Methicillin-resistant *Staphylococcus aureus* (MRSA) infections cause significant mortality and morbidity globally. MRSA resistance to β-lactam antibiotics is mediated by two divergents that control levels of a β-lactamate, PC1, and a penicillin-binding protein poorly acylated by β-lactam antibiotics, PBP2a. Expression of genes encoding these proteins is controlled by two integral membrane proteins, BlaR1 and MecR1, which both have an extracellular β-lactam–binding sensor domain. Here, we solved the X-ray crystallographic structures of the BlaR1 and MecR1 sensor domains in complex with avibactam, a diazabicyclooctane β-lactamate inhibitor at 1.6–2.0 Å resolution. Additionally, we show that *S. aureus* SF8300, a clinically relevant strain from the USA300 clone of MRSA, responds to avibactam by up-regulating the expression of the *blaZ* and *pbp2a* antibiotic-resistance genes, encoding PC1 and PBP2a, respectively. The BlaR1–avibactam structure of the carbamoyl-enzyme intermediate revealed that avibactam is bound to the active-site serine in two orientations ~180° to each other. Although a physiological role of the observed alternative pose remains to be validated, our structural results hint at the presence of a secondary sulfate-binding pocket that could be exploited in the design of future inhibitors of BlaR1/MecR1 sensor domains or the structurally similar class D β-lactamases. The MecR1–avibactam structure adopted a singular avibactam orientation similar to one of the two states observed in the BlaR1–avibactam structure. Given avibactam up-regulates expression of *blaZ* and *pbp2a* antibiotic resistance genes, we suggest further consideration and research is needed to explore what effects administering β-lactam–avibactam combinations have on treating MRSA infections.

*Staphylococcus aureus* is an important Gram-positive pathogen infecting humans and livestock around the world (1). Although *S. aureus* commonly forms part of the human microbiome as a commensal species, it also causes serious disease as an opportunistic pathogen in both nosocomial and community settings (2). Methicillin-resistant *S. aureus* (MRSA) strains cause particularly notorious infections, due to their virulence and the reduced treatment options available (1).

Although the β-lactam class of antibiotics has long been successfully used to inhibit penicillin-binding proteins (PBPs) and continues to be the most commonly prescribed class of antibiotics (3), this antibiotic class is now frequently ineffective in treating MRSA infections. *S. aureus* resistance to β-lactam antibiotics is facilitated by the β-lactamase PC1 (4, 5) and penicillin-binding protein 2a (PBP2a) (5, 6). PC1 is a class A β-lactamase, which protects the bacterium by catalyzing the hydrolysis of the β-lactam ring, thereby preventing inhibition of PBPs (4). Expression of *pbp2a* enables broad-spectrum β-lactam antibiotic resistance via its sterically hindered active site (6), which is proposed to open when peptidoglycan binds to its allosteric site (7). Expression of *blaZ* (the gene coding for PC1) and *pbp2a* are encoded and regulated by the bla and mec divergents in MRSA with similar pathways existing in *Bacillus licheniformis* (8) and the pathogen *Clostridium botulinum* (9).

A schematic of the bla and mec pathways is shown in Fig. 1. Expression of *blaZ* is regulated by two proteins: BlaR1 and Blal. BlaR1 is a 69.3-kDa polytopic α-helical membrane protein with a predicted amino-terminal zinc-metalloprotease domain and carboxyl-terminal penicilloyl serine transferase extracellular domain that functions as a β-lactam sensor. Blal is a transpositional repressor that regulates the expression of *blaZ*, and *pbp2a* by binding to the operator and restricting transcription of the divergon (10). The mec divergon is closely analogous to the bla divergon with a sensor/transducing protein, MecR1, and a repressor protein MecI, both regulating the expression of *pbp2a* (11). The close protein sequence identity of the corresponding proteins in the pathway (MecR1/BlaR1, 35%; MecI/Blal, 61%) suggests the mec and bla pathways are similarly regulated and indeed, BlaR1 can regulate the mec pathway in the absence of MecR1 (12). Acylation of BlaR1/MecR1 sensor domains (here denoted BlaR1<sup>SD</sup> and MecR1<sup>SD</sup>, respectively) by a β-lactam antibiotic is hypothesized to activate the zinc metalloprotease domain of both proteins, leading to the cleavage of the repressor (BlaI/MecI).

Searches of the PDB using the Dali server reveal BlaR1<sup>SD</sup>/MecR1<sup>SD</sup> most closely resemble the Ambler class D β-lacta-
The top match for both sensor domains is the class D β-lactamase, YbxI, from Bacillus subtilis (PDB ID 5E2F) (Dali Z scores of 30.5/34 and C-α RMSD of 3.7/3.0 Å over 212/216 atoms for BlaR1SD and MecR1SD, respectively). BlaR1SD/MecR1SD are also structurally very similar to the Gram-negative class D β-lactamases, OXA-10 from Pseudomonas aeruginosa (e.g. PDB ID 4S2O) and OXA-48 from Klebsiella pneumoniae (e.g. PDB ID 4S2K) (14).

Both sensor domains have the three canonical catalytic motifs found in all penicillin-binding proteins and β-lactamases: SXX, (S/Y/F)X(N/C), and (K/H)(S/T)G (15, 16). Despite close structural similarity to OXA-10/OXA-48 both the sensor domains have an SXN motif instead of (Y/F)XN common in the class D β-lactamases (16).

The sole function of a β-lactamase is to hydrolyze β-lactam antibiotics as efficiently as possible, affording drug resistance to the pathogen. In contrast, BlaR1 and MecR1 function as receptors and therefore it is presumably advantageous to have a slow deacylation rate to allow signal propagation even in the presence of low concentrations of β-lactams. Although both class D β-lactamases and the sensor domains described here bind β-lactams, a key difference between the BlaR1/MecR1 sensor domains and β-lactamases is their deacylation rates. Deacylation in class D β-lactamases is thought to occur when the N-ζ carboxy-lysine of the SXXK motif abstracts a proton from the hydrolytic water (17). This activated nucleophile then attacks the α-carbon of the acyl-enzyme intermediate, causing the enzyme to be regenerated (17). The BlaR1 sensor domain has also been found to have a carboxylated SXK motif lysine in the ligand-free form (18). However, lysine carboxylation is lost upon acylation of the BlaR1 SXK motif serine (19). The sensor domains are thought to retard the regeneration of the carboxylysine via hydrogen bonding between the N-ζ of the lysine and a nearby asparagine side chain (Asn-339 in BlaR1 and Asn-341 in MecR1) (18, 20).

Efforts to re-potentiate β-lactams rendered ineffective by the presence of the class A serine β-lactamases have been made using a combination therapy of β-lactam antibiotic and β-lactam–based β-lactamase inhibitor (21). Further research with diazabicyclooctane-based inhibitors led to the development of the first clinically used non-β-lactam β-lactamase inhibitor, avibactam (22). Once avibactam is carbamylated to the β-lactamase active site serine it has a very slow rate of decarbamylation (23). Furthermore, upon eventual processing, intact avibactam is produced instead of a hydrolyzed product as is found with conventional β-lactam–based β-lactamase inhibitors (Fig. 2) (23, 24).

New broad-spectrum antibiotics are critically needed to allow treatment prior to the identification of the bacterial
species causing the infection. Avibactam in combination with the β-lactam ceftazidime has since been approved in the United States by the FDA to treat complicated urinary tract infections, intra-abdominal infections, hospital-acquired bacterial pneumonia, and ventilator-associated bacterial pneumonia (25).

Although ceftazidime/avibactam combination therapy is currently indicated in the treatment of Gram-negative organisms, studies have shown that combination therapy with ceftaroline and avibactam is additionally effective against clinical isolates of MRSA and pathogenic Gram-negative bacteria such as Enterobacteriaceae, P. aeruginosa, and Acinetobacter spp. suggesting avibactam could be useful in treating infections where the causative pathogen has yet to be identified or where a broad-spectrum antibiotic is needed (26–28).

Although avibactam has shown promise as a β-lactamase inhibitor, it has also been shown to influence transcription of the β-lactamase gene ampC found in Enterobacter cloacae with effects ranging from strong up-regulation of ampC transcription to no detectable up-regulation in other strains (29, 30). For these reasons, we are interested whether avibactam, a non-β-lactam, can also activate the bla and mec pathways of MRSA in a similar way as for conventional β-lactam antibiotics or whether it inhibits these pathways by preventing β-lactams from binding.

In this paper, we show evidence that avibactam binds to the BlaR1SD and MecR1SD and up-regulates blaZ and phe2a expression in the S. aureus SF8300, a USA300 clone. The USA300 MRSA clone is a leading cause of MRSA infections in the USA (31, 32). Additionally, we use X-ray crystallography to visualize the molecular interactions between avibactam and the sensor domains of BlaR1 and MecR1. Although avibactam adopts a single conformation in MecR1SD, it is oriented in two, sensor domains of BlaR1SD and MecR1SD and up-regulates these pathways. The two avibactam crystal structures were solved to 2.0 and 1.6 Å resolution, respectively, with excellent statistics and no Ramachandran outliers (full data collection and refinement statistics shown in Table 1). BlaR1SD crystals were obtained in potassium citrate and PEG3350 at pH 8.1 while MecR1SD crystals formed in ammonium sulfate at pH 7.5. Avibactam was modeled with a total occupancy of 1 in both structures with clear electron density supporting modeled positions as shown by 2mFo–DFc maps (Fig. 5) and mFo–DFc volume omit maps (Fig. S1). In both structures, avibactam is covalently bound to the catalytic serine of BlaR1 (Ser-389) or MecR1 (Ser-391) SXXK motifs with the C7 carbonyl group coordinated in the oxyanion hole by the backbone nitrogen atoms of Ser-389 and Thr-529 in BlaR1 (Ser-391 and Thr-531 in MecR1) (Fig. 5).

Results

Effect of avibactam on gene expression of phe2a and blaZ

Avibactam activates expression of the bla and mec divergents in S. aureus SF8300, a USA300 clone of MRSA (Fig. 3). S. aureus SF8300 lacks MecR1 from the mec divergon so phe2a expression is under the control of BlaR1. Both phe2a and blaZ mRNA transcripts were up-regulated by avibactam at concentrations of ≥8 μg ml⁻¹ with further increased expression at 16, 32, and 64 μg ml⁻¹. Expression levels of phe2a and blaZ mRNA transcripts at 2 μg ml⁻¹ were comparable with the DMSO control used.

Thermal stability of BlaR1SD and MecR1SD with avibactam and β-lactam antibiotics

Avibactam binding to the BlaR1SD and MecR1SD affects their thermal stability differently. The thermal stability of the sensor domains was measured in the presence of a range of concentrations of different β-lactam antibiotics and avibactam using differential static light scattering. Avibactam appears to increase the thermal stability of MecR1SD by 2.6 °C, whereas minimally affecting BlaR1SD thermal stability (0.4 °C of stabilization) (Fig. 4). Similarly, nafcillin also appears to confer different effects on the two proteins, but with the opposite effect, stabilizing BlaR1SD but having little effect on MecR1SD. As expected, both sensor domains show an increase in ΔTagg when acylated by ampicillin and were not affected by the control antibiotic kanamycin for which they are not a target.

BlaR1SD and MecR1SD avibactam crystal structures

BlaR1SD and MecR1SD avibactam X-ray crystallographic co-structures were solved to 2.0 and 1.6 Å resolution, respectively, with excellent statistics and no Ramachandran outliers (full data collection and refinement statistics shown in Table 1). BlaR1SD crystals were obtained in potassium citrate and PEG3350 at pH 8.1 while MecR1SD crystals formed in ammonium sulfate at pH 7.5. Avibactam was modeled with a total occupancy of 1 in both structures with clear electron density supporting modeled positions as shown by 2mFo–DFc maps (Fig. 5) and mFo–DFc volume omit maps (Fig. S1). In both structures, avibactam is covalently bound to the catalytic serine of BlaR1 (Ser-389) or MecR1 (Ser-391) SXXK motifs with the C7 carbonyl group coordinated in the oxyanion hole by the backbone nitrogen atoms of Ser-389 and Thr-529 in BlaR1 (Ser-391 and Thr-531 in MecR1) (Fig. 5). Interactions between avibactam and BlaR1SD or MecR1SD are also depicted in figures created with LigPlot+ (Figs. S2–S4). Remarkably, avibactam is observed covalently bound in two alternate orientations to BlaR1SD, whereas in MecR1SD, it is only present in one orientation (Fig. 5). Despite MecR1SD being co-crystallized initially with oxacillin (oxacillin was included to aid in the initial formation of crystals), the resulting electron density shows unequivocally that soaking with high concentrations of avibactam post-crystallization successfully displaced the oxacillin. Although backsoaking is a commonly used technique for hard to crystallize proteins, there is always the possibility that this method prevented a second conformation of avibactam binding. However, we do point out the close similarity of the MecR1SD ligand-free structure determined in a different space group, the prior oxacillin structure, and our avibactam structure here (Table 2) would suggest the latter’s conformation is not being influenced or modulated by the initially present oxacillin.

The two conformations of avibactam in the BlaR1SD structure are orientated ~180° to the other (Fig. 5, A–C). The occupancy of the two avibactam conformations was allowed to float during refinement in Phenix.refine (34) resulting in occupancies of 0.58 and 0.42 for conformation A (Fig. 5A) and...
conformation B (Fig. 5B), respectively. Thr-529 forms hydrogen bonds with both conformations but at opposite termini of avibactam, interacting with the sulfate of conformation A and the C2 carboxamide of conformation B. The avibactam sulfate in conformation A, in keeping with the electronegative carboxylate of typical β-lactam substrates, projects toward an electronegative pocket formed by Lys-526 and Thr-527 of the KTG motif as well as Thr-529. The sulfate in conformation B on the other hand, is coordinated by hydrogen bonds to the d-N of Asn-388, the backbone nitrogen of Ile-531, and via a water coordinated by the backbone carbonyl of Ile-531 (Fig. 5, B – C).

Avibactam is bound to MecR1SD in a similar orientation as conformation A in the BlaR1SD structure with analogous conserved residues Ser-439, Thr-529, Thr-531, and Lys-528 forming hydrogen bonds with the sulfate moiety of avibactam, whereas the C2 carboxamide at the opposite termini hydrogen bonds with a water, Asn-441, Asn-478, and Thr-531 (Fig. 5D). There is unambiguously no evidence of a secondary orientation of avibactam in the active site of MecR1SD that we see in BlaR1SD (Fig. 5D and Fig. S1).

Due to the orientation of the two BlaR1SD molecules in the ASU, the avibactam ligands are in close proximity to residues of the opposite chain (Fig. S5). This juxtaposition allows the formation of the inter-chain hydrogen bonds to the avibactam sulfate oxygen and C2 carboxamide nitrogen (Fig. S5). However, as suggested by the in silico docking and molecular dynamics simulations discussed below, we believe these inter-

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**Table 1**

Data collection and structure refinement statistics for the BlaR1SD and MecR1SD in covalent complex with avibactam

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

| Structure  | BlaR1SD-avibactam | MecR1SD-avibactam |
|------------|------------------|------------------|
| PDB ID     | 6O9W             | 6O9S             |
| Beamline   | CLS-08ID-1       | CLS-08ID-1       |
| Cell dimensions |                 |                  |
| a, b, c (Å) | 53.4, 92.6, 56.5 | 58.7, 58.7, 147.6 |
| α, β, γ (°) | 90.0, 104.6, 90.0 | 90.0, 90.0, 90.0 |
| Wavelength (Å) | 0.9793          | 0.9794           |
| Resolution (Å) | 46.32-1.95      | 45.93-1.59       |
| Rmerge      | 0.094 (0.915)    | 0.049 (0.502)    |
| CC1/2       | 0.997 (0.639)    | 0.999 (0.812)    |
| Rfree       | 1.06 (1.31)      | 2.13 (2.55)      |
| Completeness (%) | 99.58 (99.51)  | 99.1 (92.9)      |
| Redundancy  | 3.4 (3.4)        | 6.7 (4.4)        |

**Refinement**

| Resolution (Å) | 2.0 | 1.6 |
| No. reflections | 38,592 (3,840) | 35,297 (3,214) |
| Rwork/Rfree (Å) | 0.185/0.225 | 0.158/0.197 |
| No. Nonhydrogen atoms | 5944 | 3490 |
| Macromolecules | 340 | 175 |
| Water | 168 | 159 |
| B-factors (Å²) | 39.8 | 27.2 |
| Protein | 32.4 | 33.2 |
| Ligand | 105.9 | 38.3 |
| Ion | 41.3 | 38.3 |
| RM5D | 0.010 | 0.007 |
| Bond lengths (Å) | 1.02 | 0.95 |
| Bond angles (°) | 97.4/2.6/0.0 | 98.0/2.1/0.0 |

1Highest resolution shell is shown in parentheses.
**Table 2**

| PDB ID | Ligand present     | Cα RMSD (Å) | Number of atoms aligned | Ref. |
|--------|--------------------|-------------|-------------------------|------|
| 2IWB   | Inhibitor free     | 0.56        | 246                     | 33   |
| 2IWC   | Benzylpenicillin   | 0.31        | 246                     | 33   |
| 2IWD   | Oxacillin          | 0.37        | 246                     | 33   |

*Figure 5. Active site of BlaR1<sup>SD</sup> and MecR1<sup>SD</sup> in complex with avibactam. Avibactam is shown in the active site of BlaR1<sup>SD</sup> in either (A) conformation A, (B) conformation B, (C) conformation A and B together, or (D) in the MecR1<sup>SD</sup> active site. Selected residues from BlaR1<sup>SD</sup> and MecR1<sup>SD</sup> are depicted in gray and white wire, respectively, on the left, whereas the 2mF<sub>o</sub> − DF<sub>c</sub> map around avibactam is shown in blue mesh and contoured at 1σ on the right. Avibactam is shown in ball and stick form with carbon atoms shown in green, water molecules are shown in cyan, and other atoms with conventional coloring.*
chain hydrogen bonds are not necessary for the observed dual orientation of avibactam in the BlaR1SD active site.

Size-exclusion chromatography multiangle light scattering (SEC-MALS) of both BlaR1SD and MecR1SD show both constructs are monomeric in solution and provide molecular weights that reflect theoretical predictions (Fig. S6). Additionally, as the N terminus of BlaR1SD is located on opposite sides of the ASU dimer, it suggests this interaction would not be topologically possible when the sensor domain is expressed as a full-length protein along with the N-terminal zinc-metalloprotease domain in the cell membrane (Fig. S5). The most significant buried interface between the two monomers in the BlaR1 crystal ASU as calculated using PISA (35) is 979 Å², also supporting the observed pair as not reflective of a physiological interaction.

The BlaR1SD and MecR1SD active sites are clearly similar but do display differences in hydrogen bonding between the general base lysine and nucleophilic serine of the SXKX motif (Fig. 6A). The number of hydrogen bonds between avibactam and the active site is listed for each structure in Table 3. Briefly, MecR1 appears to have more hydrogen bonding with avibactam than BlaR1 does in either conformation of avibactam. In the BlaR1SD avibactam structure, the N-ε of the lysine is far (3.9 Å) from the O-γ of the serine for hydrogen bonding and base-mediated extraction of the serine O-γ bound hydrogen in the decarbamylated state. In contrast, the same residues in MecR1SD are only 3.1 Å away and in a position to facilitate both hydrogen bonding and extraction of the aforementioned hydrogen in the decarbamylated state. Additionally, the distance between the δ-N of the asparagine (Asn-439 in BlaR1 and Asn-441 in MecR1) and the C2 carboxamide oxygen of avibactam in the MecR1 structure is 3.0 Å, whereas it is 3.7 Å in the BlaR1 structure.

The BlaR1SD and MecR1SD avibactam structures closely align (Cα RMSD: 3.5 Å over 321 common residues) excepting the same loop (residues His-403–Gln-428 in BlaR1 and residues Asn-405–Gln-430 in MecR1). The loop formed by BlaR1 residues His-403–Gln-428 in the avibactam structure is in a completely different orientation compared with previous BlaR1SD structures (Figs. S7 and S8), whereas the overall MecR1SD avibactam complex structure is highly similar to existing MecR1SD structures (Tables 2 and 4 and Fig. S8). Residues 408–427 were not modeled in chain A of the BlaR1SD avibactam structure due to disorder/poor electron density, whereas more defined electron density in chain B allowed the majority of the loop to be modeled excepting residues 413–414.

In silico docking of avibactam, nacubactam, and relebactam into BlaR1SD and MecR1SD avibactam crystal structures

We also performed in silico docking to further our understanding of the binding energies associated with each avibactam conformation in the BlaR1SD and MecR1SD structures (Table 5). Covalent docking was performed in the Molecular Operating Environment (MOE, version 2009; Chemical Computer Group Inc.). For all in silico modeling described here, three different docking algorithms were used based on the active-site ligand shape, atomic contact count, and force field (38). The calculated energies of binding for avibactam to BlaR1 were the same for conformations A and B using the Affinity dG and ASE docking algorithms (Table 5). However, for the GBVI/WSA dG, force field-based docking algorithm the calculated
Structural analysis of BlaR1 and MecR1 with Avibactam

Table 3
Number of hydrogen bonds between the active site and avibactam in BlaR1SD, MecR1SD, and OXA-10 (PDB ID 4520) structures

| Complex and conformation | Number of hydrogen bonds to protein in the same chain | Number of hydrogen bonds to the active site via a water | Number of hydrogen bonds to the active site via a water |
|--------------------------|-----------------------------------------------------|------------------------------------------------------|------------------------------------------------------|
| BlaR1SD-avibactam         | Conformation A: 7                                   | Conformation B: 6                                    | Conformation A: 0                                     | Conformation B: 1                                    |
| MecR1SD-avibactam         | Conformation A: 9                                   | Conformation B: -                                    | Conformation A: 1                                     | Conformation B: -                                    |
| OXA-10-avibactam          | Conformation A: 6                                   | Conformation B: -                                    | Conformation A: 2                                     | Conformation B: -                                    |

Table 4
Ca RMSD values for existing BlaR1SD structures (from S. aureus unless otherwise noted) compared with the BlaR1SD structure with avibactam (PDB ID 609W)

| PDB ID | Ligand                      | Ca RMSD (Å) | Number of atoms aligned | Notes                          | Ref. |
|--------|-----------------------------|-------------|-------------------------|--------------------------------|------|
| 1NRF   | Inhibitor free              | 4.45        | 232                     | BlaR1 from B. licheniformis    | 8    |
| 1XKZ   | Ceftazidime                 | 2.44        | 242                     |                                | 36   |
| 1XA1   | Inhibitor free              | 2.28        | 242                     |                                | 44   |
| 1XA7   | Benzylpenicillin            | 2.48        | 237                     |                                | 44   |
| 3UY6   | Inhibitor free              | 2.39        | 245                     | With N439V mutation            | 20   |
| 3Q7V   | Inhibitor free              | 2.40        | 245                     | With carboxylated Lys-392      | 18   |
| 3Q7Z   | -lactam benzoyl-6 amino-penicillin acid | 2.45 | 245 |                     | 18   |
| 3Q81   | Imipenem                    | 2.41        | 245                     |                                | 18   |
| 3Q82   | Meropenem                   | 2.41        | 245                     |                                | 18   |

Docking energy of avibactam to BlaR1 for conformation A (−4.7 kcal mol⁻¹) was slightly more favorable than for conformation B (−4.3 kcal mol⁻¹). The generally similar binding energies for avibactam in both conformations support our observed dual orientation, ~equal occupancy of avibactam in the BlaR1SD X-ray crystal structure and suggests crystal packing effects are likely not causing the dual orientation of avibactam as only one of the two protein chains in the ASU were used in the docking calculation. Interestingly, in the docking trials for MecR1SD, the energy of binding was reduced for avibactam in conformation B compared with conformation A, which follows our observation that avibactam adopts only conformation A in the crystal structure. The reported docking poses closely match the avibactam crystal structure poses for MecR1SD with avibactam in the A conformation and for both orientations of avibactam in BlaR1SD (RMSD < 1 Å over 17 atoms) (Fig. S9). Additionally, we docked two avibactam derivatives that are of clinical interest, nacubactam and relebactam, into the BlaR1SD structure with avibactam (PDB ID 609W).

Molecular dynamics simulations of BlaR1SD and MecR1SD interactions with avibactam

Molecular dynamics (MD) simulations over 40 ns were used to further explore the interactions between the BlaR1/MecR1 sensor domains and avibactam in poses A and B. As in the in silico docking experiments, the MD simulations were performed with a single protein monomer. In BlaR1 5 stable contacts (maintained for ≥30% of the simulation) were observed between active site residues and the avibactam terminal sulfate and carboxamide moieties in either conformation (Fig. S11). In MecR1 there were 6 contacts between active site residues and the avibactam terminal sulfate and carboxamide moieties in conformation A and 3 stable contacts with the same avibactam moieties in conformation B (Fig. S11). The interaction trajectories for the residues interacting with avibactam are shown for each frame of the simulation in Fig. S11.

Discussion

Although studies have shown that avibactam may be a suitable Β-lactam inhibitor for use in new broad-spectrum treatments for Gram-positive and -negative infections (26–28), the effect of avibactam on the mec and bla resistance pathways in MRSA was previously unknown. Here we show avibactam induces up-regulation of antibiotic-resistance genes in a clinical strain of MRSA, and our X-ray crystallographic models provide a molecular basis for avibactam binding to the BlaR1 and MecR1 sensor domains.

Gene expression studies performed here in S. aureus SF8300, a USA300 clone, suggest avibactam is able to trigger an antibiotic-resistance phenotype by causing increased expression of pbp2a and blaZ transcripts (Fig. 3). As this strain of S. aureus lacks a functional copy of mecR1, this also supports previous evidence that pbp2a expression can be controlled via BlaR1 and Blal when MecR1 is not present (12). Cotreatment of MRSA infections with avibactam and ceftaroline, a late generation cephalosporin, has not been found to be inferior to treatment with ceftaroline alone (26); however, given that avibactam does trigger up-regulation of MRSA resistance genes we provide evidence that caution should be exercised when using avibactam in combination with Β-lactam antibiotics.

Following the initial evidence that avibactam binds the sensor domains of BlaR1SD and MecR1SD, we became interested in the specific interactions mediating avibactam binding. The BlaR1SD avibactam structure displays a novel, dual orientation of avibactam in the active site with both conformers forming a carbamoyl link with the catalytic serine (Ser-389) (Fig. 5 and Fig. S1). Given the structural and sequence similarity between BlaR1SD and MecR1SD, it was surprising that the BlaR1SD avibactam structure appears to have two orientations of avibactam, whereas the MecR1SD structure only has...
avibactam in a single orientation, similar to that observed in serine-based class A and D b-lactamases. To our knowledge this dual orientation of avibactam binding has not been previously observed. Further work is needed to determine whether one or both avibactam poses are responsible for activating BlaR1 as this could be important for guiding the development
of future diazabicyclooctane \(\beta\)-lactam inhibitors and drugs to combat MRSA.

Avibactam co-structures typically have residues of the signature KTG motif coordinate the sulfate moiety of avibactam while the asparagine residue side chain from the SXX motif coordinates the C2 carboxamide group on the opposite side of the diazabicyclooctane scaffold (21). Avibactam in conformation A of the BlaR1\(^{SD}\) structure presented here partially follows this trend with Lys-526 and Thr-527 of the KTG motif coordinating the avibactam sulfate moiety but lacks SXX motif coordination of the C2 carboxamide (Fig. 5A). Instead, the \(\gamma\)-O of Ser-437 of the SXX motif forms a hydrogen bond with N6 of avibactam. Predictably, avibactam in the B conformation, orientated \(\sim 180^\circ\) to conformation A, does not have the canonical conformation either. Instead, Ser-437 of the SXX motif coordinates the carboxamide and Asn-388 and Ile-531 coordinates the sulfate (Fig. 5B). The MecR1\(^{SD}\) structure shows typical avibactam coordination with Asn-441 of the SXX motif coordinating the carboxamide and Lys-528 and Thr-529 of the KTG motif forming electrostatic contacts with the sulfate (Fig. 5D).

Equivalent residues, Asn-439 in BlaR1 and Asn-441 in MecR1, both hydrogen bond similarly with the lysine \(\zeta\)-nitrogen of the SXXX motif but interact differently with the C2 carboxamide oxygen of avibactam. Residue Asn-439 of the SXX motif in BlaR1 has been shown to be instrumental in allowing the sensor domain of BlaR1 to function as a receptor rather than a \(\beta\)-lactamase (20). Hydrogen bond interactions between the Asn-439 \(\delta\)-O and the lysine \(\zeta\)-N of the SXXX motif are thought to prevent the carboxylation of the same lysine when the catalytic serine is acylated by a \(\beta\)-lactam antibiotic, thereby inhibiting release of bound inhibitors (18, 20). We observe no evidence of SXXX lysine carboxylation in both avibactam co-structures described here, which follows given the observed hydrogen bonding between the asparagine and lysine of the aforementioned motifs. Additionally, as shown above, avibactam causes the up-regulation of \(pbp2a\) and \(blaZ\) in \(S. aureus\), suggesting the BlaR1 sensor domain is working as an avibactam receptor rather than a \(\beta\)-lactamase. Despite the similarity of Asn-439—Lys-392 (Asn-441—Lys-394 in MecR1) hydrogen bonding in both structures, in the MecR1\(^{SD}\) structure the avibactam C2 carboxamide oxygen hydrogen bonds (3.0 Å) with the same asparagine (Asn-441 in MecR1) \(\delta\)-nitrogen, whereas in the BlaR1 structure this interaction is long in avibactam conformation A (3.7 Å) and not present with avibactam conformation B.

From our crystallographic analysis, and the very similar structures of ligand-free and avibactam forms, we surmise that crystal packing effects are not the underlying basis for our observations, although subtle or longer-range influences cannot be unequivocally ruled out. Following on this we wanted to better understand how the observed avibactam conformations might be predicted to behave in solution. As mentioned above, there are inter-chain interactions involving the avibactam ligands in the BlaR1\(^{SD}\) structure (Fig. S5). However, only a single protein chain of the BlaR1\(^{SD}\) structure was used in the \emph{in silico} docking and MD experiments, allowing us to explore whether these inter-chain electrostatic interactions seen in the crystal structure were likely significantly perturbing the avibactam ligand position. The closely aligning poses for avibactam in the BlaR1\(^{SD}\) structure and the \emph{in silico} docking experiments (Fig. S9) suggest the electrostatic interactions between avibactam from one chain and protein in the neighboring chain (Fig. S5) do not dramatically influence the binding of the avibactam in the crystal structure.

\textbf{In silico} docking and MD simulations hint as to why there are two main binding poses for avibactam in BlaR1 and only one in MecR1. The top two \emph{in silico} predicted avibactam-binding poses for BlaR1 show avibactam binding in conformations A and B as seen in the crystal structure with similar binding energies for both conformations (Table 5 and Fig. S9). Additionally, MD simulations suggest there are an equal number of stable contacts (interactions maintained for \(\geq 30\%\) of the simulation) between BlaR1 active site residues and both poses of avibactam terminal moieties, further supporting the presence of two avibactam-binding sites in solvated BlaR1 (Fig. S11). In contrast, only half as many stable contacts were present in the MecR1 MD simulation with avibactam terminal moieties in conformation B as compared with conformation A, providing support for the single observed conformation A in the crystal structure (Fig. S11).

Although \emph{in silico} docking and MD results were supportive of the crystallographic observations, it led us to consider the molecular basis for this finding. Upon alignment of the BlaR1\(^{SD}\) and MecR1\(^{SD}\) avibactam structures, we deduce MecR1 residues Asn-478 and Asn-390 (corresponding to residues Met-476 and Asn-388 in BlaR1) may play a role in favoring avibactam conformation A. In the MecR1\(^{SD}\) structure we modeled Asn-478 in silico docking and MD experiments were supportive of the calculated and MD simulation energies for both conformations (Table 5 and Fig. S9). Additionally, the position of Asn-478 influences the hydrogen-bonding network such that the side chain of Asn-390 in MecR1 is flipped and would clash with avibactam sulfaate in orientation B. Fewer hydrogen bonds between the BlaR1\(^{SD}\) active site and both conformers of avibactam versus avibactam in the MecR1\(^{SD}\) structure may also facilitate the multiple orientations of avibactam in the BlaR1\(^{SD}\) structure (Table 3). Furthermore, BlaR1\(^{SD}\) has a more positively charged electrostatic surface in and surrounding the catalytic cleft than MecR1\(^{SD}\), which may contribute to accommodating the dual orientation of the avibactam ligand with its negatively charged sulfate moiety (Fig. S12).

Although we do not eliminate the possibility that the dual orientation of avibactam in the BlaR1\(^{SD}\) structure or the singular pose of avibactam in the MecR1\(^{SD}\) structure are influenced by crystal packing, we believe the \emph{in silico} docking and MD simulations detailed above provide more likely explanations. Together, these experiments demonstrate the significant changes in ligand binding that can arise from subtle changes in hydrogen bonding networks and highlight the challenges of rational drug design.

To probe whether the dual orientation of avibactam observed with the BlaR1 structure is likely to be present with other clinically relevant diazabicyclooctane \(\beta\)-lactamase inhibitors, we also docked relebactam and nacubactam into the BlaR1\(^{SD}\) and MecR1\(^{SD}\) crystal structures. Relebactam and
nacubactam are in current or recently completed clinical trials in combination with β-lactam antibiotics (clinicaltrials.gov, NCT03182504 and NCT03293485) (39). Both relebactam and nacubactam are derivatized at the C2 carboxamide with the addition of a 2-aminoethoxy and piperidinium substituents respectively (Fig. S10). Interestingly, our in silico docking experiments show more favorable binding energies for the nacubactam- and relebactam-binding pose analogous to the A conformation seen with avibactam (Table 5 and Fig. S10). This result is not entirely surprising given that binding in two orientations would cause the positively charged 2-aminoethoxy or piperidinium moieties to be in close proximity to the binding site of one of the two negatively charged avibactam sulfates.

Although BlaR1<sup>SD</sup> and MecR1<sup>SD</sup> have closely analogous structures with the class D β-lactamasess (particularly OXA-10 and OXA-48 commonly found in <i>P. aeruginosa</i> (40) and carbapenem-resistant Enterobacteriaceae (41), respectively), there are key differences in how the active sites coordinate avibactam. Here we compare the avibactam BlaR1/MecR1 sensor domain structures with the previously solved OXA-10–avibactam structure (PDB ID 4S2O) (Fig. 6), but similar differences occur in the OXA-48–avibactam structure (PDB ID 452K) (14). The two largest differences in avibactam binding between the two sensor domains and OXA-10 are the coordination of the avibactam sulfate and carboxamide. The guanidinium cation moiety of Arg-250 in OXA-10 directly coordinates the avibactam sulfate (2.7 and 3.1 Å away) in addition to the ζ-N of Lys-205 (3.2 Å away) (Fig. 6, B and C). The two sensor domains examined here lack an arginine residue in this position and instead use a lysine-threonine cradle to stabilize the avibactam sulfate moiety in conformation A. The second, B conformation of avibactam seen in the BlaR1<sup>SD</sup> structure is stabilized by an asparagine side chain nitrogen and the backbone nitrogen of an isoleucine residue as previously discussed. Notably, the C2 carboxamide at the opposite termini of the avibactam sulfate is coordinated directly by residues of the active site in the sensor domain structures, whereas in the OXA-10 structure it is oriented toward solvent with only a single bridging water. The MecR1<sup>SD</sup> structure appears to have the most contacts to avibactam, with BlaR1 and OXA-10 having fewer contacts (Table 3). Although the BlaR1/MecR1 sensor domain, OXA-10, and OXA-48 active site cavities are closely similar, only BlaR1 has avibactam bound in two orientations. Further research is needed to determine whether improved class D β-lactamase inhibitors can be developed to take advantage of this secondary sulfate-binding site found in the BlaR1<sup>SD</sup> active site.

As has been previously observed with the β-lactam class of inhibitors, there is no global change in either sensor domain structure following binding of avibactam compared with either inhibitor-free or β-lactam bound structures (Fig. S8 and Tables 2 and 4) (33, 44). However, the BlaR1<sup>SD</sup> avibactam structure has a loop consisting of residues His-403–Asp-429 that adopts a radically different orientation than observed in all previous inhibitor-free and β-lactam–acylated BlaR1<sup>SD</sup> structures even when compared with crystals from the same space group and grown in similar, high molecular weight PEG-based crystallization conditions (Figs. S7 and S8). Given this, it is inferred that the binding of avibactam may be influencing the relative position of this loop. This loop showing considerable variation in positions, borders the active site and is analogous to the P-loop found in class D β-lactamases (42, 43). In some class D β-lactamases, residues of the P-loop interact directly with covalently bound β-lactam (45) which is hypothesized to contribute to increased affinity for the ligand (46). In the BlaR1<sup>SD</sup> avibactam structure described here, the analogous P-loop is retracted from the active site such that the catalytic groove is widened by 6-8 Å compared with other BlaR1<sup>SD</sup> and MecR1<sup>SD</sup> structures with and without ligand (Fig. 7). Although only one conformation of the BlaR1 loop was observed and modeled, the B-factors of this loop are obviously higher than the corresponding residues in the MecR1<sup>SD</sup> structure, suggesting that they may be more dynamic (Fig. S13). This is corroborated by previous research examining the ligand-free and acylated structures of BlaR1<sup>SD</sup> using NMR, which found the P-loop experienced local dynamic changes upon acylation (47). Finally we note the P-loop has been predicted to pack against with the L2 loop of the closely related full-length MecR1 zinc metalloprotease domain (48) opening the possibility it may play a role in signal transduction between the sensor domain and the zinc metalloprotease domain.

The thermal stability of the sensor domains depends on the ligand added. Although both sensor domains were stabilized by 2-9 °C by ampicillin, avibactam appeared to have little effect on the stability of BlaR1<sup>SD</sup>, whereas conferring stability at higher temperatures to MecR1<sup>SD</sup>. Although nafcillin provided a small increase in ΔT<sub>agg</sub> with BlaR1<sup>SD</sup> it appeared to slightly destabilize MecR1<sup>SD</sup>. It is not known how the alternate positioning of the analogous P-loop consisting of residues 403-439 would affect BlaR1 sensing or activation capability, but its position may contribute to the absence of thermal stability gained upon avibactam binding to BlaR1<sup>SD</sup> (Fig. 4). The apparent dynamic nature of the avibactam ligand in the active site where more than one binding orientation is present could also be contributing to the lack of thermal stabilization effect on BlaR1<sup>SD</sup> by avibactam. In contrast, the MecR1<sup>SD</sup> structure with avibactam shows that one orientation of the ligand is preferred, which combined with the increased number of hydrogen bonds between the ligand and protein, may aid in providing the increase in thermal stability seen here.

Given avibactam is already used in the clinic, and the substantial investment in this drug by the pharmaceutical industry, a thorough examination of its off-target effects is needed. This research begins to address this unmet need by exploring the interaction between avibactam and a major determinant of drug resistance in Staphylococcal strains, BlaR1/MecR1. Additionally, work presented here could facilitate the development of inhibitors that do not activate and/or inhibit the bla/mec pathways. Developing an inhibitor that is still able to bind the sensor domain active site but at the same time blocks activation of the pathway could be a useful way to circumvent β-lactam resistance in MRSA. Finally, more research is needed to determine the structure of full-length BlaR1 and MecR1, with and without sensor domain inhibitors, to illuminate the mechanism of signal propagation from the sensor domain to the zinc metalloprotease domain.
Experimental procedures

Expression and purification of BlaR1SD and MecR1SD

Both S. aureus mecR1 (coding for residues Ser-334–Ile-585) (UniProt ID P0A0B0) and S. aureus blaR1 (coding for residues Met-330–Gln-585) (UniProt ID P18357) were expressed from a pET28a vector with a cleavable N-terminal deca-histidine tag in *Escherichia coli* BL21 (DE3). Cells were grown in LB media supplemented with 50 μg ml\(^{-1}\) of kanamycin at 37 °C to OD\(_{600}\) = 0.5-0.8 with 225 rpm shaking before being cooled at 4 °C for 30-60 min and induced with a final concentration of 100 μM isopropyl β-D-1-thiogalactopyranoside. Cells were incubated overnight at 17 °C with shaking before being harvested by centrifugation and stored at −80 °C.

Cell pellets from 4.5-9 liters were thawed and resuspended in buffer A (20 mM Tris, pH 7.5, 20 mM imidazole, 500 mM sodium chloride) with either cComplete, EDTA-free protease inhibitor mixture tablet (Roche) or Protease Inhibitor mixture Set II, EDTA free (Calbiochem) and bovine DNase Type I (Roche Applied Science) to a final concentration of ~10 ng ml\(^{-1}\).

Future steps were carried out at 4 °C unless noted otherwise. Cells were lysed in a homogenizer (Avestin) followed by centrifugation at 45,000 rpm for 45 min in a Type 70Ti rotor (Beckman Coulter). The supernatant was filtered through a 0.45-μm membrane and loaded onto a 1-ml HisTrap HP column (GE Healthcare Life Sciences) pre-equilibrated with buffer A. The column was washed with buffer A until the flow-through absorbance at 280 nm stabilized and the protein was eluted with a linear gradient of buffer B (20 mM Tris, pH 7.5, 1 x imidazole, 500 mM sodium chloride) to 500 mM imidazole over 40 min at a flow rate of 1 ml min\(^{-1}\). Fractions were analyzed via SDS-PAGE and those containing high levels of the protein of interest were pooled. The polyhistidine tag on MecR1SD was removed with a 40:1 (mol:mol) protein to TEV protease ratio before, while being dialyzed in buffer A overnight. This produced BlaR1SD protein with an amino terminus GHM sequence before the amino terminus sequence starting with GSH followed by the amino terminus sequence starting with GSH followed by the sequence Ile-585. The polyhistidine tag on the BlaR1 construct was cleaved overnight with a 1:1000 (v/v) ratio of bovine α-thrombin (Hematologic Technologies Inc.) to protein ratio following desalting of the sample into buffer C (20 mM Tris, pH 8, 200 mM NaCl). This produced BlaR1SD protein with an amino terminus sequence starting with GSH followed by the BlaR1SD protein. The sample was then concentrated on a 10-kDa molecular mass cut off Centricon (Amicon) and imidazole was added to 20 mM if necessary, before being run on a 1-ml HisTrap HP column (GE Healthcare Life Sciences) pre-equilibrated with buffer A. The flow-through was collected and concentrated as above before being loaded on a Superdex 75 10/300 column (GE Healthcare Life Sciences) pre-equilibrated with buffer C. Fractions with purified protein were pooled, concentrated as above, flash frozen in liquid nitrogen, and stored at −80 °C.

Crystallization of BlaR1SD and MecR1SD

Avibactam-bound BlaR1SD crystals were grown using the sitting drop vapor diffusion method at 23 °C in 24-well plates. Drops contained 1 μl of 20 mg ml\(^{-1}\) of protein preincubated with 4 mM avibactam and an equal volume of precipitant (200 mM tripotassium citrate, 20% PEG3350). The BlaR1SD precipitant solution was made without addition of acid or base, but the final pH was ~8.1. Crystals were seeded by twirling a housecat whisker in a drop containing avibactam-bound BlaR1SD crystals and then moving the whisker though the freshly set up drop.

MecR1SD was crystallized in a 1:1 volume ratio of MecR1 protein at 7.5 mg ml\(^{-1}\) preincubated with 1 mM oxacillin and precipitant solution (2.5 mM ammonium sulfate, 50 mM HEPES, pH 7.5) producing a total drop volume of 2 μl. Drops were set up in 24-well sitting drop plates and incubated at 23 °C.

Soaking and harvesting crystals

Avibactam (Fedora Pharmaceuticals or Cayman Chemical Company) stock solution was made in DMSO. Avibactam was soaked into MecR1SD crystals by adding 3 μl of avibactam soaking solution (9 mM avibactam diluted in an equivolume ratio of Buffer C and precipitant solution) to the 2-μl crystallization drop. The crystals were soaked for 2 to 40 min before the crystals were looped and passed through cryoprotectant solution (3 mM avibactam and 20% glycerol diluted in a 1:1 ratio of crytal buffer and precipitant solution) before vitrification in liquid nitrogen. This avibactam concentration and soaking time was sufficient to outcompete the existing oxacillin in the crystallization drop.

BlaR1SD avibactam co-crystals were cryoprotected by adding 10 μl of cryoprotectant solution (30% glycerol and 5 mM avibactam diluted in mother liquor) directly to the 2-μl crystallization drop before loopimg and vitrification in liquid nitrogen.

Data collection and processing

All X-ray diffraction data were collected at the Canadian Light Source, beamline ID-08. Data were processed with Xia2 (49) using XDS (50), and Aimless (51) from the CCP4 (52) program suite. The avibactam-bound BlaR1SD and MecR1SD structures were solved by molecular replacement using Phaser (53), with chain A of PDB ID 1XAI and 2IW, respectively. The Phenix program suite (54) was used for model building and refinement with AutoBuild (55) initially being used. Models were built with several cycles of manual rebuilding in Coot (56), followed by refinement using phenix.refine (34). TLS groups determined using the TLS Motion Determination server (57, 58) were used later in refinement. Avibactam was added manually after several rounds of refinement by examination of the F\(_o\) – F\(_c\) and 2 F\(_o\) – F\(_c\) electron density maps. Coordinates and structure factors were deposited to the PDB with accession codes (6O9W) and (6O9S) for BlaR1SD and MecR1SD avibactam structures, respectively. Figs. 5–7 and Figs. S1, S5, S7–S10, S12, and S13 were designed using PyMol (Schroedinger, LLC, New York), whereas Figs. S2–S4 were created using LIGPLOT\(^+\) (59). Electrostatic potential surfaces in Fig. S12 were calculated using PDB2PQR (60) and APBS (61) plugins in PyMol. Chain B of the BlaR1SD avibactam structure was used for all analysis.
**In silico ligand docking into BlaR1<sup>SD</sup> and MecR1<sup>SD</sup> avibactam structures**

All preparation and covalent docking calculations were performed in the Molecular Operating Environment (MOE, version 2009, Chemical Computing Group Inc., Canada). Covalent docking was performed using atomic contact count, force field-based, and shape-based scoring functions (38). For all in silico docking calculations, chain B of the BlaR1<sup>SD</sup> avibactam structure and chain A of the MecR1<sup>SD</sup> avibactam structure were used. Protein modules were prepared for docking by including any missing sidechains, removing the avibactam ligand, and protonating the model. The catalytic serine (Ser-389 in BlaR1 and Ser-391 in MecR1) side chain was restored to its unreacted form for docking with the hydrolyzed form of the ligand and MOE was used to create a reaction file for the β-lactam ring opening.

**Molecular dynamic simulations of avibactam with BlaR1<sup>SD</sup> and MecR1<sup>SD</sup>**

Protein models were prepared as described for the in silico docking. MD simulations were performed using Desmond package from Schrödinger (62). The setting for each simulation: an SPc water-solvent model; orthorhombic simulation box shape; NPT ensemble with a pressure of 1.01325 bar; and temperature 300 K. The simulations were run for 40 ns with ~1000 frames. Fig. S11 was created with output from the Desmond package and edited to show all residues interacting with avibactam for at least 20% of the duration of the simulation.

**SEC-MALS**

Purified *S. aureus* BlaR1<sup>SD</sup>, with or without 5 mM avibactam, or MecR1<sup>SD</sup> applied to a size exclusion column (Superdex 75 10/300 column (GE Healthcare) for BlaR1<sup>SD</sup> and Superdex 200 10/300 column (GE Healthcare) for MecR1<sup>SD</sup> using an Agilent 1100 series HPLC (Agilent Technologies), which was coupled in-line to a Dawn<sup>®</sup> Heleos<sup>TM</sup>II 18-angle MALS light scattering detector, and Optilab<sup>®</sup> T-rEX<sup>TM</sup> differential refractometer protein detector (both from Wyatt Technology). The light scattering detectors were first normalized using monomeric BSA (Sigma-Aldrich). A total of 100 µg of purified protein sample was injected on the column, pre-equilibrated in running buffer (20 mM Tris, pH 7.5 or 8, 150 mM NaCl). Data were collected and analyzed using the Astra 6 software. The protein absolute molecular weight was calculated assuming a dn/dc value of 0.185 ml/g and a theoretical extinction coefficient of 2.04 ml (mg cm)<sup>-1</sup> for BlaR1<sup>SD</sup> and 1.94 ml (mg cm)<sup>-1</sup> for MecR1<sup>SD</sup>.

**Thermal aggregation assays**

BlaR1 and MecR1 sensor domain protein was thawed on ice and diluted in assay buffer (100 mM sodium phosphate, pH 7.0) to a final concentration of 0.5 mg ml<sup>−1</sup>. Ampicillin (Fisher), avibactam (Cayman Chemical), nafcillin (Sigma), and kanamycin (Gold-Bio) were serially diluted in assay buffer and mixed with the protein samples. 9 µl of sample was added to each well of a 384-well plate (Corning, 3540).

The four replicates of each condition were pipetted into the plate, the plate was briefly centrifuged, 11 µl of mineral oil was added to overlay the samples, and the plate was centrifuged again. The plate was then assayed with differential static light scattering (Stargazer<sup>®</sup>, Epiphyte Three Inc.) while increasing the temperature at 1 °C min<sup>−1</sup> from 25 to 85 °C. The data were analyzed using Stargazer AIR (Epiphyte3) software and the temperature of aggregation (Tagg) was found using Boltzmann regression. By subtracting the Tagg at a given drug concentration from the Tagg in the absence of drug the ΔTagg was calculated to give an idea of the stability gained or lost with a particular compound.

**Quantitative real-time PCR of blaZ and pbp2a**

Quantitative real-time PCR (qRT-PCR) was carried out as before (63) with a few modifications. Briefly, overnight cultures of *S. aureus* SF8300 strain was subcultured in TSB media and grown for 2 h at 37 °C with constant shaking. 10 ml of the bacterial culture was aliquoted in 50-ml conical tubes and antibiotics were added to attain the desired condition. The resultant bacterial cultures were incubated for an additional 1 h and ~10<sup>9</sup> bacterial cells were harvested for RNA isolation. Bacterial total RNA was isolated using Qiagen RNeasy Mini kit and following treatment of the RNA with DNase (Ambion), cDNA was synthesized using Superscript IV (Thermo Fischer Scientific). Absolute quantification of genes was carried out using SYBR Green qRT-PCR master mix (Thermo Fischer Scientific) and the primers indicated in Table S1. Each experiment was carried out in triplicate and the gyrB gene was used as housekeeping control. Groups given avibactam or nafcillin were compared with the DMSO control using a one-way analysis of variance with the Dunnet multiple comparison test in GraphPad Prism version 8.3.1.

**Data availability**

The structures presented in this paper have all been deposited in the Protein Data Bank (PDB) with the following codes: 6O9W and 6O9S. All remaining data are contained within the article.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: MRSA, methicillin-resistant *Staphylococcus aureus*; ASU, asymmetric unit; PBP, penicillin-binding protein; Ca, α-carbon; PDB, Protein Data Bank; RMSD, root mean square deviation; qRT-PCR, quantitative real-time polymerase chain reaction; SEC-MALS, size-exclusion chromatography multiangle light scattering; Tagg, temperature of aggregation; MD, molecular dynamics; TEV, tobacco etch virus.

References

1. Lee, A. S., de Lencastre, H., Garau, J., Klyutmans, J., Malhotra-Kumar, S., Peschel, A., and Harbarth, S. (2018) Methicillin-resistant *Staphylococcus aureus*. *Nat. Rev. Dis. Prim.* 4, 18003 CrossRef Medline

2. Wertheim, H. F., Melles, D. C., Vos, M. C., Van Leeuwen, W., Van Belkum, A., Verbrugh, H. A., and Nouwen, J. L. (2005) The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect. Dis.* 5, 751–762 CrossRef Medline

3. Bush, K., and Bradford, P. A. (2016) β-lactams and β-lactamase inhibitors: an overview. *Cold Spring Harb. Perspect. Med.* 6, a002547 CrossRef

4. Herzberg, O., and Moult, J. (1987) Bacterial resistance to beta-lactam antibiotics: crystal structure of β-lactamase from *Staphylococcus aureus* PC1 at 2.5 Å resolution. *Science* 236, 694–701 CrossRef Medline

5. Fishovitz, J., Hermoso, J. A., Chang, M., and Mobashery, S. (2014) Penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. *J. Bacteriol.* 166, 572–577 CrossRef Medline

6. Lim, D., and Strynadka, N. C. J. (2002) Structural basis for the beta lactam resistance of PBP2a from methicillin-resistant *Staphylococcus aureus*. *Nat. Struct. Biol.* 9, 870–876 CrossRef Medline

7. Otero, L. H., Rojas-Altuve, A., Llarrull, L. I., Carrasco-Lopez, C., Kumarasiri, M., Lastochkin, E., Fishovitz, J., Dawley, M., Hesek, D., Lee, M., Johnson, J. W., Fisher, J. F., Chang, M., Mobashery, S., Hermoso, J. A., et al. (2013) How allosteric control of *Staphylococcus aureus* penicillin binding protein 2a enables methicillin resistance and physiological function. *Proc. Natl. Acad. Sci. U.S.A.* 110, 16808–16813 CrossRef Medline

8. Kerff, F., Charlier, P., Colombo, M.-L., Sauvage, E., Brans, A., Frère, J.-M., Joris, B., and Fonze, E. (2003) Crystal structure of the sensor domain of the BlaR penicillin receptor from *Bacillus licheniformis*. *Biochemistry* 42, 12835–12843 CrossRef Medline

9. Mazuet, C., Yoon, E.-J., Boyer, S., Pignier, S., Blanc, T., Doehring, I., Meziane-Cherif, D., Dumant-Forest, C., Sautereau, J., Legeay, C., Bouvet, P., Boucher, C., Quijano-Roy, S., Pestel-Caron, M., Courvalin, P., et al. (2016) A penicillin- and metronidazole-resistant *Clostridium botulinum* strain responsible for an infant botulism case. *Clin. Microbiol. Infect.* 22, 644.e7–644.e12 CrossRef Medline

10. Zhang, H. Z., Hackbarth, C. J., Chansky, K. M., and Chambers, H. F. (2001) A proteolytic transmembrane signaling pathway and resistance to β-lactams in *staphylococci*. *Science* 291, 1962–1965 CrossRef Medline

11. García-Castellanos, R., Mallorquí-Fernández, G., Marrero, A., Potempa, J., Coll, M., and Gomis-Ruth, F. X. (2004) On the transcriptional regulation of methicillin resistance: Mecl repressor in complex with its operator. *J. Biol. Chem.* 279, 17888–17896 CrossRef Medline

12. Hackbarth, C. J., and Chambers, H. F. (1993) BlaI and blaR1 regulate beta-lactamase and PBP 2a production in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 37, 1144–1149 CrossRef Medline

13. Holm, L., and Laakso, L. M. (2016) Dali server update. *Nucleic Acids Res.* 44, W351–355 CrossRef Medline

14. King, D. T., King, A. M., Lal, S. M., Wright, G. D., and Strynadka, N. C. J. (2001) Critical involvement of a carbamylated lysine in catalytic function of class D β-lactamases. *Proc. Natl. Acad. Sci. U.S.A.* 98, 14280–14285 CrossRef Medline

15. Lovering, A. L., Safadi, S. S., and Strynadka, N. C. J. (2012) Structural perspective of peptidoglycan biosynthesis and assembly. *Annu. Rev. Biochem.* 81, 451–478 CrossRef Medline

16. Poirel, L., Naas, T., and Nordmann, P. (2010) Diversity, epidemiology, and genetics of class D β-lactamases. *Antimicrob. Agents Chemother.* 54, 24–38 CrossRef Medline

17. Otero, L. H., Rojas-Altuve, A., Llarrull, L. I., Carrao-Lopez, C., Kumarasiri, M., Lastochkin, E., Fishovitz, J., Dawley, M., Hesek, D., Lee, M., Johnson, J. W., Fisher, J. F., Chang, M., Mobashery, S., Hermoso, J. A., et al. (2013) How allosteric control of *Staphylococcus aureus* penicillin binding protein 2a enables methicillin resistance and physiological function. *Proc. Natl. Acad. Sci. U.S.A.* 110, 16808–16813 CrossRef Medline

18. Kerff, F., Charlier, P., Colombo, M.-L., Sauvage, E., Brans, A., Frère, J.-M., Joris, B., and Fonze, E. (2003) Crystal structure of the sensor domain of the BlaR penicillin receptor from *Bacillus licheniformis*. *Biochemistry* 42, 12835–12843 CrossRef Medline

19. Mazuet, C., Yoon, E.-J., Boyer, S., Pignier, S., Blanc, T., Doehring, I., Meziane-Cherif, D., Dumant-Forest, C., Sautereau, J., Legeay, C., Bouvet, P., Boucher, C., Quijano-Roy, S., Pestel-Caron, M., Courvalin, P., et al. (2016) A penicillin- and metronidazole-resistant *Clostridium botulinum* strain responsible for an infant botulism case. *Clin. Microbiol. Infect.* 22, 644.e7–644.e12 CrossRef Medline

20. Zhang, H. Z., Hackbarth, C. J., Chansky, K. M., and Chambers, H. F. (2001) A proteolytic transmembrane signaling pathway and resistance to β-lactams in *staphylococci*. *Science* 291, 1962–1965 CrossRef Medline

21. García-Castellanos, R., Mallorquí-Fernández, G., Marrero, A., Potempa, J., Coll, M., and Gomis-Ruth, F. X. (2004) On the transcriptional regulation of methicillin resistance: Mecl repressor in complex with its operator. *J. Biol. Chem.* 279, 17888–17896 CrossRef Medline
27. Karlsowoy, J. A., Adam, H. J., Baker, M. R., Lagacé-Wiens, P. R. S., Walkty, A. J., Hoban, D. J., and Zhan, G. G. (2013) In vitro activity of cefaroline-avibactam against Gram-negative and Gram-positive pathogens isolated from patients in Canadian hospitals from 2010 to 2012: results from the CANIVARD Surveillance Study. Antimicrob. Agents Chemother. 57, 5600–5611 CrossRef Medline

28. Castanheiro, M., Sader, H. S., Farrell, D. J., Mendes, R. E., and Jones, R. N. (2012) Activity of cefaroline-avibactam tested against Gram-negative and Gram-positive organism populations, including strains expressing one or more β-lactamases and methicillin-resistant *Staphylococcus aureus* carrying various staphylococcal cassette chromosome mec types. Antimicrob. Agents Chemother. 56, 4779–4785 CrossRef Medline

29. Miossec, C., Caudron, M., Lefevre, P., Black, M. T., Caudon, M., Levasseur, P., and Black, M. T. (2013) The β-lactamase inhibitor avibactam (NXL104) does not induce ampC β-lactamase in Enterobacter cloacae. *Infect. Drug Resist.* 6, 235–240

30. Livermore, D. M., Jamrozy, D., Mushtaq, S., Nichols, W. W., Young, K., and Woodford, N. (2017) AmpC β-lactamase induction by avibactam and relebactam. *J. Antimicrob. Chemother.* 72, 3342–3348 CrossRef Medline

31. Thrall, L. R., Joshi, G. S., and Richardson, A. R. (2012) Virulence strategies of the dominant USA300 lineage of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA). *FEMS Immunol. Med. Microbiol.* 65, 5–22 CrossRef Medline

32. Challagunda, L., Luo, X., Tickler, I. A., Didelot, X., Coleman, D. C., Shore, A. C., Coombs, G. W., Sordelli, D. O., Brown, E. L., Skov, R., Larsen, A. R., Reyes, J., Robledo, I. E., Vazquez, G. J., Rivera, R., et al. (2018) Range expansion and the origin of USA300 North American epidemic meticillin-resistant *Staphylococcus aureus*. *MBio* 9, CrossRef

33. Marnoto, R., Mallosorri-Fernández, G., Guevara, T., García-Castellanos, R., and Geimis-Rüth, F. X. (2006) Unbound and acylated structures of the MecR1 extracellular antibiotic-sensor domain provide insights into the signal-transduction system that triggers methicillin resistance. *J. Mol. Biol.* 361, 506–521 CrossRef Medline

34. Afonine, P. V., Grosse-Kunstleve, R. W., Echols, N., Headd, J. J., Moriarty, N. W., Murshudov, G. N., Adams, P. D. (2012) Towards automated crystallographic structure refinement with phenix.refine. *Acta Crystallogr. D Biol. Crystallogr.* 68, 352–367 CrossRef Medline

35. Krissinel, E., and Henrick, K. (2007) Inference of macromolecular assemblies from crystalline state. *J. Mol. Biol.* 372, 774–797 CrossRef Medline

36. Birck, C., Cha, J. Y., Cross, J., Schulze-Briese, C., Meroueh, S. O., Schlégel, H. B., Mobashery, S., and Samama, J.-P. (2004) X-ray crystal structure of the acylated β-lactam sensor domain of BlaR1 from *Staphylococcus aureus* and the mechanism of receptor activation for signal transduction. *J. Biol. Chem.* 279, 1610–1616 CrossRef Medline

37. Castanheira, M., Sader, H. S., Farrell, D. J., Mendes, R. E., and Jones, R. N. (2014) OXA-24 lin-resistant *Staphylococcus aureus* and the mechanism of receptor activation for signal transduction. *J. Mol. Biol.* 423, 4926–4941 CrossRef Medline

38. Szarecka, A., Lesnock, K. R., Ramirez-Mondragon, C. A., Nicholas, H. B., Mobashery, S., and Samama, J.-P. (2015) X-ray crystal structure of *Acinetobacter baumannii* pose an emerging threat through increased hydrolytic activity against carbapenems. *Antimicrob. Agents Chemother.* 60, 6155–6164 CrossRef Medline

39. Zare, A., Lesnock, K. R., Ramirez-Mondragon, C. A., Nicholas, H. B., and Wymore, T. (2011) The class D β-lactamase family: residues governing the maintenance and diversity of function. *Protein Eng. Des. Sel.* 24, 801–809 CrossRef Medline

40. Wilke, M. S., Hills, T. L., Zhang, H.-Z., Chambers, H. F., and Strynadka, N. C. J. (2004) Crystal structures of the Apo and penicillin-acetylated forms of the BlaR1 β-lactamase of *Staphylococcus aureus*. *J. Biol. Chem.* 279, 47278–47287 CrossRef Medline

41. June, C. M., Muckenhalter, T. J., Schroder, E. C., Klamr, Z. L., Wawrzak, Z., Powers, R. A., Zarecka, A., and Leonard, D. A. (2016) The structure of a doripenem-bound OXA-51 class D β-lactamase variant with enhanced carbapenemase activity. *Protein Sci.* 25, 2152–2163 CrossRef Medline

42. Santillana, E., Beceiro, A., Bou, G., and Romero, A. (2007) Crystal structure of the carbapenemase OXA-24 reveals insights into the mechanism of carbapenem hydrolysis. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5354–5359 CrossMedline

43. Stauder, M. W., Frederick, T. E., Natarajan, S. V., Wilson, B. D., Tanner, C. E., Ruggiero, S. T., Mobashery, S., and Peng, J. W. (2015) Investigation of signal transduction routes within the sensor/transducer protein BlaR1 of *Staphylococcus aureus*. *Biochemistry* 54, 1600–1610 CrossRef Medline

44. Belluso, B. S., Abatia, L. A., Giannini, E., Mihovilcevic, D., Dal Peraro, M., and Llarrull, L. I. (2019) An experiment-informed signal transduction model for the role of the *Staphylococcus aureus* MecR1 protein in β-lactam resistance. *Sci. Rep.* 9, 19558 CrossRef

45. Winter, G., Lobley, C. M. C., and Prince, S. M. (2013) Decision making in xia2. *Acta Crystallogr. D Biol. Crystallogr.* 69, 1260–1273 CrossRef Medline

46. Kabsch, W. (2010) XDS. *Acta Crystallogr. Sect. D Biol. Crystallogr.* 66, 125–132 CrossRef Medline

47. Evans, P. R., and Murshudov, G. N. (2013) How good are my data and what is the resolution? *Acta Crystallogr. Sect. D Biol. Crystallogr.* 69, 1204–1214 CrossRef Medline

48. Winn, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., Keegan, R. M., Krissinel, E. B., Leslie, A. G. W., McCoy, A., McNicholas, S. J., Murshudov, G. N., Pannu, N. S., Potterton, E. A., Powell, H. R., et al. (2011) Overview of the CCP4 suite and current developments. *Acta Crystallogr. Sect. D Biol. Crystallogr.* 67, 235–242 CrossRef Medline

49. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. *J. Appl. Crystallogr.* 40, 658–674 CrossRef Medline

50. Adams, P. D., Afonine, P. V., Cowtan, K. D., Emsley, P., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. *J. Appl. Crystallogr.* 40, 658–674 CrossRef Medline

51. Antunes, N. T., Lamoureux, T. L., Toth, M., Stewart, N. K., Frase, H., and Vakulenko, S. B. (2014) Class D β-lactamases: are they all carbapenemases? *Antimicrob. Agents Chemother.* 58, 2119–2125 CrossRef Medline

52. Evans, B. A., and Ameyes, S. G. B. (2014) OXA β-lactamases. *Clin. Microbiol. Rev.* 27, 241–263 CrossRef Medline

53. Schroder, E. C., Klamr, Z. L., Saral, A., Sugg, K. A., June, C. M., Wymore, T., Zarecka, A., and Leonard, D. A. (2016) Clinical variants of the native class D β-lactamase of *Acinetobacter baumannii* pose an emerging threat
60. Dolinsky, T. J., Nielsen, J. E., McCammon, J. A., and Baker, N. A. (2004) PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Res.* CrossRef

61. Jurrus, E., Engel, D., Star, K., Monson, K., Brandi, J., Felberg, L. E., Brookes, D. H., Wilson, L., Chen, J., Liles, K., Chun, M., Li, P., Gohara, D. W., Dolinsky, T., Konecny, R., et al. (2018) Improvements to the APBS biomolecular solvation software suite. *Protein Sci.* 27, 112–128 CrossRef Medline

62. Schrödinger Release 2019-1 (2019) *Desmond molecular dynamics system (DESR)*, NY Maestro-Desmond Interoperability Tools, Schrödinger, New York

63. Basuino, L., Jousselin, A., Alexander, J. A. N., Strynadka, N. C. J., Pinho, M. G., Chambers, H. F., and Chatterjee, S. S. (2018) PBP4 activity and its overexpression are necessary for PBP4-mediated high-level β-lactam resistance. *J. Antimicrob. Chemother.* 73, 1177–1180 CrossRef Medline