Crystal Structures of the Apo and Penicillin-acylated Forms of the BlaR1 β-Lactam Sensor of *Staphylococcus aureus*

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*Staphylococcus aureus* is among the most prevalent and antibiotic-resistant of pathogenic bacteria. The resistance of *S. aureus* to prototypical β-lactam antibiotics is conferred by two mechanisms: (i) secretion of hydrolytic β-lactamase enzymes and (ii) production of β-lactam-insensitive penicillin-binding proteins (PBP2a). Despite their distinct modes of resistance, expression of these proteins is controlled by similar regulation systems, including a repressor (BlaI/MecI) and a multidomain transmembrane receptor (BlaR1/MecR1). Resistance is triggered in response to a covalent binding event between a β-lactam antibiotic and the extracellular sensor domain of BlaR1/MecR1 by transduction of the binding signal to an intracellular protease domain capable of repressor inactivation. This study describes the first crystal structures of the sensor domain of BlaR1 (BlaR1*) from *S. aureus* in both the apo and penicillin-acylated forms. The structures show that the sensor domain resembles the β-lactam-hydrolyzing class D β-lactamases, but is rendered a penicillin-binding protein due to the formation of a very stable acyl-enzyme. Surprisingly, conformational changes upon penicillin binding were not observed in our structures, supporting the hypothesis that transduction of the antibiotic-binding signal into the cytosol is mediated by additional intramolecular interactions of the sensor domain with an adjacent extracellular loop in BlaR1.

The evolution and dissemination of antibiotic resistance in pathogenic bacteria are a growing concern. Many of the antimicrobial “wonder drugs” society has come to rely on for the treatment of bacterial infections have been neutralized by new strains equipped with a variety of molecular countermeasures. Methicillin-resistant *Staphylococcus aureus* is a prominent example of this (1–3). Although β-lactam antibiotics such as penicillin have long been the drugs of choice for infections of *S. aureus*, current therapies for methicillin-resistant *S. aureus* infections now depend on distinct antibiotic classes such as glycopeptides to counter the increased resistance to penicillin and its derivatives. The recent emergence of glycopeptide-resistant strains of *S. aureus* (4, 5) underscores the need not only for the development of novel therapeutics, but for better understanding of the molecular mechanisms involved in antibiotic resistance.

β-Lactam antibiotics kill bacteria by inhibiting the cell wall transpeptidases (also known as penicillin-binding proteins (PBPs)) that are responsible for the essential cross-linking of peptidoglycan during synthesis of the rigid bacterial cell wall. Resistance to β-lactam antibiotics in *S. aureus* can be conferred by two mechanisms. In one case, β-lactamase enzymes are secreted from the bacterium to hydrolytically inactivate β-lactam antibiotics. Resistance of this form is encoded by the blaZ gene and is carried by >95% of *S. aureus* isolates (3). The second resistance mechanism is expression of PBP2a, a cell wall transpeptidase with broad-spectrum insensitivity to β-lactam antibiotics (6). These methicillin-resistant *S. aureus* strains carry the mecA gene and have become infamous for causing outbreaks of infections in hospitals and in the community (7). PBP2a-mediated resistance is spreading rapidly, and methicillin-resistant *S. aureus* strains currently constitute ~25–50% of clinical *S. aureus* isolates in the United States, Europe, and Asia (8). Many strains of methicillin-resistant *S. aureus* are resistant to multiple classes of antibiotics and are especially worrisome with regard to the evolution of so-called “superbugs,” pathogenic bacteria that are invulnerable to all available antibacterial drugs.

Expression of the structural genes for β-lactamases and PBP2a (i.e. blaZ and mecA, respectively) are regulated by orthogonal systems (Fig. 1) consisting of a repressor (BlaI/MecI) and a sensor/transducer protein (BlaR1/MecR1) that destroys the activity of the repressor when activated by antibiotics. The BlaI and MecI repressors form homodimers that specifically bind DNA operator/promoter sequences. BlaR1 and MecR1 are membrane-spanning multidomain proteins, each composed of three domains: (i) an extracellular C-terminal β-lactam sensor domain, (ii) an N-terminal transmembrane domain containing four predicted transmembrane α-helices (9), and (iii) an intracellular metalloprotease domain that is thought to proteolytically inactivate the BlaI/MecR1 repressor in the cytoplasm. Existing evidence suggests that binding of β-lactam antibiotics to the sensor domain causes a conformational change that results in derepression of the gene.

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The atomic coordinates and structure factors (code 1XAI and 1XAT) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).

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in autolytic activation of the intracellular protease domain (10), allowing it in turn to catalyze cleavage of BlaI/MecI (directly or indirectly) at a site critical for dimerization. This cleavage event effectively destroys the ability of the repressor to bind DNA, permitting the transcription of not only blaZ/mecA, but blaI/mecI and blaR1/mecR1 as well. In this system, expression of these proteins is efficiently terminated when the signaling antibiotic levels are reduced (primarily through hydrolysis by surrounding β-lactamase enzymes (10)). Whereas the bla divergeon (i.e. blaZ, blaR1, and blaR2) is plasmid-borne, constitutive β-lactamase expression has been observed in S. aureus strains possessing normal penicillinase plasmids (11). Several explanations for this observation have been proposed, including the involvement of an as of yet unidentified chromosomally encoded regulatory component known as BlaR2 (12, 13).

BlaR1 and MecR1 from S. aureus share significant sequence identity (sensor domains, 43%; protease domains, 33%; and full-length proteins, 34%), and the regulatory genes of bla and mec have been shown to be interchangeable in vivo (14, 15). Indeed, due to the potency of the MecI repressor, many methicillin-resistant S. aureus strains have Mec deletions that inactivate repression (16), incurring either constitutive expression of PBP2a or inducible expression regulated by BlaR1/BlaI. The recent observation that the absence of the bla or mec regulatory genes selects against PBP2a expression suggests a role for these genes in stabilizing dissemination of mecA to new host strains (17).

Crystal structures have been determined previously for the S. aureus repressor MecI (13, 18) and the apo form of the BlaR1 sensor domain (Bacillus licheniformis) (19). In addition, the NMR solution structure is available for the B. licheniformis BlaI DNA-binding domain (20). This study describes the crystal structures of the S. aureus BlaR1 β-lactam sensor domain (hereafter referred to as BlaR8) in both its apo and penicillin-acylated forms. These structures illuminate the active-site features that are responsible for the PBP activity of BlaR8 and provide mechanistic insights into the role of the BlaR1 sensor domain in detecting β-lactam antibiotics and transcoding the binding signal across the bacterial cell membrane.

**EXPERIMENTAL PROCEDURES**

Cloning, Overexpression, and Purification—BlaR1 was cloned from a plasmid containing the bla divergeon (p184R6H) as described previously (10). This plasmid was originally derived from a β-lactamase-expressing strain of *S. aureus* (67-0) isolated from a patient at San Francisco General Hospital Medical Center (21, 22). The β-lactam sensor domain (amino acids 330–555, i.e. BlaR8) was subcloned into the pET41b (+) vector (Novagen) and transformed into *Escherichia coli* DH5α (DE3) (Novagen). Cells were grown from an overnight culture at 37 °C until A600 = 0.6, heat-shocked at 42 °C for 1 h, cooled to 15 °C, and then induced to overexpress BlaR8 overnight using 1 mM isopropyl β-D-thiogalactopyranoside. This same expression procedure was adapted for the production of selenomethionine (SeMet)-substituted BlaR8 using previously described protocols (23). The cells were lysed by high pressure homogenization using an Avestin EmulsiFlex-C5. The BlaR8 protein was purified from the soluble cell fraction using three chromatographic steps performed at 4 °C. The soluble cell lysate was first bound to Fractogel EMD SO3+ resin (Novagen), pre-equilibrated in 20 mM Hepes (pH 7.5) and 50 mM NaCl, and eluted with 0.8 mM NaCl. The eluate was dialyzed overnight at 4 °C in buffer containing 0.2 M NaCl and then filtered using 0.22-µm filters. The filtrate was further purified using a Mono S HR 5/5 cation exchange column (Amersham Biosciences) and a 0.2–0.8 M NaCl elution gradient. Elution fractions corresponding to the principal Amax peak were combined, concentrated, and passed through a Superdex 200 HR 10/30 size exclusion column (Amersham Biosciences) equilibrated in 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl as the storage buffer. The single Amax peak was collected and concentrated to 20–40 mg/ml as estimated using a predicted molar absorption coefficient of 60280 M−1 cm−1 (24). Concentrated BlaR8 was either used fresh (stored at 4 °C) or flash-frozen in liquid N2 and stored at −80 °C.

**Crystalization and Data Collection**—Crystals of apo- and acyl-Bl aR8 were obtained using the hanging drop vapor diffusion method. For apo-Bl aR8 crystals, 1 µl of a 20 mg/ml protein solution was added to equal volumes of reservoir solution consisting of 18–23% polyethylene glycol 3350 and 0.2 mM NaH2PO4. Equilibration over a 0.5-ml reservoir at 18 °C for 1–2 weeks produced single crystals reaching dimensions of up to 0.7 × 0.5 × 0.4 nm. For acyl-Bl aR8, benzylpenicillin was incubated with BlaR8 at final concentrations of 20 mg/ml protein and 10 mM benzylpenicillin for 15 min at room temperature before initiating crystallization. Native acyl-Bl aR8 crystals were then grown using 1 µl of this protein solution mixed with an equal volume of reservoir solution consisting of 26–29% polyethylene glycol 3350, 0.2 M NaCl, and 0.1 M BisTris (pH 6.6). Crystals were typically observed after 1 week at 18 °C. SeMet crystals of acyl-Bl aR8 were obtained by streak-seeding native crystals into fresh drops containing SeMet-substituted BlaR8, benzylpenicillin, and crystallization reagents at the same concentrations used to grow the native crystals. Crystal clusters formed in 3–5 days with maximum dimensions of 0.4 × 0.2 × 0.15 mm for the individual crystals of the cluster. Single SeMet acyl-Bl aR8 crystals were obtained by gently breaking the clusters.

Diffraction data were collected at 100 K using cryoprotectant solu-
tions of 35% polyethylene glycol 3350 and 0.3 M NaHPO₄ for the apo-BlaR₅ crystal and 35% polyethylene glycol 3350, 0.5 M NaCl, and 0.1 M BisTris (pH 6.6) for the SeMet acyl-BlaR₅ crystal. All data sets were collected at the National Synchrotron Light Source on beamline X8-C using an ADSC Quantum Q4R CCD detector. Data were processed using the HKL package (25) and programs from the CCP4 software suite (26). The apo crystal was of space group P2₁, with four molecules/asymmetric unit and unit cell dimensions of a = 59.9 Å, b = 104.9 Å, and c = 90.3 Å and β = 107.7°. SeMet acyl-BlaR₅ crystallized in space group P4₁2₁2, with two molecules/asymmetric unit and unit cell dimensions of a = b = 88.4 Å and c = 125.1 Å. Statistics for data collection and processing are summarized in Table I.

Structure Solution and Refinement—The structure of acyl-BlaR₅ was determined using single anomalous dispersion with the peak data to locate the initial selenium sites in SOLVE (27), followed by three-wavelength multiple anomalous dispersion to generate more accurate phases. Through 100% isomorphous replacement of SeMet into BlaR₅ was indicated by mass spectrometry, only 15% of possible selenium sites were located. Phases were improved with density modification using RE-SOLVE (28). The initial model was automatically built with RESOLVE (50% complete) (29) and manually rebuilt using XTALVIEW (30). Iterations of refinement with CNS (25) and REFMAC (26) resulted in the final model. Of the 255 residues in the BlaR₅ construct, 242 residues were modeled in chain A (residues at 1–13, 18–201, and 204–251), and 241 residues were modeled in chain B (residues 8–26 and 31–252). Model quality was analyzed using PROCHECK (42% in the most favored region of the Ramachandran plot) (32). Amn₅ψ is a well ordered active-site residue, but adopts a disallowed main chain conformation due to its juxtaposition to benzylpenicillin-acylated Serₚ⁵⁵. The apo-BlaR₅ structure was solved by molecular replacement using the acyl-BlaR₅ structure as a starting model and the program MOLREP (33). Model rebuilding was performed with XTALVIEW using a prime-and-switch map generated by RESOLVE to reduce model bias. The model was refined and analyzed for quality as described above. The resulting model consisted of 244 residues for chain A (residues 8–166 and 169–253), 247 residues for chain B (residues 8–254), 243 residues for chain C (residues 5–15, 17–76, 78–84, 86–202, and 206–253), and 240 residues for chain D (residues 7–76, 80–82, 84–166, and 169–252). The four molecules of the apo form, a pyrophosphate was also modeled into the active site. This ligand is only partially occupied in three of the four molecules of the asymmetric unit, as revealed by the close proximity of a partially occupied water molecule to one of the phosphorus atoms. Considering that the crystallization conditions for apo-BlaR₅ contained 0.2 M sodium phosphate, the unknown electron density could also be modeled to two partially occupied phosphate ions, but the staggered geometry of the tetrahedral phosphorus atoms and the bent P–O–P bond indicate a molecule of pyrophosphate, introduced as a sodium phosphate impurity. The final apo-BlaR₅ model had 88% of the residues in the most favorable region of the Ramachandran plot and none in the disallowed region. The multiple anomalous dispersion phasing and refinement statistics for both structures are provided in Table I. Fig. 3 was prepared with MOLSCRIPT (34) and rendered with Raster3D (35). All other protein graphics (Figs. 5–7) were prepared and rendered with PyMOL (36).

Static Light Scattering—Static light scattering experiments were performed at 25 °C on a Superdex 75 HR 10/30 size exclusion column (Amersham Biosciences) using 50 mM HEPES (pH 7.5) and 100 mM NaCl. Protein concentration was 1 mg/ml. Refractive index and multiDAWN static light scattering—were measured at 2.5 ml of brain/heart infusion broth containing 15 μg/ml chloramphenicol to maintain the plasmid vector with and without 10 μg/ml CBB as an inducer on a reciprocal shaker platform at 250 rpm. 1-ml volumes were removed; cells were pelleted by centrifugation at 10,000 × g for 3 min; the broth was discarded; and cells were resuspended in 0.1 M sodium phosphate buffer (pH 6.0) to achieve a cell suspension of A₅₉₀ = 0.95–1.05. A 0.9-mL volume of bacterial suspension was added to 0.1 mL of 1 mM cephaloridine (final cephaloridine concentration of 100 μM) in sodium phosphate buffer and incubated at 37 °C. At 30 and 60 min, 1-ml samples were centrifuged to pellet cells. The cephaloridine concentration in the supernatant was determined spectrophotometrically at 254 nm using buffer and the cephaloridine sodim chloride as the blank.

RESULTS AND DISCUSSION

Functional Characterization of Recombinant BlaR₅—The C-terminal extracellular sensor of the BlaR₁ signal transducer from Staphylococcus aureus (BlaR₅) was expressed in the cytoplasm of Escherichia coli as a soluble domain, spanning amino acids 331–585 and lacking an N-terminal Met. To verify the activity of our recombinant protein, the ability of BlaR₅ to be covalently modified by β-lactam antibiotics was demonstrated using mass spectrometric analysis before and after incubation with benzylpenicillin. This experiment yielded a homogeneous mass shift of 334 Da, corresponding closely to the expected mass shift of 334 Da for a benzylpenicillin adduct. Similar deletion mutants containing the sensor domain of BlaR₁ have been expressed in E. coli previously for both S. aureus (38) and B. licheniformis (39) and have been shown to fully retain their activities as highly sensitive PBPs.

Overall Fold and Oligomerization State—BlaR₅ was crystallized in the absence and presence of benzylpenicillin to facilitate determination of the three-dimensional structures of the apo- and acyl-BlaR₅ proteins by x-ray crystallography. The crystal structure of acyl-BlaR₅ was solved first using multiple anomalous dispersion data collected from a SeMet-substituted crystal. The final model of acyl-BlaR₅ consisted of two molecules/asymmetric unit, which superposed with a root mean square deviation (r.m.s.d.) of 0.58 Å on the 232 commonly observed Cα atoms. The model was refined to 2.4 Å resolution with R and Rfree values of 22.0 and 27.5%, respectively (Table 1). The atomic coordinates from the acyl-BlaR₅ structure were then used to solve the crystal structure of apo-BlaR₅ by molecular replacement. The resulting model had four molecules in the asymmetric unit and was refined to 1.8 Å resolution with R and Rfree values of 18.6 and 22.7%, respectively (Table 1). Four molecules superposed closely with r.m.s.d. values of 0.33–0.45 Å on the 234–244 commonly observed Cα atoms.

The main chain fold of BlaR₅ consists of two domains, one helical and one mixed α/β with the penicillin-binding site residing at the interface (Fig. 2). The mixed α/β domain includes a seven-stranded β-sheet, composed of six antiparallel strands and a short parallel strand, sandwiched between a pair of α-helices on either side. The helical domain is composed of six helices with the active site situated in an interdomain cleft centered on helix C, which consists of a single 3₁-helical turn followed by three α-helical turns. An α-loops connects helices G and H and forms one edge of the active site. There is no obvious

### Table I

| Structure | Resolution (Å) | R-value (%) | Rfree (%) |
|-----------|----------------|-------------|-----------|
| Apo-BlaR₅ | 2.4            | 22.0        | 27.5      |
| Acyl-BlaR₅ | 1.8            | 18.6        | 22.7      |
Aromatic or hydrophobic patch on the surface of BlaRS, and addition of detergent or lipid was not required for solubility or activity, suggesting no close association between the sensor domain of BlaR1 and the bacterial membrane.

As observed previously for the B. licheniformis BlaR1 sensor domain (19), the S. aureus BlaRS structure resembles that of the class D β-lactamases, with overall r.m.s.d. values in the range of 1.17–1.41 Å on 192–200 common C-α residues for the available OXA structures. Overlapping the apo form of the sensor domains of B. licheniformis and S. aureus gives similar r.m.s.d. values of 1.23–1.27 Å on 214 common C-α atoms (36% amino acid sequence identity). The closest match of BlaRS and the class D enzymes occurs with OXA-1 from E. coli, which shares 28% amino acid sequence identity with BlaRS. This is consistent with the fact that OXA-1 is the only class D β-lactamase that prefers a monomeric state (40). Other class D β-lactamases have been shown to exist predominantly as dimers in solution, an oligomeric form that promotes maximum catalytic activity and that can be mediated by ion binding (41). BlaRS was observed as a monomer in either crystal form as well as in solution as determined by static light scattering at similar protein concentrations (data not shown). Examination of the BlaRS structure indicates that it has lost the prominent dimeric interface observed in the class D crystal structures. A comparison of BlaRS with the OXA-10 dimer reveals that many of the residues responsible for stabilizing the OXA dimer are different in BlaRS, precluding the formation of two salt bridges, three hydrophobic interactions, and three metal ligands. Although a pseudo 2-fold symmetry axis that resembles the OXA dimer is created by crystal packing in both of our structures, the strands of the intermolecular β-sheet meet at a steep angle of ∼30°, and only a total of four hydrogen bonds join the two molecules.

**Active-site Architecture and the Benzylpenicillin Adduct**—Three conserved sequence motifs define the active site of all penicilloyl serine transferases/hydrolases, including the class A, C, and D β-lactamases; the cell wall transpeptidases; and BlaR1/MecR1 (Fig. 3). In BlaRS, these motifs correspond to Ser389-X-X-Lys392 (with Ser389 the proposed nucleophile in acylation), Ser437-X-Asn439, and Lys526-Thr527-Gly528 (Fig. 4). Within the α-helical domain, the SXXK motif lies at the center of the active-site cleft at the N-terminal end of helix C. The SXN element is adjacent on a short loop connecting helices E and F, and the KTG motif is situated nearby on strand H of the α/β domain. Flanking Lys392 and opposite Ser389 are several hydrophobic residues, including Leu389 and Phe112 as well as Trp145 and Met146, which are positioned on a proximate β-sheet (Asn439-Lys440, Ser437-Trp475, Lys484-Cys485, and Ser437-Lys526). From sequence information, it is expected that the other species of the BlaR1/MecR1 sensor domain should share highly similar active-site features with BlaRS. The only comparison that is possible at this time is with the apo form of the sensor domain of B. licheniformis. As predicted, the two active sites are highly similar (overall r.m.s.d. on all atoms of common active-site residues, i.e. Ser389, Ser437, and Ser439) and of the same conformation (0.68–0.70 Å). The unique substitution of threonine for Asn439 in the B. licheniformis species (19). An additional feature observed in the active site of our apo-BlaRS is a molecule...
of partially occupied pyrophosphate that hydrogen bonds with the O-\(\cdot\)H of Ser389 (2.4 Å), the N-\(\cdot\)H of Lys526 (2.9 Å), the side chains of Thr527 and Thr529, the backbone carbonyl of Thr529, and the amide nitrogens of Ser389 and Thr529 that comprise the oxyanion hole.

In the acyl-BlaR\(^S\) crystal, benzylpenicillin is unambiguously observed as a covalent adduct of Ser389 in both molecules of the asymmetric unit, with C-7 bound to the O-\(\cdot\)H of the proposed nucleophile Ser389 via an ester linkage (Fig. 5A). The adduct has been refined at full occupancy in each molecule, with average B-factors of 34.8 and 37.8 Å\(^2\) (similar to the average B-factor of 31.1 Å\(^2\) observed for the surrounding active-site residues). The backbone nitrogens of Thr529 and Ser389 form hydrogen bonds with the carbonyl oxygen of the adduct ester, creating the oxyanion hole typical of \(\beta\)-lactamases and serine proteases. The thiazolidine methyls are stabilized by hydrophobic interactions with the side chains of Phe421 and Thr529, whereas hydrogen bonds with the side chain of Asn439 and the backbone carbonyl of Thr529 position the adduct amide. The thiazolidine carboxylate is fixed by hydrogen bonds with the side chains of Thr527 and Thr529, a feature typical of PBPs.

Interestingly, the extra arginine residue utilized by the various classes of serine \(\beta\)-lactamases to form electrostatic interactions with the thiazolidine carboxylate of the substrate is absent in the BlaR\(^S\) structure, a scenario also typical of PBPs. The binding mode of benzylpenicillin is highly similar in both molecules of the asymmetric unit, except for the less ordered side chain phenyl substituent (Fig. 5B), which adopts alternative conformations in each case (making hydrophobic interactions with the side chains of Ile531, Thr529, Phe421, and Tyr536 or with the side chains of Ile531 and Met476 and the C-\(\cdot\) and C-\(\cdot\) of Glu477).

**Role of Lys392 as the General Base in Acylation**—The active sites of the PBPs and class A, C, and D \(\beta\)-lactamases center on a common serine nucleophile and are well conserved, but the general base involved in catalysis is apparently different in each (e.g. Lys73 (42–44) and/or Glu166 (45–47) in class A \(\beta\)-lactamases, Tyr150 in class C \(\beta\)-lactamases (48), carboxylated Lys70 in class D \(\beta\)-lactamases (49), and unprotonated Lys392 in PBPs (50, 51)) (Fig. 6). Whichever the class, the role of the general base in acylation is to activate the nucleophile that attacks the \(\beta\)-lactam ring by deprotonation of the catalytic S\(\cdot\)XXK serine. Deprotonation requires either an additional general base to activate water for hydrolysis of the acyl-enzyme intermediate (e.g. class A \(\beta\)-lactamases) or a mechanism for deprotonating/regenerating the first general base (e.g. class A, C, and D \(\beta\)-lactamases). In BlaR\(^S\), the close proximity of Lys392 (2.4–2.8 Å) and Ser437 (3.2–3.5 Å) to the Ser389 nucleophile suggests two possible candidates for a general base in acylation, the
former arguably more suitable in terms of distance and potential $pK_a$ to act either in an unprotonated state similar to the PBPs (19) or in a carboxylated state similar to the class D/β-lactamases (38).

Carboxylation is favored at basic pH, but has been observed in crystal structures of the class D/β-lactamases at as low as pH 6.0 (49) and perhaps even at pH 5.5 with low occupancy (40). The structures of apo- and acyl-BlaRS presented here were determined at pH 4.7 and 6.6, respectively. In either case, carboxylation of Lys392 was not observed at any contour level in our electron density maps. In the class D/β-lactamase structures, the non-carboxylated Lys70 (corresponding to Lys392 in BlaR1) seems to encourage an “inactive” conformation of Ser115 (Ser437 in BlaR1), in which the serine hydroxyl (presumed to shuttle a proton from the carboxylated lysine to the leaving group nitrogen of the β-lactam substrate) is somewhat displaced from its typical position in the active site (49, 52). In contrast with many of the non-carboxylated structures of the class D/β-lactamases, Kerff et al. (19) noted that the active site of the apo form of the B. licheniformis BlaR1 sensor domain (which also lacks a carboxylated active-site lysine despite the fact that the crystals were grown at pH 7.0) closely resembles the “active” conformation. Similarly, we observed the active sites of both our apo- and acyl-BlaRS forms to adopt the active conformation. These results are surprising since Lys392 of BlaRS has been shown to specifically bind CO$_2$ with a dissociation constant of 0.6 mM (38), only a 2–3-fold increase versus the class D/β-lactamase OXA-10 (49). It should be noted, however, that the carboxylation of Lys392 has not yet been directly shown to be a requirement for the β-lactam binding activity of BlaR1 in S. aureus, and preliminary evidence in B. licheniformis suggests no significant increase in acylating activity under conditions that would promote carboxylation of Lys392 in that system (19).

With that in mind, the active site of BlaRS differs from those of class D/β-lactamases such as OXA-10 in several interesting ways (Fig. 6B). The carboxylate of the carboxy-Lys70 of OXA-10 is held in position by hydrogen bonding interactions with Ser67, Trp154, and Asn73 (through one molecule of water) and hydrophobic interactions with Phe120, Ile155, and Val117. Two of these interactions have been disrupted in the BlaRS active site. Strictly conserved in the OXA structures, Val117 has been replaced by Asn439 in the BlaRS structure, which now forms a hydrogen bond with the N-ζ of Lys392 (an interaction found in all class A and C β-lactamases as well as in the PBPs, none of which utilize a carboxylated lysine). Likewise, Asn73 from the structure of OXA-10 has been replaced with the aliphatic Leu395 in BlaR1, eliminating the possibility for hydrogen bonding with Lys392 in a putative carboxylated state. Although the other available structures of class D/β-lactamases show mutations at this position, they represent residues capable of hydro-
gen bonding (i.e. His73 in OXA-2 (Protein Data Bank code 1k38) and Ser73 in OXA-13 (52)) or utilize a neighboring residue as a hydrogen bond donor (i.e. Ser120 in OXA-1 (40)). Collectively, these differences in BlaRS create an environment that may discourage carboxylation of Lys392. It is fascinating that these two distinctions are conserved between BlaRS and class A \(\beta\)-lactamases such as SHV-2 (Fig. 6C) as well as PBPs such as PBP2x (Fig. 6D). In this sense, the active site of BlaRS best resembles a hybrid between those of the PBPs and the class D \(\beta\)-lactamases.

**Formation of the Stable Penicilloyl Adduct—**BlaRS shares the greatest resemblance with the class D \(\beta\)-lactamases in terms of its fold and sequence identity, but its sluggish deacylation activity is that of a PBP. Although carboxylation of Lys392 was not observed in the crystal structures of BlaRS, the possibility for carboxylation of this residue under physiological conditions cannot be dismissed at this time. As such, a discussion of the mechanism of BlaRS must consider both scenarios.

In the observed case, in which Lys392 is not carboxylated, the mechanism resembles that of the PBPs and requires an unprotonated Lys392 (Fig. 7A) (51). Deprotonation of the lysine could be accomplished through the relatively hydrophobic environment of the Lys392 side chain consisting of Leu395, Met434, Phe442, Trp475, and Met476. Considering the hydrogen bond network surrounding Lys392 in the apo-BlaRS structure, including a tight hydrogen bond with the proposed nucleophile Ser389, a proton shuttling scheme similar to that proposed for the PBPs can be envisaged (Fig. 7A). The pathway is initiated by positioning of the \(\beta\)-lactam antibiotic in the active site and abstraction of a proton from Ser389 by Lys392. The tetrahedral transition state (stabilized by the backbone nitrogens of Thr529 and Ser437) collapses to break the scissile \(\beta\)-lactam amide. The close proximity of Ser437 to two protonated lysines (Lys392 and Lys526) facilitates the abstraction of its hydroxyl hydrogen by the lone pair electrons of the \(\beta\)-lactam amide nitrogen. Ser437 finishes the cycle by abstracting a proton from Lys392. Following acylation, the N-\(\gamma\) of Lys392 is observed rotated away from the adduct. With Ser437 protonated and in the absence of a suitable general base for deacylation, BlaRS is stabilized in an acylated state.

**Fig. 6.** Superpositions of the apo-BlaRS active site (tan) with the corresponding residues in a representative class C \(\beta\)-lactamase (AmpC from E. coli; yellow; Protein Data Bank code 1KE4) (A), class D \(\beta\)-lactamase (OXA-10 from P. aeruginosa; green; code 1K57) (B), class A \(\beta\)-lactamase (SHV-2 from Klebsiella pneumoniae; purple; code 1N9B) (C), and PBP (PBP2x from Streptococcus pneumoniae; light blue; code 1QME) (D). Active-site overlays were generated with Swiss-Pdb Viewer (31) by fitting the C-\(\alpha\) atoms of Ser389, Lys392, and Asn439 from BlaRS with the corresponding C-\(\alpha\) atoms from the other structures.
In an alternative mechanism and as in the class D β-lactama-
ses, the carboxylation of Lys392 in BlaR1 could provide a
general base not only for acylation, but for deacylation as well.
For this reason, a mechanism utilizing carboxylysine for acy-
lation in BlaR1 must include a mode of preventing regenera-
tion (i.e. deprotonation) of this residue as the general base for
decaylation. Following a mechanistic scheme for the class D
β-lactamases (40), deprotonation by the carboxylate of carboxy-
Lys392 could activate Ser389 for nucleophilic attack of the β-lac-
tam carbonyl (Fig. 7B). Were BlaR an OXA-like hydrolase, a
deprotonated Ser437 could then subsequently deprotonate car-
boxy-Lys392 to regenerate its nucleophilicity and to permit
decaylation by activation of a bound water (53). Without a
structure showing a carboxylated Lys392 in BlaR1, it is difficult
to rationalize how the position of the carboxylysine carboxylate
would be perturbed in BlaR1 versus a class D β-lactamase.
Indeed, the effect of the lack of a stabilizing hydrogen bond at
Leu439 and the substitution of asparagine for valine at position
439 in BlaR1 can only be surmised. Still, one possibility is that
Asn439 may prevent deprotonation of carboxy-Lys392 by accept-
ing a hydrogen bond from the now protonated carboxylic acid
group (Fig. 7B). A molecule of water bound to Lys426 ultimately
provides the proton for the shuttle instead, generating a stabi-
lized acyl-enzyme. Although Asn439 is not absolutely conserved
in BlaR1/MecR1, this position appears to always be occupied by
a polar residue capable of accepting a hydrogen bond (e.g. Thr452 in the B. licheniformis BlaR (19)).

Structural Differences between Apo- and Acyl-BlaR<sup>S</sup>—The
structures determined in this study provide us with the first
opportunity to compare the two forms of the sensor domain of
the BlaR1 receptor. The gross apo- and acyl-BlaR<sup>S</sup> structures
are highly similar, possessing a r.m.s.d. in the range of 0.59–
0.74 Å on the 231–239 C-terminus atoms. The four molecules that form
the asymmetric unit of the apo-BlaR<sup>S</sup> structure are shown
superposed along with the two molecules that compose the asym-
metric unit of the acyl-BlaR<sup>S</sup> structure (Fig. 8A). It is clear
from this superposition that the only regions that differ in
position between the apo- and acyl-BlaR<sup>S</sup> structures are sur-
face-exposed loops that likely vary due to thermal motion/
mobility as opposed to significant conformational differences
between the apo- and acyl-BlaR<sup>S</sup> forms. In the case of acyl-
BlaR<sup>S</sup>, these loops exhibit weak electron density and higher
than average B-factors. These disordered regions include (i)
the β-hairpin connecting strands 5 and 6 (residues 531–537), (ii)
the large loop connecting the short α-helix D (residues 406–427), and (iii) the N-terminal
region up to β-strand 2 (residues 334–360). Although these
loops are substantially better ordered in the apo structure, they
make numerous stabilizing interactions with neighboring mol-
ecules in the P<sub>2</sub>₁ crystal lattice.

The differences between the active sites of apo- and acyl-
BlaR<sup>S</sup> are similarly subtle (Fig. 8B). Indeed, besides the actual
acylation of Ser<sup>389</sup>, the largest structural difference appears to
be a slight repositioning of β-strand 5 due to hydrogen bonds
with the benzylpenicillin adduct. The change in position of
Ile<sup>521</sup> is not surprising since it composes the C-terminal end of
β-strand 5 and the beginning of a hairpin turn that is poorly
ordered in the acylated structure. A rotation about the χ₁ of
Thr<sup>527</sup> is a consequence of a hydrogen bond with the carboxyl-
Carboxylation of Lys392 is required not only for acylation, but also for acylation of BlaRS using a battery of biophysical techniques, including infrared spectroscopy, and deuterium/hydrogen exchange. Antibiotics using a pair of biophysical techniques, including infrared spectroscopy, and deuterium/hydrogen exchange. Antibiotics using a battery of biophysical techniques, including infrared spectroscopy, and deuterium/hydrogen exchange.

BlaRS using pairwise comparisons with the molecules of the apo- and acyl-BlaRS strongly suggests that the L2 loop plays an integral role as a molecular trigger in signal transduction. This result suggests the disruption of a tonic interaction between BlaRS and the L2 loop that determines basal levels of \( \beta \)-lactamate production, as was observed for the wild-type ZRI transformant, which hydrolyzed some cephaloridine in the absence of inducer. These prolines constitute part of a conserved PXXP sequence motif located at the N terminus of the L2 loop. We propose that the conformational rigidity inherent to proline residues serves to anchor the L2 loop appropriately for interaction with and regulation of the sensor domain. The lack of apparent conformational differences between the apo- and acylated forms of BlaRS supports the prediction that the L2 loop is responsible for modulation of BlaR1 activity by interaction with the BlaRS active site. Indeed, integrity of the L2 loop appears to be required for transmitting the \( \beta \)-lactam-binding signal and de-repressing \( \beta \)-lactamase expression. We have also observed that the fusion of either a Myc or hexahistidine tag to the C terminus of BlaR1 confers a phenotype of constitutive high level \( \beta \)-lactamate expression (Table II), perhaps because this tag interferes with the binding of the L2 loop to the BlaR1 sensor domain.

| Phenotype | -I | +I | -I | +I |
|-----------|----|----|----|----|
| Controls  | 50 ± 10 | 50 ± 4 | 50 ± 10 | 50 ± 4 |
| RN4220    | 100 ± 9 | 98 ± 7 | 100 ± 9 | 98 ± 7 |
| ZRI       | 71 ± 6 | 47 ± 14 | 51 ± 10 | 35 ± 7 |
| C-terminal tags | Myc | 39 ± 4 | 43 ± 7 | 36 ± 3 | 36 ± 1 |
|           | His45 | 45 ± 4 | 48 ± 4 | 38 ± 2 | 40 ± 8 |
| Point mutations in L2 loop | P49A | 98 ± 5 | 102 ± 2 | 94 ± 9 | 98 ± 5 |
|           | P52A | 101 ± 9 | 100 ± 7 | 99 ± 5 | 99 ± 3 |

* The S. aureus host strain RN4220 was transformed with a chloramphenicol-selectable S. aureus vector, pHN5542 (indicated as RN4220); with vector plus cloned wild-type blaR1-blaI-blaZ (ZRI); and with vector plus wild-type blaI and blaZ and one of four blaR1 mutations: a C-terminal tag, a C-terminal hexahistidine tag, an Ala-for-Pro substitution at residue 49 (P49A), and Ala-for-Pro substitution at residue 52 (P52A).

P represents the inducer CBAP at 10 \( \mu \)g/ml.

**Table II**

Summary of mutations and their effects on CBAP-inducible \( \beta \)-lactamate expression assayed by hydrolysis of 100 \( \mu \)m cephaloridine

Acylations of the benzylpenicillin adduct. Acylation also reorients the N-\( \xi \) of Lys392 away from Ser389, breaking the hydrogen bond observed in the apo structure.

**Transmembrane Signal Transduction**—There are no major conformational differences observed between the structures of the apo- and acyl-BlaRS forms to provide an explanation as to how \( \beta \)-lactam binding at the extracellular face of BlaR1 initiates a signal capable of being transduced to the BlaR1 cytosolic protease domain. Based on the results of previous circular dichroism experiments that indicated an enhancement of secondary structure upon acylation (38), it was expected that acylation of BlaRS by benzylpenicillin would result in a change in conformation that could alter the interaction of the BlaR1 sensor domain with the transmembrane domain (Fig. 1). Although we cannot rule out the possibility that the unexpected binding of pyrophosphate in the active site of our apo-BlaRS structure triggered the “acyl” conformation (indeed, several of the interactions of pyrophosphate with the BlaRS active site closely resemble the interactions made with the benzylpenicillin adduct), if this were the case, distinct conformational differences should then be observed between these structures and the structure of B. licheniformis apo-BlaRS (19). However, superpositions of the B. licheniformis apo structure with the apo- and acyl-BlaRS structures presented here reveal no significant conformational differences (r.m.s.d. of 1.23–1.27 Å on 214 C-α atoms for apo-BlaRS using pairwise comparisons with the molecules of the asymmetric unit and r.m.s.d. of 1.07–1.22 Å for the same set of comparisons in acyl-BlaRS). Moreover, recent experiments using B. licheniformis apo-BlaRS revealed no significant alterations in secondary or tertiary structure upon acylation with \( \beta \)-lactam antibiotics using a battery of biophysical techniques, including circular dichroism, fluorescence spectroscopy, Fourier transform infrared spectroscopy, and deuterium/hydrogen exchange kinetics (54). Although an argument can be made that the carboxylation of Lys392 is required not only for acylation, but also for maintaining the acyl conformation, no significant conformational changes have been observed in the class D \( \beta \)-lactamases upon \( \beta \)-lactam acylation. Accordingly, it is more likely that the BlaR1 “sensor” remains missing. Recent evidence points to the 56-residue extracellular loop (L2) connecting the second and third transmembrane helices in BlaR1 as the missing trigger (Fig. 1). Phage display experiments with components of B. licheniformis BlaR1 predict an interaction between the L2 loop and BlaRS that is interrupted by the acylation of BlaRS with \( \beta \)-lactam antibiotics (54).

To address this possibility in vivo in the S. aureus BlaR1 system, we have performed site-specific mutagenesis on highly conserved residues within the L2 loop. Mutation of either Pro49 or Pro52 to alanine in the L2 loop was observed to negate inducibility of \( \beta \)-lactamase expression (Table II). In fact, \( \beta \)-lactamase activity was not even detectable in the proline mutants because the cephaloridine concentrations were virtually identical to those of the \( \beta \)-lactamase-negative RN4220 control. This result suggests the disruption of a tonic interaction between BlaRS and the L2 loop that determines basal levels of \( \beta \)-lactamate production, as was observed for the wild-type ZRI transformant, which hydrolyzed some cephaloridine in the absence of inducer. These prolines constitute part of a conserved PXXP sequence motif located at the N terminus of the L2 loop. We propose that the conformational rigidity inherent to proline residues serves to anchor the L2 loop appropriately for interaction with and regulation of the sensor domain. The lack of apparent conformational differences between the apo- and acylated forms of BlaRS supports the prediction that the L2 loop is responsible for modulation of BlaR1 activity by interaction with the BlaRS active site. Indeed, integrity of the L2 loop appears to be required for transmitting the \( \beta \)-lactam-binding signal and de-repressing \( \beta \)-lactamase expression. We have also observed that the fusion of either a Myc or hexahistidine tag to the C terminus of BlaR1 confers a phenotype of constitutive high level \( \beta \)-lactamate expression (Table II), perhaps because this tag interferes with the binding of the L2 loop to the BlaR1 sensor domain.

The first structures of the sensor domain of apo- and acyl-BlaR1 from S. aureus have provided mechanistic predictions that can be probed further using active-site mutants. Of special import is concluding whether or not carboxylation of Lys392 is required for acylation because this detail will clarify the PBP import is concluding whether or not carboxylation of Lys392 is required for acylation because this detail will clarify the PBP import is concluding whether or not carboxylation of Lys392 is required for acylation because this detail will clarify the PBP import is concluding whether or not carboxylation of Lys392 is required for acylation because this detail will clarify the PBP import is concluding whether or not carboxylation of Lys392 is required for acylation because this detail will clarify the PBP activity of BlaRS. The absence of a conformational change upon acylation of BlaRS strongly suggests that the L2 loop plays an integral role as a molecular trigger in signal transduction. This idea is supported by in vivo site-directed mutagenesis of the L2 loop. From the perspective of structure-based drug design, a detailed description of this interaction could inspire new drug leads. In the context of the entire BlaR1 signal transduction process, many questions remain that would clearly benefit from future structural studies. An understanding of the regulation machinery of \( \beta \)-lactam resistance not only will provide the possibility of artificially down-regulating resistance in dangerous pathogens such as S. aureus, but will resolve a novel and fascinating mode of regulating protein expression.

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Crystal Structures of the Apo and Penicillin-acylated Forms of the BlaR1 β-Lactam Sensor of Staphylococcus aureus
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