceftobiprole, PBP4-ceftaroline, PBP4-nafcin, apo-PBP4CRB, PBP4CRB-ceftobiprole, PBP4CRB-ceftaroline, and PBP4CRB-nafcin, respectively. Search of the Dali server (26) with chain B of apo-PBP4 suggests the PBP4-fold is most similar to the fold of several con-
firmed carboxypeptidases, PBPs, which preferentially catalyze the trimming of terminal D-Ala residues from PG peptides rather than cross-linking transpeptidation. The closest matches between the PBP4 apostructure solved here (residues 25–383) and other proteins in the PDB are PBP3 from *Streptococcus pneumoniae* and PBP5 and PBP6, both from *Escherichia coli*, as measured by Dali Z-scores of 39.2, 34.5, and 34.5, respectively (27–29). A Cα alignment of PBP3 from *S. pneumoniae* and PBP5 from *E. coli* show the N-terminal transpeptidase domains are broadly similar, whereas the cylindrical C-terminal domains composed of several β-strands show more variation (Fig. 5). The closest matches to the PBP4 C-terminal domain (residues 315 to 383) using a Dali search (26) arise from the C-terminal domains of PBP3 from *S. pneumoniae*, a D-alanyl-D-alanine carboxypeptidase from *Bacillus subtilis* (PDB ID 3MFD),4 and a deacylation-defective mutant of PBP5 from *E. coli* as measured by Dali Z-scores of 5.3, 5.0, and 4.8, respectively (27, 30).

Apo-PBP4 and PBP4CRB structures were found to be highly similar (Table 3). In both, the side chain N-ζ of residue Lys-78, the proposed general base, is hydrogen bonded with the O-γ of the Ser-75 serine nucleophile (2.9 Å for both PBP4 and PBP4CRB), presenting the optimal hydrogen bond distance and angle in the native enzyme for subsequent necessary deprotonation/activation of the serine hydroxyl during the acylation step.

In all acyl−enzyme intermediate structures, with the various substrates directly captured and observed, the 2mFo−DFc electron density maps clearly show the expected covalent linkage between the O-γ of the catalytic serine (Ser-75) and C8 of ceftobiprole and ceftaroline and C7 of nafcillin (Figs. S1 and S2), the first captured acyl−enzyme intermediates in this important MRSA antibiotic resistance mediator. Additionally, the mFo−DFc volume omit maps provide unambiguous evidence for the presence of the well-occupied ligand in the active site (Figs. S1 and S2) and Figs. S3–S5 show detailed 2D depictions of ligand−protein interactions generated using LigPlot+ (31). The structures also show the backbone amide nitrogens of residues Ser-75 and Ser-262 in PBP4 form the oxyanion hole, allowing for highly similar and optimal coordination and polarization of the substrate carbonyl (distances and angles nearly identical among the various structures), an electrostatic feature that enhances electrophilicity at the carbon center, and subsequent stabilization of the oxyanion tetrahedral transition state during acylation. WT PBP4 and mutant PBP4CRB structural differences of potential significance are depicted in Figs. 3, 4, and Fig. S6, whereas Co r.m.s.d. values comparing the structures and particular regions of the structures are listed in Table 3.

**PBP4 structures with ceftobiprole**

PBP4 and PBP4CRB structures in complex with ceftobiprole (22, 23) show antibiotic binding is stabilized via hydrogen bonds between the side chain O-γ of residue Ser-262 (2.6 Å for

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4 M. E. Cuff, E. Rakowski, K. Buck, and A. Joachimiak, unpublished data.

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![Figure 3. Separate structural alignments of (A) PBP4 structures and (B) PBP4CRB structures. Apo-PBP4 and PBP4 acyl−enzyme intermediate structures in complex with ceftobiprole, ceftaroline, and nafcillin are shown in light brown, blue, green, and teal, respectively. Apo-PBP4CRB and PBP4CRB acyl−enzyme intermediate structures in complex with ceftobiprole, ceftaroline, and nafcillin are shown in red, dark blue, purple, and light blue, respectively. The structures are depicted as cartoons and the catalytic serine (Ser-75), Ser-139 of the SxN motif, and mutated residue in PBP4CRB (F241R) are displayed as sticks in the bottom panel. Ligands are not shown for greater clarity.](image-url)
Figure 4. PBP4 and PBP4^{CRB} active-site residues in complex with ceftobiprole, ceftaroline, and nafcillin. Alignments of PBP4 and PBP4^{CRB} are shown in a covalent complex with (A) ceftobiprole, (B) ceftaroline, and (C) nafcillin along with 2D representations of the chemical structures of the β-lactam antibiotics used. PBP4 is shown as light gray lines and its covalently linked ligands are shown in teal, whereas PBP4^{CRB} is shown in dark gray and its covalently linked ligands are shown in orange. Selected residues involved in coordinating the ligands are displayed as lines with coloration according to atom type and water molecules are shown as cyan spheres. Ligands are displayed as thin stick and ball structures, whereas hydrogen bonding and electrostatic interactions are represented as black dashes.

Table 1
Data collection and structure refinement statistics for PBP4

| Ligand (PDB code) | Apo (6C39) | Ceftaroline (5TW8) | Ceftobiprole (5TXI) | Nafcillin (5TY7) |
|-------------------|------------|---------------------|---------------------|-----------------|
| **Data collection** |            |                     |                     |                 |
| Beamline          | CLS-081D-1 | CLS-081D-1          | CLS-081D-1          | CLS-081D-1      |
| Space group       | C21        | C21                 | C21                 | C21             |
| Cell dimensions   |            |                     |                     |                 |
| \(a, b, c (\AA)\) | 116.4, 92.4, 79.2 | 116.4, 92.2, 79.2 | 90.0, 99.3, 90.0 | 90.0, 99.2, 90.0 |
| \(\alpha, \beta, \gamma (\degree)\) | 90.0, 99.3, 90.0 | 90.0, 99.2, 90.0 | 90.0, 100.0, 90.0 | 90.0, 98.9, 90.0 |
| Wavelength (\AA) | 0.9795     | 0.9795              | 0.9795              | 0.9795          |
| Resolution (\AA)^a | 46.20–1.69 (1.75–1.69) | 43.10–1.72 (1.78–1.72) | 46.15–1.60 (1.66–1.60) | 50.41–1.89 (1.96–1.89) |
| No. unique reflections | 92,121 (9,120) | 87,245 (8,633) | 108,425 (10,241) | 63,836 (6,053) |
| CC1/2             | 0.998 (0.627) | 0.997 (0.584) | 0.998 (0.541) | 0.999 (0.846) |
| \(I/\sigma I\)    | 10.0 (1.72) | 9.40 (1.57) | 9.33 (1.19) | 12.19 (2.61) |
| Completeness (%)  | 99.1 (99.6) | 99.7 (99.7) | 98.2 (93.6) | 97.5 (93.4) |
| Redundancy        | 3.8 (3.8) | 3.8 (3.8) | 3.7 (3.3) | 3.8 (3.8) |
| **Refinement**    |            |                     |                     |                 |
| Resolution (\AA) | 46.20–1.69 | 43.10–1.72 | 46.15–1.60 | 39.69–1.89 |
| \(R_{work}/R_{free}\) | 0.172/0.205 | 0.186/0.215 | 0.171/0.202 | 0.185/0.222 |
| No. non-hydrogen atoms | 5,672 | 5,555 | 5,687 | 5,592 |
| Protein           | 0/17       | 78/5               | 78/20              | 58/6            |
| Water             | 667        | 481                | 580                | 388             |
| B-factors (\AA^2) |            |                     |                     |                 |
| Protein           | 26.8       | 32.2               | 23.0               | 37.8            |
| Ligand/ion        | 43.6       | 43.7               | 53.3               | 43.6            |
| Water             | 37.8       | 38.5               | 36.6               | 39.3            |
| R.m.s. deviations |            |                     |                     |                 |
| Bond lengths (\AA) | 0.005 | 0.006 | 0.015 | 0.007 |
| Bond angles (\degree) | 1.15 | 0.86 | 1.42 | 0.78 |
| Ramachandran favored/allowed/disallowed (%) | 98.0, 2.0, 0.0 | 97.6, 2.3, 0.1 | 98.3, 1.7, 0.0 | 97.7, 2.1, 0.1 |

^a Highest resolution shell is shown in parentheses.
as well as the side chain O- of Thr-260 (3.0 Å for PBP4 and 3.3 Å for PBP4\textsuperscript{CRB}) and the carboxyl group attached to C4 on the dihydrothiazine ring (Fig. 3A). Additionally, the side chain hydroxyl of Tyr-268 (3.1 Å for PBP4 and 3.0 Å for PBP4\textsuperscript{CRB}) is hydrogen bonded to the carbonyl oxygen in the R2 motif of ceftobiprole (Fig. 4A). The carboxyl group bonded to the C4 of the dihydrothiazine ring of ceftobiprole contributes to water-mediated hydrogen bond interactions via water W1 with the side chain N- of Arg-300, and the nitrogen of the oxime group in ceftobiprole similarly interacts via water-mediated hydrogen bonds with the backbone amides of Glu-183 and Ala-183 (via waters W2 and W3, respectively) in PBP4 and PBP4\textsuperscript{CRB}. In both structures, there are additional water-mediated hydrogen bonds between the R1 carbonyl oxygen at C8 and the backbone amide of Leu-115 (not shown in Fig. 4A for clarity), which could be contributing to the L1 loop adopting the closed position in both structures (Fig. 3). We also note a hydrogen bond between N1 of the ceftobiprole pyrrolidinyl group and the side chain carboxyl group of Asp-351 (2.7 Å for PBP4 and 2.8 Å for PBP4\textsuperscript{CRB}) in the neighboring monomer of the asymmetric unit in both PBP4 and PBP4\textsuperscript{CRB} complexes, potentially influencing the positioning of the ligand we observe. Furthermore, a sulfate and chloride ion (average B-factors 79.5 and 21.5 Å\textsuperscript{2}, respectively, at full occupancy) were modeled (see “Experimental procedures”) into the active site cleft in the PBP4 ceftobiprole structure, whereas interestingly the PBP4\textsuperscript{CRB} ceftobiprole structure appeared to lack both ions despite similar crystallization conditions.

**PBP4 structures with ceftaroline**

In both PBP4 and PBP4\textsuperscript{CRB} ceftaroline structures, a carboxyl group protruding from C4 of the dihydrothiazine ring of cef-

### Table 2

Data collection and structure refinement statistics for PBP4\textsuperscript{CRB}

Data corresponds to diffraction from a single crystal for each structure.

| Ligand (PDB code) | Apo (6C3K) | Ceftaroline (STW4) | Ceftobiprole (STX9) | Nafcillin (STY2) |
|-------------------|------------|-------------------|---------------------|-----------------|
| **Data collection** |            |                   |                     |                 |
| Beam line        | CLS-08ID-1 | CLS-08ID-1        | CLS-08ID-1          | CLS-08BM-1      |
| Space group      | C121       | C121              | C121                | C121            |
| Cell dimensions  |            |                   |                     |                 |
| a, b, c (Å)      | 116.9, 92.6, 79.2 | 115.7, 92.6, 79.6 | 116.9, 92.3, 79.4   | 113.9, 92.2, 79.0 |
| α, β, γ (°)      | 90.0, 99.3, 90.0 | 90.0, 99.8, 90.0   | 90.0, 100.2, 90.0   | 90.0, 98.4, 90.0 |
| Wavelength (Å)   | 0.9797     | 0.9795            | 0.9795              | 1.1046          |
| Resolution (Å)\textsuperscript{*} | 43.19–1.60 (1.66–1.60) | 46.28–1.57 (1.63–1.57) | 32.84–1.68 (1.74–1.68) | 42.80–1.70 (1.76–1.70) |
| No. unique reflections | 108,891 (10,914) | 114,289 (11,268) | 95,439 (9,378) | 85,749 (7,345) |
| R\textsubscript{merge} | 0.063 (1.158) | 0.074 (0.937) | 0.081 (0.731) | 0.057 (0.651) |
| CC1/2            | 0.999 (0.498) | 0.999 (0.654) | 0.998 (0.768) | 0.999 (0.630) |
| I/σ (Å)          | 12.45 (1.26) | 13.21 (1.66) | 10.83 (1.88) | 13.65 (1.54) |
| Completeness (%) | 99.1 (99.8) | 99.3 (98.3) | 97.8 (99.7) | 96.7 (83.4) |
| Redundancy       | 3.7 (3.7)  | 3.8 (3.8)  | 3.7 (3.8)  | 3.8 (3.4)  |

### Table 3

Cα r.m.s.d. values for PBP4 structures

| Structures or regions of structures compared | Cα r.m.s.d. (Å) | number of atoms aligned |
|---------------------------------------------|-----------------|------------------------|
| **Comparison of PBP4 and PBP4\textsuperscript{CRB} between structures** |                  |                        |
| PBP4 Apo, Chain B                           | PBP4\textsuperscript{CRB} Apo, Chain B | 0.13 | 355 |
| PBP4 Ceftobiprole, Chain B                  | PBP4\textsuperscript{CRB} Ceftobiprole, Chain B | 0.14 | 356 |
| PBP4 Ceftaroline, Chain B                   | PBP4\textsuperscript{CRB} Ceftaroline, Chain B | 0.57 | 353 |
| PBP4 Nafcillin, Chain B                     | PBP4\textsuperscript{CRB} Nafcillin, Chain B | 0.54 | 356 |
| **Comparison of chain A and B within the same structure** |                  |                        |
| PBP4 Apo, Chain A                           | PBP4\textsuperscript{CRB} Apo, Chain B | 0.33 | 360 |
| PBP4\textsuperscript{CRB} Apo, Chain B     | PBP4\textsuperscript{CRB} Apo, Chain B | 0.32 | 358 |
| PBP4 Ceftobiprole, Chain A                  | PBP4\textsuperscript{CRB} Ceftobiprole, Chain B | 0.28 | 355 |
| PBP4 Ceftaroline, Chain A                   | PBP4\textsuperscript{CRB} Ceftaroline, Chain B | 0.29 | 356 |
| PBP4 Nafcillin, Chain A                     | PBP4\textsuperscript{CRB} Nafcillin, Chain B | 0.22 | 355 |
| PBP4\textsuperscript{CRB} Nafcillin, Chain B | PBP4\textsuperscript{CRB} Nafcillin, Chain B | 0.33 | 350 |
| **Comparison of loops 1 and 2 between different structures** |                  |                        |
| PBP4 Apo, Chain B, loop 1 (residues 112–122) | PBP4\textsuperscript{CRB} Apo, Chain B, loop 1 (residues 112–122) | 0.13 | 11 |
| PBP4 Apo, Chain B, loop 2 (residues 138–140) | PBP4\textsuperscript{CRB} Apo, Chain B, loop 2 (residues 138–140) | 0.08 | 3 |
| PBP4 Ceftobiprole, Chain B, loop 1 (residues 112–122) | PBP4\textsuperscript{CRB} Ceftobiprole, Chain B, loop 1 (residues 112–122) | 0.25 | 11 |
| PBP4 Ceftaroline, Chain B, loop 1 (residues 112–122) | PBP4\textsuperscript{CRB} Ceftaroline, Chain B, loop 1 (residues 112–122) | 0.27 | 3 |
| PBP4 Ceftaroline, Chain B, loop 2 (residues 138–140) | PBP4\textsuperscript{CRB} Ceftaroline, Chain B, loop 2 (residues 138–140) | 2.59 | 11 |
| PBP4 Nafcillin, Chain B, loop 1 (residues 112–119) | PBP4\textsuperscript{CRB} Nafcillin, Chain B, loop 1 (residues 112–119) | 2.44 | 3 |
| PBP4 Nafcillin, Chain B, loop 2 (residues 138–140) | PBP4\textsuperscript{CRB} Nafcillin, Chain B, loop 2 (residues 138–140) | 2.59 | 11 |

\* Highest resolution shell is shown in parentheses.
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taroline is hydrogen bonded with the O-γ on Thr-260 (2.6 Å for PBP4 and 2.9 Å for PBP4\textsuperscript{CRB} (Fig. 4B). This carboxyl group is also within hydrogen-bonding distance of the O-γ Ser-262 in PBP4\textsuperscript{CRB} but long in PBP4 (2.6 versus 3.5 Å, respectively). The R2 group of ceftaroline in PBP4 engages in a π-stacking interaction between Phe-241 and the 1,3-thiazole ring (4.0 Å distance). In contrast, the R2 group of ceftaroline in PBP4\textsuperscript{CRB} is somewhat displaced out of the active site, such that the O-ε of Glu-297 is 4.8 Å away from N3 of the 1,3-thiazole ring in the PBP4\textsuperscript{CRB} structure compared with only 3.5 Å away in the PBP4 structure. This shift in ligand position is likely due to the introduction of a repulsive positive charge in the F241R mutation with the inherent positive charge on the 1-methylpyridinium nitrogen of ceftaroline (~4.8 Å away) and in parallel the abrogation of the π-stacking interaction between the 1,3-thiazole ring and Phe-241. In the PBP4 ceftaroline structure there are hydrogen bond (2.7 Å) contacts between the amine at C5 of the 1,2,4-thiadiazole ring and the O-ε of Glu-183. In contrast, the E183A mutation in PBP4\textsuperscript{CRB} eliminates this possibility. Instead it is replaced by a water (W4)-mediated interaction between the C5 amine substituent and the side chain amide N-ε of Asn-72. A chloride ion (average B-factor = 18.0 Å\textsuperscript{2}) was modeled in the PBP4\textsuperscript{CRB} nafcillin active site structure (see “Experimental procedures”) coordinated between the side chain nitrogen atoms of Lys-78 and Lys-258 at a distance of 3.0 and 2.8 Å, respectively. In the PBP4 structure with nafcillin, the chloride ion position is occupied by the side chain hydroxyl of Ser-139 due to variation in the position of the L2 loop.

Two loops bordering the PBP4 active site display alternate conformations

Although all PBP4 and PBP4\textsuperscript{CRB} structures shown here have similar overall architecture, there are differences in two loops bordering the active site. Loop 1 (L1; ordered residues 112–122 in apo, ceftaroline, and ceftobiprole bound structures and ordered residues 112–118 in the nafcillin bound structures) appears to adopt either an “open” or “closed” conformation that differs by 5.0–5.6 Å when comparing the positions of the Leu-115 Ca for PBP4 or PBP4\textsuperscript{CRB} structures (Fig. 3, Table 3). Interestingly, of the PBP4 structures, only the ceftobiprole acyl-enzyme intermediate structure appears to adopt the open position, whereas the apo, ceftaroline, and nafcillin structures all adopt the closed position. In contrast, only in apo-PBP4\textsuperscript{CRB} does the L1 loop adopt the closed position, whereas PBP4\textsuperscript{CRB} in complex with ceftobiprole, ceftaroline, and nafcillin all adopt the open position of the L1 loop. Loop 2 (L2; residues 138–140), which contains the SXN motif, also displays a similar pattern of differences in position between PBP4 or PBP4\textsuperscript{CRB} structures as shown in Fig. 3 and Table 3. In both PBP4 and PBP4\textsuperscript{CRB} structures displaying the open conformation, the perturbation of L2 causes a radical repositioning of the SXN motif serine hydroxyl such that it points away from the active site (Fig. 3).

Steady-state kinetic analysis of PBP4 and PBP4\textsuperscript{CRB}

Steady-state kinetic parameters for each of ceftaroline, ceftobiprole, and nitrocefin were calculated from plots of initial velocity versus substrate concentration (Fig. S7). All three β-lactams were poorly hydrolyzed by PBP4 and PBP4\textsuperscript{CRB}, with $k_{cat}$ values of less than 0.008 s\textsuperscript{−1} (Table 4), presumably reflecting slow deacylation rates previously observed for PBP4 (32). Although $k_{cat}$ values were similar (<4-fold difference) between PBP4 and PBP4\textsuperscript{CRB} for each of the three drugs, the $k_{cat}/K_{m}$ values differed between the two enzymes. More specif-
Table 4
Steady-state kinetic parameters for PBP4 and PBP4CRB

| β-Lactam       | PBP4        | PBP4CRB     |
|----------------|-------------|-------------|
|                | $k_{cat}$   | $K_m$       | $k_{cat}/K_m$ | $k_{cat}$ | $K_m$       | $k_{cat}/K_m$ |
| Cefotibrope    | $1.9 \pm 0.2$ | $1.1 \pm 0.7$ | $2000 \pm 1000$ | $7.2 \pm 0.4$ | $170 \pm 20$ | $43 \pm 6$ |
| Ceftaroline    | $2.50 \pm 0.06$ | $27 \pm 2$ | $91 \pm 8$ | $1.1 \pm 0.07$ | $21 \pm 6$ | $50 \pm 10$ |
| Nitrocefin     | $5.1 \pm 0.3$ | $2.1 \pm 0.4$ | $2500 \pm 500$ | $5.3 \pm 0.1$ | $100 \pm 6$ | $53 \pm 3$ |

Discussion

An improved understanding of *S. aureus* antibiotic resistance is needed to develop new antimicrobials and reduce patient mortality. Here we shed light on the mechanisms of PBP4-mediated β-lactam resistance using X-ray crystallography to characterize apo and acyl-enzyme intermediate complexes of native PBP4 and PBP4CRB from the drug-resistant CRB *S. aureus* strain with three clinically relevant β-lactam antibiotics: cefotibrope, ceftaroline, and nitrocefin. Additionally, we collected steady-state kinetic parameters for PBP4 and PBP4CRB with cefotibrope, ceftaroline, and nitrocefin. Together, these data advance our understanding of PBP4-mediated β-lactam resistance in *S. aureus*.

Despite differences in the positioning of loops bordering the active site in the PBP4 and PBP4CRB structures, the overall fold is preserved (Figs. 3, Fig. S6, Table 3). In general, we observe the PBP4CRB active-site cleft to be more closed and with lower B-factors for both the ligand and the surrounding residues compared with the PBP4 active-site cleft (Figs. S8 and S9). Given the isomorphous nature of the crystal structures determined here, one might suggest this apparent thermal order of a more closed state may facilitate less promiscuous interaction of this enzyme variant with antibiotics; whether this translates to heightened resistance *in vivo* remains to be verified.

Our data show the PBP4 structure aligns most closely to structures of known carboxypeptidases (Fig. 5), enzymes that trim rather than cross-link PG stem peptides. This is interesting as earlier *in vivo* experiments showed PBP4 acts to increase PG cross-linking and stiffness (5, 33), corroborating *in vitro* experiments suggesting transpeptidation rather than terminating carboxypeptidation is the primary action of PBP4 (34). It has been hypothesized that the C-terminal domains of *S. aureus* PBP4 and *E. coli* PBP5 may play a role in determining the preference for transpeptidase or carboxypeptidase activity (35). The *S. aureus* PBP4 C-terminal domain, annotated as DUF1958 (Pfam, PF09211), is distinct from the *E. coli* PBP5 C-terminal domain (Pfam, PF07943) and appears to be associated with transpeptidase activity in contrast to the carboxypeptidase activity of the latter (35). Interestingly, the *E. coli* PBP5 is N terminally anchored to the membrane, whereas *S. aureus* PBP4 is C terminally anchored. As shown in Fig. 5, the C-terminal domain has the most structural variation between *S. aureus* PBP4 and closely related PBP5 in the PDB. The implications of structural variations in these potentially dynamic accessory domains are currently not fully understood in terms of ultimate enzymatic specificity and activity, hindered by the hurdle of isolating homogenous PG substrates for atomic resolution analysis. Future experiments identifying and structurally characterizing the binding site of the PG acceptor strand in transpeptidases will help elucidate the mechanism by which the PG acceptor strand is selected to participate in decapoylation over water, as is used in carboxypeptidases.

Movement of two loops (L1 and L2) bordering the active site causes reorientation of the serine hydroxyl of the SxN motif in PBP4CRB structures (Fig. 3 and Fig. S6). L1 includes residues that directly interact with the SxN motif, whereas L2 includes the SxN motif, common to PBP5s and containing key mechanistic residues as described above (15, 16, 30, 36, 37). It is intriguing to note that in all the ligand-bound PBP4CRB structures, the open conformation of L1 and L2 is adopted with the SxN motif serine (Ser-139) hydroxyl pointing away from the active site. In PBP5s, the SxN motif serine hydroxyl is typically positioned between the serine of the SxXK and lysine of the KTG motifs. This provides the hydrogen bonding and electrostatic environment for catalysis allowing the necessary serine hydroxyl mediated protonation of the leaving group nitrogen of the D-Ala–D-Ala peptide substrate or alternatively of the β-lactam antibiotic ring (substrate analogs). Indeed, the PBP4CRB structures with ceftaroline and nafcillin both indicate significant displacement of the SxN motif located in L2, compared with the PBP4 structure with the same ligands (Figs. 3 and Fig. S6, C and D).

Although the sequence is not conserved, analogous structures to the L1 loop can be found in class A, B, and C PBPs and perturbation of this loop has been shown to play a role in β-lactam resistance (30, 38, 39). Notably, PBP2x from a highly mutated, penicillin-resistant strain of *S. pneumoniae* PBP2x, was also found to have variation in positioning of the SxN motif with the serine hydroxyl displaced away from the active site when compared with penicillin-sensitive PBP2x variants (40). In contrast, with PBP4 and PBP4CRB the movements of the SxN motif appear to be mediated by a combination of the particular drug bound to the catalytic serine and the presence of the mis-sense mutations in PBP4CRB underlying the complexity of predicting resistance patterns in these variants.

The similar catalytic constants ($k_{cat}/K_m$ values) of PBP4 and PBP4CRB for the three tested β-lactam antibiotics (Table 4) suggests that the two substitutions in PBP4CRB do not affect the rate-limiting step of β-lactam hydrolysis. Previous studies have established that decapoylation is rate-limiting in PBP4 (32) with
rate constants for deacylation ($k_d$) from several penicillin-sensitive and -resistant strains ranging from 0.4 to $3.4 \times 10^{-3}$ s$^{-1}$ (32). These values are very similar to the $k_{cat}$ values of PBP4 and PBP4$^{CRB}$ found here, suggesting that deacylation is also rate-limiting in the turnover of ceftaroline, ceftobiprole, and nitrocefin. Despite the variation in the positioning of the SXX motif in the eight crystal structures presented here, and the overall higher $B$-factors suggesting potential dynamic motion (Fig. S9), we do not observe significant changes in deacylation rates of the associated variants. It therefore follows that the $S. aureus$ PBP4 SXX motif may primarily be involved in acylation. In the structures presented here the distances between the SXX serine (Ser-139) and the leaving group $\beta$-lactam nitrogen ranges from 3.5 to 5.7 Å, suggesting that it is in a position to take part in catalysis when loops L1 and L2 are in the closed confirmation but not when they are in the open confirmation (Table 5 and Fig. 3). Although it has been suggested that this motif plays a role in deacylation in the structurally similar PBP5 of $E. coli$ (30, 36, 37), perturbation of the SXX motif in PBP2, a transpeptidase from *Neisseria gonorrhoeae*, has been previously shown to reduce penicillin acylation rates (39). Future work is needed to determine the individual rate constants of acylation and deacylation in PBP4 and PBP4$^{CRB}$ to better understand the role of the displaced and/or dynamic motion of the SXX motif in $S. aureus$ PBP4 and the general resistance phenomenon it mediates.

Our 8 isomorphous high-resolution structures allow comparison of hydrogen bonding between the ligand and protein or solvent in the PBP4 and PBP4$^{CRB}$ structures, as summarized in Table 6. Interestingly, in the PBP4$^{CRB}$ structures all three ligands examined here appear to have additional hydrogen bonds to water compared with ligands in the PBP4 structures. Although the number of hydrogen bonds between the ligand and protein residues are relatively similar there are differences in the ligand hydrogen-bonding networks to water and protein that may facilitate resistance.

The steady-state kinetic parameters we determined for PBP4$^{CRB}$ and ceftobiprole are consistent with the $S. aureus$ CRB-resistance phenotype for this $\beta$-lactam drug. The lower catalytic efficiency of PBP4$^{CRB}$ for ceftobiprole suggests it does not compete as effectively with the enzyme’s physiological substrate and further supports the important role of the $pbp4$ missense mutations (E183A and F241R) in our proposed mode of ceftobiprole resistance. Given this, we note, the $K_m$ values of PBP4 and PBP4$^{CRB}$ with ceftobiprole determined here (1.1 ± 0.7 and 170 ± 20 μM, respectively) agree remarkably well with the minimum inhibitory concentrations (MIC) of $S. aureus$ COLnex and $S. aureus$ CRB (1 (2 μM) and 128 μg/ml (239 μM), respectively) (5). Furthermore, the MIC for $S. aureus$ COLnex $P_{CRB} pbp4$ (a COLnex strain with the same $pbp4$ promoter mutation found in $S. aureus$ CRB) was 4 μg/ml for ceftobiprole (5), suggesting this promoter mutation only plays a minor role in $S. aureus$ CRB ceftobiprole resistance.

Our data also indicate the mechanisms of PBP4-mediated resistance in $S. aureus$ may differ depending on the $\beta$-lactam antibiotic challenge. The similar kinetic parameters of PBP4 and PBP4$^{CRB}$ for ceftaroline may indicate that the missense mutations in PBP4$^{CRB}$ may not be the sole determinant of resistance to this drug. Instead, the previously described $S. aureus$ CRB $pbp4$ promoter mutation ($P_{CRB} pbp4$) may play a more pivotal role in this case (5). The ceftaroline MICs were 64 and 32 μg/ml for $S. aureus$ CRB and $S. aureus$ COLnex $P_{CRB} pbp4$ (5), supporting the significance of $P_{CRB} pbp4$ in ceftaroline resistance. As $S. aureus$ COLnex was passaged in ceftobiprole to generate the CRB strain (19), the mutations in PBP4$^{CRB}$ may not be optimal for ceftaroline resistance, particularly considering that passaging of $S. aureus$ in ceftaroline resulted in different $pbp4$ mutations than those seen for $S. aureus$ CRB (6).

Collectively, we provide evidence $S. aureus$ CRB employs at least two different PBP4-mediated mechanisms of resistance. High-level ceftobiprole resistance in this strain is heavily reliant on two PBP4 missense mutations, E183A and F241R. In contrast, the CRB strain resistance to ceftaroline appears less influenced by PBP4$^{CRB}$ mutations and is instead at least partially conferred by increased expression of PBP4. These results have implications for screening and diagnostics of $S. aureus$ infections as well as monitoring programs. As neither of these resistance mechanisms utilize PBP2a, even advanced PCR screening for mecA, mecC, or the staphylococcal cassette chromosome mec element, as has previously been used (41), may not identify $S. aureus$ infections with high-level $\beta$-lactam resistance. Indeed, mecA negative strains with a high-level of $\beta$-lactam resistance have already been observed in the clinic (42). Thus, our studies here recommend a more thorough investigation of potential resistance genes rather than simply looking for the presence of PBP2a when screening for MRSA. This work also emphasizes the importance of PBP4 in $S. aureus$ high-level $\beta$-lactam resistance and indicates the potential of combination therapies targeting PBP4 and other PBPs as noted previously (11). Additionally, our high-resolution acyl-enzyme intermediate structures of PBP4 and PBP4$^{CRB}$ provide a starting point for structure-aided drug design of improved PBP4 inhibitors.

**Experimental procedures**

**Expression and purification of PBP4 and PBP4$^{CRB}$**

The expression and purification of PBP4 and PBP4$^{CRB}$ was performed as described previously (5).
Antibiotics

Antibiotics for crystallization and kinetic experiments were prepared as follows. Cefotibiprole (Basilea Pharmaceutica), was dissolved in DMSO with 0.2% (v/v) TFA. Ceftaroline (Forest Labs) and nitrocefin (Toku-E) stocks were prepared in DMSO, whereas nafcillin (Sigma) was dissolved in water.

Crystallization, structure determination, and modeling of PBP4 and PBP4CRB structures

PBP4 crystals were obtained via the sitting drop vapor diffusion method in 24-well plates with streak seeding and incubation at 23 °C. Streak seeding was performed by stroking PBP4 crystals in mother liquor with a housecat whisker before drawing the whisker through freshly set up drops. PBP4 in buffer C (20 mM MES, pH 6, 300 mM sodium chloride) was crystallized in a 1:1 volume ratio of protein at 30 mg/ml and precipitant solution (8 mM zinc chloride, 80 mM sodium acetate, pH 5, 400 mM dimethyl(2-hydroxyethyl)ammonium propane sulfonate, and 16% PEG 6000), with a total drop volume of 2 μl. PBP4CRB was crystallized in a 1:1 volume ratio of protein at 15–20 mg/ml and precipitant solution (8 mM zinc chloride, 80 mM sodium acetate, pH 5, 100 mM sodium fluoride, and 16% PEG 6000), with a total drop volume of 2 μl. Antibiotics were soaked into the crystals for 40–180 min at final concentrations of 2 mM for ceftaroline, 5 mM for nafcillin, and 0.75 mM for cefotibiprole. Cryoprotectant (1:1 precipitant solution and buffer C, with a final concentration of 15% glycerol, and antibiotic at the soaking concentrations indicated above) was added to the crystals prior to looping, vitrification, and storage in liquid nitrogen.

Data were collected at the Canadian Light Source Synchrotron beam lines 08ID-1 and 08B1-1 under cryogenic conditions (100 K). Data were processed using Xia2 (43) and XDS (44) with a space group of C121 and merged with Aimless (45) in the CCP4 software package (46). The structures were solved via molecular replacement using Phaser (47), with chain A of PDB ID 1TVF as the starting model. Model building and refinement were conducted with the Phenix suite of programs (48). Particularly, AutoBuild (49) was used with iterative rounds of manipulating the model into the electron density with Coot (50), followed by refinement with Phenix.refine (51) with TLS being used in the later stages of refinement. The same set of Rfree flags were used for cross-validation purposes in all eight structures. All structures were refined using isotropic B-factors. Ligand coordinates and restraints were generated using ACE-DRG (distributed within the CCP4 package) and ligands were refined with an occupancy of 1 in all structures. Ions were included in the models based on the electron density, refined B factors, and the surrounding chemical environment. Structures were validated with Molprobity (52) and PDB redo (53). The interfacial buried surface area PBP4COIL was calculated using PISA (25). Figs. 1, 3–5, and S1, S2, S6, S8, and S9 were generated using PyMOL (54) using chain B of the structures solved here. R.m.s.d. calculations comparing the Cα alignment of the structures were performed in PyMOL (54) also using chain B. The sequence alignment in Fig. 1C was produced using the ESPript 3.0 server (55). Figs. S3–S5 were generated using LigPlot + (31). Coordinates and structure factors for PBP4-apo, PBP4-ceftobiprole, PBP4-ceftaro-

Analysis of S. aureus PBP4 structure and kinetics

Protein was prepared as previously described (5) and aliquots were thawed on ice as needed. PBP4 was used at a final concentration of 0.5 μM, whereas PBP4CRB was used at concentrations of 1 to 5 μM. Protein concentrations were measured via absorbance at 280 nm using an extinction coefficient of 45,270 M⁻¹ cm⁻¹ for PBP4 and PBP4CRB calculated using ProtParam (56). All enzymatic reactions were carried out at 25 °C using a plate reader (Bio-tek Synergy H4) in 384-well plates (Corning 3540). Enzyme assays were carried out in reaction buffer (40 mM HEPES, 100 mM sodium chloride, pH 7.5) in a total volume of 20–30 μl. Hydrolysis of the β-lactam ring in ceftobiprole, ceftaroline, and nitrocefin was monitored as previously described by measuring absorbance at 290 (Δε290 = 6,970 M⁻¹ cm⁻¹), 306 (Δε306 = −6,300 M⁻¹ cm⁻¹), and 486 nm (Δε486 = 14,600 M⁻¹ cm⁻¹), respectively (57, 58). State-kinetic parameters were calculated via nonlinear least-squares regression to the Michaelis-Menten-Henri equation (GraphPad Prism7) with data from initial velocities. For each substrate concentration, data were collected using protein from two separate protein purifications.

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