Shared Functional Attributes between the *mecA* Gene Product of *Staphylococcus sciuri* and Penicillin-Binding Protein 2a of Methicillin-Resistant *Staphylococcus aureus*[†]

Cosimo Fuda, Maxim Suvorov, Qicun Shi, Dusan Hesek, Mijoon Lee, and Shahriar Mobashery*

Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

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ABSTRACT: The genome of *Staphylococcus aureus* is constantly in a state of flux, acquiring genes that enable the bacterium to maintain resistance in the face of antibiotic pressure. The acquisition of the *mecA* gene from an unknown origin imparted *S. aureus* with broad resistance to β-lactam antibiotics, with the resultant strain designated as methicillin-resistant *S. aureus* (MRSA). Epidemiological and genetic evidence suggests that the gene encoding PBP 2a of MRSA might have originated from *Staphylococcus sciuri*, an animal pathogen, where it exists as a silent gene of unknown function. We synthesized, cloned, and expressed the *mecA* gene of *S. sciuri* in *Escherichia coli*, and the protein product was purified to homogeneity. Biochemical characterization and comparison of the protein to PBP 2a of *S. aureus* revealed them to be highly similar. These characteristics start with sequence similarity but extend to biochemical behavior in inhibition by β-lactam antibiotics, to the existence of an allosteric site for binding of bacterial peptidoglycan, to the issues of the sheltered active site, and to the need for conformational change in making the active site accessible to the substrate and the inhibitors. Altogether, the evidence strongly argues that the kinship between the two proteins is deep-rooted on the basis of many biochemical attributes quantified in this study.

Antimicrobial resistance entails the continuous change of a flexible bacterial genome that in the face of the antibiotic pressure moves inexorably toward better fitness. *Staphylococcus aureus* exemplifies this change, thriving as a result of its considerable genomic plasticity with respect to antibacterial resistance mechanisms. The quintessential example is the acquisition of the *mecA* gene, which set in motion the emergence of methicillin-resistant *S. aureus* (MRSA)[1] in the early 1960s (*1*). The clinical difficulty with this organism escalated when variants of MRSA became resistant to vancomycin, one of the last resort antibiotics in treatment of the organism (*2*, *3*). Major resistance to vancomycin emerged when the enterococcal *vanA* gene was acquired by MRSA.

The acquisition of the non-native *mecA* gene by *S. aureus* has occurred several times over the past 40 years from an unknown species (*4*, *5*). The search for this original gene led to a structural homologue of *mecA* as a native gene in the animal commensal species *Staphylococcus sciuri* (*6*−*8*). Different variants of the *mecA* homologue (*mecA1* and *mecA2*) have been detected in both methicillin-resistant and -susceptible isolates, suggesting that the homologue of the *mecA* gene in *S. sciuri* might serve distinct and yet unknown functions in this organism, representing a natural reservoir of the methicillin resistance gene (*6*, *8*−*11*). There are several lines of evidence that indicate that *mecA* of *S. sciuri* might be the predecessor to *mecA* of MRSA. The *mecA* gene of *S. aureus* and the *mecA* homologue of *S. sciuri* both encode a protein with a predicted transpeptidase domain that has the typical sequence of high-molecular weight penicillin-binding proteins (*12*). Introduction of the *mecA* homologue from methicillin-resistant *S. sciuri* into *S. aureus* produced quantities of the requisite protein that reacted with monoclonal antibodies prepared against PBP 2a of MRSA (*13*). The argument became even more compelling when a *mecA* homologue from a methicillin-resistant strain of *S. sciuri* was introduced into a susceptible strain of *S. aureus* and strengthened antibiotic resistance (*14*). However, the similarities between the two *mecA* genes are counterbalanced by differences as well, the most profound being the ubiquitous presence of the *mecA* homologue in all genetically and epidemiologically unrelated isolates of *S. sciuri* (*6*). This is in contrast to *mecA* of *S. aureus*, which is found only in resistant strains. Moreover, the *mecA* homologue is believed to perform an unknown physiological function in *S. sciuri* unrelated to antibiotic resistance (*6*, *11*). It is likely that *S. sciuri* in its natural environment is not challenged by β-lactam antibiotics.

These tantalizing observations at the genetic level argue for the kinship of the two genes; however, biochemical characterization necessary for the demonstration of the

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* To whom correspondence should be addressed: Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556. Telephone: (574) 631-6652. Fax: (574) 631-2933. E-mail: mobashery@nd.edu.

[1] Abbreviations: CD, circular dichroism; IPTG, isopropyl β-D-thiogalactoside; LB, Luria-Bertani; MRSA, methicillin-resistant *S. aureus*; NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramic acid; PBP 2A, penicillin-binding protein 2a; PCR, polymerase chain reaction.

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similarity between the two at the protein level is lacking at present. We recently reported on cloning, purification, and kinetic analyses of the interactions of β-lactam antibiotics with PBP 2a of MRSA to document the origins of its unfavorable inhibition by these antibiotics (*15*). Furthermore, we have documented that the protein has an allosteric site for binding of the cell wall and that binding cell wall fragments to the allosteric site allows the active site of the enzyme to become more accessible to the β-lactam antibiotics (*16*, *17*). Both binding of the β-lactam antibiotics to the active site and that of the cell wall to the allosteric site are prompted by conformational changes in the protein. On the basis of the consideration that the crystal structure of PBP 2a of *S. aureus* exhibits a sheltered active site, we have argued that the conformational change is necessary to make the active site accessible to the cell wall substrate for the physiological function of this protein in vivo (*15*, *16*).

We report herein the cloning, expression, and purification of the gene product of *mecA* from *S. sciuri*. We also describe a homology-based computational structure for the protein. We present evidence in this report that suggests many of the aspects of the biochemical properties of PBP 2a of *S. aureus* are shared with its counterpart from *S. sciuri*. At a quantitative level, the kinetic parameters for the aforementioned interactions are quite similar in both proteins. The biochemical evidence documents the direct kinship between the two *mecA* gene products. The argument for the possibility of the transfer of the *mecA* gene from the animal commensal species *S. sciuri* to the human pathogen *S. aureus* would appear plausible at both the genetic and biochemical level of collective knowledge.

EXPERIMENTAL PROCEDURES

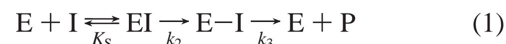
Cloning of the mecA Gene of S. sciuri. The sequence for the *mecA* gene of *S. sciuri* ATCC29062 was optimized for *Escherichia coli* codon usage using Protein2DNA (DNA2, Inc.) software. The optimized gene was synthesized by EZBioLAB with the gene missing the sequence for the 24-amino acid N-terminal membrane anchor. The sites for the restriction endonucleases *Nco*I and *Xho*I were added at opposite ends of the sequence for cloning purposes. The synthetic *mecA* gene was cloned into the *Sma*I site of the pUC57 plasmid. The resulting construct was used to transform *E. coli* JM83, and cells were grown overnight on LB agar plates. Plasmids were isolated from overnight colonies. The fragment of the plasmid containing the truncated *mecA* was excised from the original construct using *Nco*I and *Hin*dIII endonucleases and cloned into the *Nco*I and *Hin*dIII sites of the pET24d(+) vector, and *E. coli* JM83 was transformed by this plasmid. Both strands of the entire *mecA* gene from four transformants were sequenced by primer walking, starting with the M13 universal and reverse primers.

Expression of the S. sciuri MecA Protein. E. coli BL21-(DE3) was transformed with the *mecA* gene of *S. sciuri* contained within the multiple cloning site of the pET24d-(+) plasmid. An overnight seed culture (3 mL) was used to inoculate 500 mL of LB medium supplemented with kanamycin (30 μg/mL), and cells were grown at 37 °C with shaking (120 rpm) until the optical density at 600 nm (OD_{600}) reached ~0.8 (~8 h). Expression was induced within the bacterial culture by addition of 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG), followed by incubation at 25 °C for an additional 18 h. Cells were harvested by centrifugation at 14000*g* for 20 min at 4 °C, and the pellet was resuspended in 10 mM Tris-HCl buffer (pH 8) (buffer A).

Purification of the MecA Protein of S. sciuri. The MecA protein was purified using a two-step purification protocol with an LP chromatography system (Amersham) at 4 °C. Cells were disrupted by 30 cycles of sonication (20 s of burst and 20 s of rest for each cycle). The resulting supernatant was centrifuged at 14000*g* for 30 min using a Beckman-Coulter centrifuge. Pelletting, suspension in buffer A, and sonication were repeated twice to optimize the yield. The resulting cell-free extract was loaded at 2 mL/min onto a Q-Sepharose column (2.5 cm × 20 cm; 80 mL of High Q support resin, Bio-Rad) equilibrated with buffer A. The column was washed with 200 mL of buffer and was eluted with a linear gradient from 0 to 0.3 M NaCl in buffer A at a rate of 2 mL/min (total volume of 400 mL). The MecA protein was eluted at ~0.08−0.12 M NaCl.

The fractions containing the protein were combined and concentrated, and the buffer was exchanged with 50 mM sodium phosphate and 0.2 M NaCl (pH 7.0) (buffer B). The sample was loaded at a rate of 1.0 mL/min onto a Sepharose column (2.5 cm × 50 cm; 160 mL of High S support resin, Bio-Rad) equilibrated with buffer B. The protein was eluted with a linear gradient from 0.2 to 1.0 M in buffer B at a rate of 2 mL/min to a final volume of 1500 mL. The MecA protein eluted from the column at ~0.4−0.6 M NaCl. The fractions were combined, concentrated, and dialyzed against 25 mM Hepes in 1 M NaCl (pH 7.0). The protein concentration was determined with the BCA protein assay kit (Pierce). The yield from a 500 mL cell culture was ~10 mg.

Determination of the Kinetic Parameters for Interactions of β-Lactam Antibiotics with the MecA Protein of S. sciuri in the Absence and Presence of a Cell Wall Fragment. The kinetic parameters for interactions of β-lactam antibiotics with the MecA protein were determined by known methods (*15*, *18*). The MecA protein experiences acylation at the active site serine (Ser401), and the acyl−enzyme species slowly undergoes deacylation according to eq 1.



$$E + I \underset{K_S}{\rightleftarrows} EI \underset{k_2}{\rightarrow} E{-}I \underset{k_3}{\rightarrow} E + P \quad (1)$$

where E represents the MecA protein, EI is the noncovalent pre-acylation complex, E−I is the covalent acyl−enzyme species, and P is the product of hydrolysis of the β-lactam antibiotic. The first-order rate constants for protein acylation with different β-lactam antibiotics were determined using a Cary 50 UV spectrophotometer (Varian Inc.) at room temperature (~25 °C). The experiments were carried out in 25 mM Hepes and 1 M NaCl (pH 7.0). The reaction between the MecA protein and nitrocefin was assessed by monitoring the formation of the acyl−enzyme species at 500 nm ($\Delta\epsilon_{500}$ = 15 900 cm^{-1} M^{-1}). The observed first-order rate constants (k_{obs}) were measured at a protein concentration of 3.0 μM with different concentrations of nitrocefin (20−500 μM). Each was monitored for 45 min, at which time the protein was invariably acylated. Nitrocefin (80 μM) was used as the reporter molecule to determine the apparent first-order rate constants for acylation by other nonchromogenic (or poorly

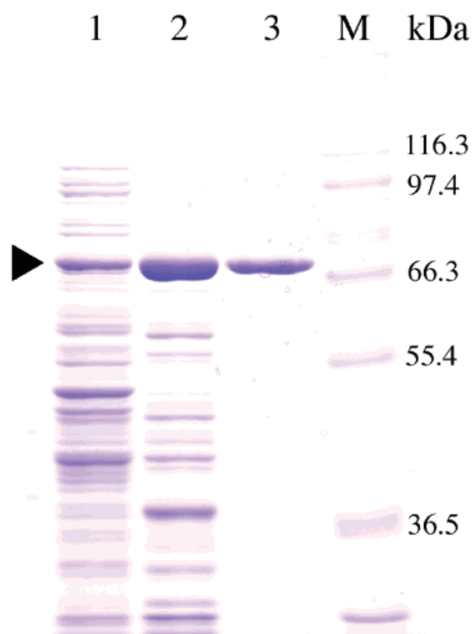


FIGURE 1: SDS−PAGE (8.5%) analysis during the purification of the MecA protein: lane 1, crude supernatant; lane 2, after the Q-Sepharose column; lane 3, after the S-Sepharose column; lane M, molecular weight ladder. The arrowhead indicates the position of the MecA protein.

chromogenic) β-lactams at several concentrations in competition experiments.

The observed first-order rate constants (k_{obs}) were also measured for the MecA protein of *S. sciuri* (3 μM) with several concentrations of nitrocefin (10−120 μM) in the presence of cell wall surrogate compound **1** (0.5−2 mM) using the same parameters that were described above.

The deacylation rate constants for the MecA protein were determined using BOCILLIN FL (Molecular Probes, Inc.) as a reporter molecule (compound **2** shown in Figure 3), both in the absence and in the presence of cell wall surrogate compound **1**. A typical reaction mixture (60 μL) contained 20 μM MecA and a β-lactam antibiotic concentration at least 2-fold higher than its K_d value. The mixture was incubated at room temperature for 45 min in 25 mM Hepes and 1 M NaCl (pH 7.0). The excess β-lactam was removed by passing the mixture through a Micro Bio-Spin6 column (Bio-Rad). For experiments performed in the presence of compound **1**, it (0.5−2.0 mM) was added to the resultant mixture immediately following the removal of excess antibiotic by the aforementioned step. An aliquot (3 μL) of the mixture was diluted 5-fold with the same buffer and incubated at room temperature for different time intervals. The amount of free protein, liberated from the acyl−protein species, was assayed by the addition of BOCILLIN FL to give a final concentration of 50 μM and incubated for an additional 45 min at room temperature. The SDS sample buffer (15 μL) was added to the reaction mixture, which was then boiled for 3 min. The samples (30 μL in total) were loaded onto a 10% SDS−polyacrylamide gel, which was developed and then scanned using a Storm840 Fluorimager.

Circular Dichroic Measurements. The circular dichroic (CD) spectra of MecA [9 μM in 50 mM KH_2PO_4 and 0.1 M NaCl (pH 7.5)] were recorded in the absence and presence of 30 μM oxacillin or 30 μM ceftazidime at 25 °C on a stopped-flow circular dichroism spectrometer (AVIV Instruments Inc. 202 SF) with a 2 mm path length. The CD readings for the β-lactams were negligible compared with those of the protein and were subtracted in each case.

The CD spectra of MecA [9 μM in 50 mM KH_2PO_4 and 0.1 M NaCl (pH 7.5)] were recorded in the absence and presence of compound **1** in incremental amounts (from 0.5 to 2 mM) at 25 °C. The spectrophotometric contribution of compound **1** was subtracted in each case.

The K_d^P parameter for the interaction between the MecA homologue and compound **1**, assuming a 1:1 complex, was determined using CD spectra by expressing the change in molar ellipticity at 222 nm as a function of the degree of saturation α. The degree of saturation is defined by the relation $\alpha = \Delta\Theta/\Delta\Theta_{max}$, where $\Delta\Theta$ is the change in ellipticity of the MecA protein in the presence of a subsaturating amount of ligand (compound **1**) and $\Delta\Theta_{max}$ is the change in ellipticity of the MecA protein in the presence of a saturating amount of ligand. The dissociation constant for the peptidoglycan (K_d^P) is determined from the slope ($1/K_d^P$) of the plot of $1/(1 - \alpha)$ versus [ligand]/α (*19*).

Homology Model Construction and Molecular Dynamics Simulations. The amino acid sequences for *mecA* of *S. sciuri* (target) and *mecA* of *S. aureus* (template) were analyzed with Swiss-Model (a protein homology modeling server) to determine the initial geometry of the target sequence and its quantitative homology to the known template structure (*20*). The structural coordinates for PBP 2a, the *mecA* gene product of MRSA, were obtained from the Protein Data Bank (entry 1VQQ). This template amino acid sequence was chosen due to its high level of identity in sequence (∼87%) to the template. The target structure was modeled in standard PDB format, comprised of heavy atoms (i.e., C, O, N, and S), while the addition of light atoms (i.e., hydrogen) was carried out using the protonation package in the Amber8 Molecular Dynamics Program. The protein was encapsulated by TIP3P water molecules within a cubic box of ∼94 Å × 85 Å × 159 Å. The distance from the protein surface to the closest face of the box was least 10 Å. Following this step, the Amber xLEaP program was used to generate topological and geometrical information for the target structure. The topology calculations integrated charges, force fields, connectivity, and other essential information for molecular dynamics simulations.

The molecular dynamics protocol has been reported by us previously (*21*). With atoms restrained in harmonic potential, the structure was energy minimized within 5000 steps in Cartesian space using the steepest descent method. This was followed by 4 ps of molecular dynamics at constant pressure (1 atm), where the temperature was increased from 0 to 300 K. Subsequently, six steepest descent energy minimizations, each consisting of 1000 steps, were carried out by gradually relaxing the restraints. The fully relaxed system was then subjected to three molecular dynamics runs, 12 ps while heating from 0 to 100 K and 4 ps equilibrated at 100 K. Similar procedures were applied for temperatures ranging between 100−200 and 200−300 K. After equilibration, 10 iterative simulations were carried out at 200 ps each, with the averaged structure from each simulation used as the basis for the next simulation. Coordinates were sampled every 0.2 ps.

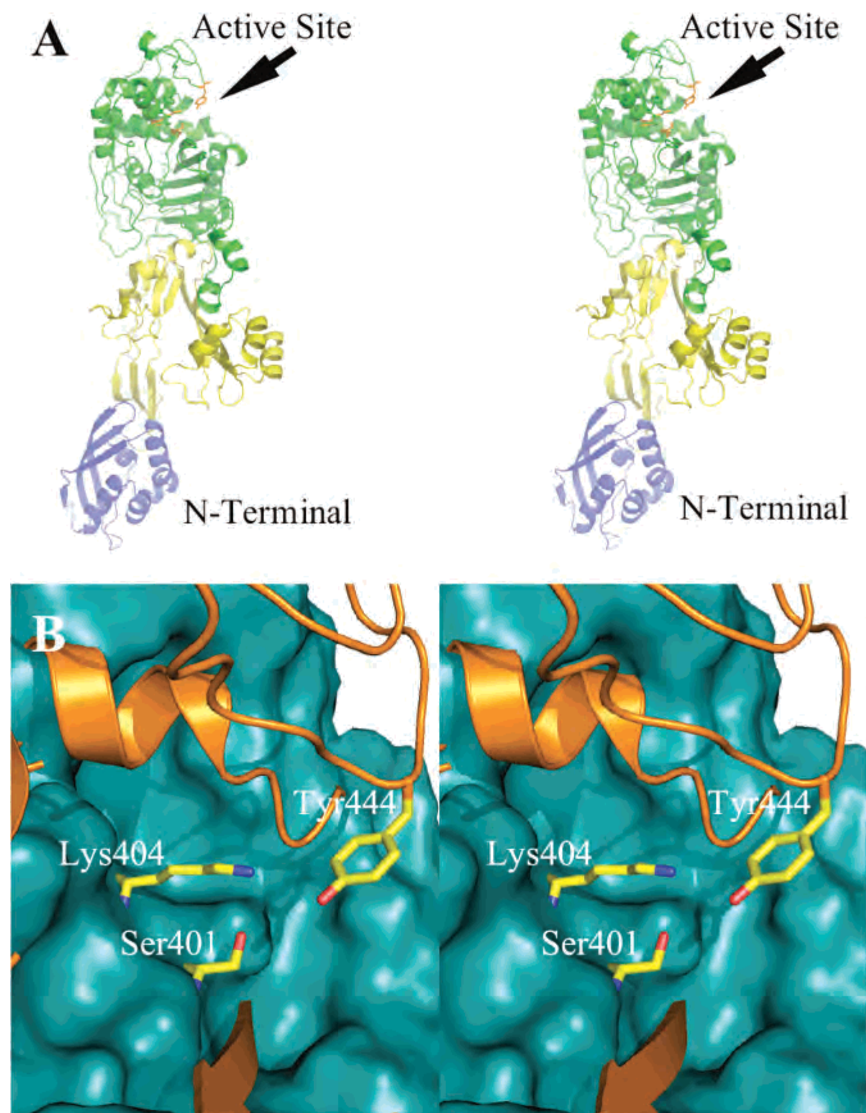


FIGURE 2: (A) Stereoview of the ribbon representation for the homology-based three-dimensional structural model of MecA of *S. sciuri*. The structure is colored green for the transpeptidase domain (Gly343−Asp656), yellow for the non-penicillin-binding domain (Pro145−Ile312), purple for the N-terminal extension domain (Ala23−Gln139), and orange for active site (indicated by arrows) residues Ser401, Lys404, and Tyr444. (B) Stereoview of the active site of MecA of *S. sciuri* showing the Ser-Lys dyad (Ser401 and Lys404) and Tyr444. The side chains of the active site residues are shown in capped-stick representation with colors according to atom type (C, N, and O colored yellow, blue, and red, respectively). A Connolly solvent accessible surface is constructed around the active site.

Table 1: Kinetic Parameters for Interactions of β-Lactam Antibiotics with the MecA Protein of *S. sciuri* and with PBP 2a of MRSA

	S. sciuri				*S. aureus*			
cephalosporin	k_2 ($\times 10^3$ s^{-1})	k_3 ($\times 10^6$ s^{-1})	K_d (μM)	k_2/K_d (M^{-1} s^{-1})	k_2 ($\times 10^3$ s^{-1})	k_3 ($\times 10^6$ s^{-1})	K_d (μM)	k_2/K_d (M^{-1} s^{-1})
nitrocefin	16 ± 1	4.0 ± 0.1	142 ± 21	114 ± 16	3.7 ± 0.3	7.2 ± 0.1	192 ± 24	19 ± 3.0
cefepime	9 ± 1	5.6 ± 1.0	1750 ± 250	6 ± 1	1.5 ± 0.1	5.9 ± 0.5	1618 ± 145	0.9 ± 0.1
ceftazidime	6 ± 1	4.4 ± 0.1	1100 ± 115	6 ± 1	1.0 ± 0.1	3.2 ± 0.2	671 ± 116	1.5 ± 0.3
ampicillin	15 ± 2	8.0 ± 1.0	475 ± 80	32 ± 6	3.4 ± 0.1	3.2 ± 0.1	668 ± 124	5.0 ± 1.0
oxacillin	5 ± 1	7.5 ± 0.3	450 ± 90	12 ± 3	1.6 ± 0.1	2.5 ± 0.1	180 ± 25	9.0 ± 1.0
imipenem	4 ± 1	7.5 ± 0.4	500 ± 30	8 ± 2	1.7 ± 0.1	3.3 ± 0.3	603 ± 93	2.8 ± 0.4

RESULTS AND DISCUSSION

A host of microbiological experiments have argued that PBP 2a proteins from *S. sciuri* and *S. aureus* should be related to each other (*5*, *9*, *11*, *13*, *14*). The high degree of amino acid sequence identity also provided support for the assertion, yet structural and biochemical information about the *mecA* product from *S. sciuri* has been missing from the literature. To provide such information for this protein, and specifically to compare and contrast these properties to those of the protein from *S. aureus*, we undertook this study.

To accomplish this task, the protein had to be cloned, expressed, and purified. Cloning of the 1998-nucleotide *mecA* gene of *S. sciuri* into an expression vector proved to be a difficult task. Initially, the *mecA* gene from *S. sciuri* was PCR-amplified from the chromosomal DNA of *S. sciuri* ATCC29062 without the sequence for the 24-amino acid N-terminal membrane anchor. The gene was cloned into the

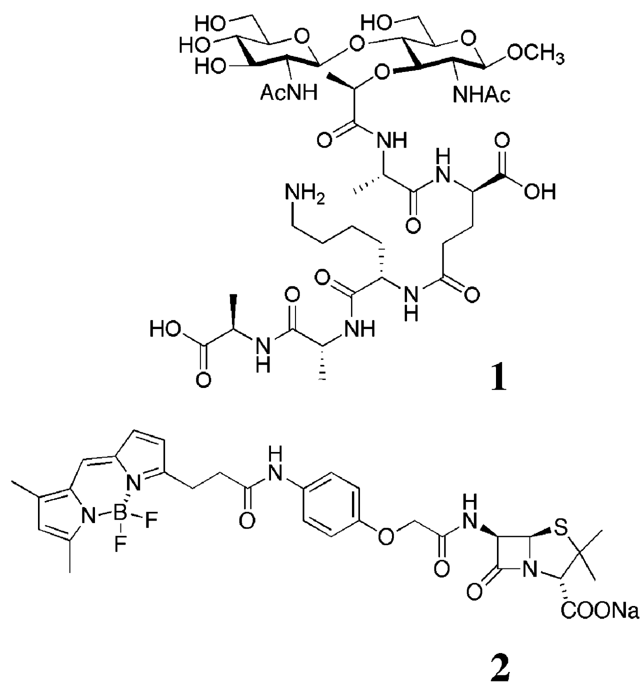


FIGURE 3: Compound **1** is a disaccharide-containing surrogate of the bacterial peptidoglycan. Compound **2** is the structure of BOCILLIN FL.

pET24d(+) expression vector under an IPTG-inducible promoter. Conditions for optimizing expression were investigated; however, either the MecA protein failed to be expressed outright, or the levels of expression were never sufficient for practical purification. We tried to simplify the process by recloning the gene into pET24d(+) with a histidine tag to purify the protein using affinity chromatography. However, the improvement in yield was minimal, and purification using affinity chromatography still could not result in homogeneous protein.

We concluded that the levels of expression were likely depressed because of the disparity of the codon usage between the staphylococci and *E. coli*. Hence, the *mecA* gene of *S. sciuri* was optimized for *E. coli* codon usage, and the rather large gene was synthesized without the portion for the 24-amino acid N-terminal membrane anchor and ultimately cloned into pET24d(+). This approach led to success. The enzyme was produced intracellularly upon induction with IPTG, with dramatically improved expression levels. The MecA protein was purified to apparent homogeneity in two chromatographic steps (Figure 1). We typically produce ∼20 mg of the protein from a liter of cell culture.

We originally had anticipated expression levels of the MecA protein of *S. sciuri* would be comparable to that of PBP 2a of *S. aureus*. This expectation was based on high degrees of nucleic acid and amino acid sequence similarity between the two proteins (*22*) and the fact that recombinant PBP 2a of MRSA was expressed well in *E. coli* and was easily purified (*15*). These expectations were wrong, and the differences in expression of the two genes proved to be problematic. Coincidentally, the *mecA* gene is present in all genetically and epidemiologically unrelated isolates of *S. sciuri* but poorly expressed in antibiotic-susceptible strains (*6*). Furthermore, the physiological role of the MecA protein of *S. sciuri* is currently uncharacterized, although it is capable of participating in cell wall synthesis (*5*, *14*). This differs from the case of PBP 2a of MRSA, which is expressed well in vivo and plays an integral role in cell wall synthesis in the presence of β-lactam antibiotics (*23*, *24*).

Table 2. Kinetic Parameters for Interaction of Nitrocefin with the MecA Protein of *S. sciuri* in the Absence and Presence of Compound **1**

[**1**] (mM)	k_2 ($\times 10^3$ s^{-1})	k_3 ($\times 10^6$ s^{-1})	K_d (μM)	k_2/K_d (M^{-1} s^{-1})
0	16 ± 1	4.0 ± 0.1	142 ± 21	114 ± 16
0.5	18 ± 1	8.0 ± 0.6	122 ± 20	150 ± 22
1.0	22 ± 1		98 ± 13	230 ± 30
1.5	28 ± 4		80 ± 15	360 ± 75
2.0	36 ± 1	16 ± 1	60 ± 6	604 ± 55

The high degrees of sequence similarity between the MecA proteins of *S. sciuri* and *S. aureus* argue for structural similarities between the two proteins. However, in the absence of a three-dimensional structure for the former, we used the X-ray structure of the latter (*25*) to generate a computational homology model for the MecA protein of *S. sciuri*. The MecA protein of *S. sciuri* consists of 666 amino acid residues. The structure modeled for the *MecA* protein of *S. sciuri* includes three domains, as shown in Figure 2A. They include the N-terminal extension domain (Ala23−Gln139, colored purple), the non-penicillin-binding (nPB) domain (Pro145−Ile312, colored yellow), and the transpeptidase domain (Gly343−Asp656, colored green). The overall dimensions of the protein are ∼140 Å × 64 Å × 56 Å, close to the dimensions of PBP 2a of *S. aureus* (*25*). The resulting rms deviation for the backbone between the two structures is 2.6 Å. The transpeptidase domains of the two MecA proteins share a similar overall fold. The active site of the protein appears to be as sheltered like that of PBP 2a of *S. aureus*. This is due to the loop that lowers itself onto the active site, on which residue Tyr444 is located (Figure 2B). The centrally located Tyr444 essentially caps the active site, making access to it difficult by both the substrate and inhibitors, a feature also seen in the X-ray structure of PBP 2a of *S. aureus* (*25*).

Structural information about the MecA protein of *S. sciuri* compelled us to characterize the protein by biochemical analyses. To this end, we examined the kinetics of interaction of the MecA protein of *S. sciuri* with β-lactam antibiotics as covalent inhibitors to elucidate what mechanistic aspects it shares with PBP 2a of MRSA. β-Lactam antibiotics also acylate the enzyme active site serine (Ser401), rendering the enzyme inactive and forming a complex that is irreversible for all practical purposes. We evaluated the kinetics of interactions with three cephalosporins (nitrocefin, cefepime, and ceftazidime), two penicillins (ampicillin and oxacillin), and one carbapenem (imipenem) with MecA of *S. sciuri*, comparing them side by side in Table 1 with the corresponding values for PBP 2a of MRSA (*15*).

As in the case of PBP 2a of MRSA, the MecA protein of *S. sciuri* has a poor rate of acylation (k_2 effect) with a low affinity for β-lactams in general. The $t_{1/2}$ for acylation of the MecA protein was in the range of 0.5−3 min with the antibiotics that were tested, compared to 3−12 min with PBP 2a of MRSA (*15*). The dissociation constants for the pre-acylation complexes ranged between 142 and 1750 μM, resulting in second-order rate constants (k_2/K_d) of 6−114 M^{-1} s^{-1}. This is compared to dissociation constants in the range of 180−1618 μM and second-order rate constants (k_2/K_d) of

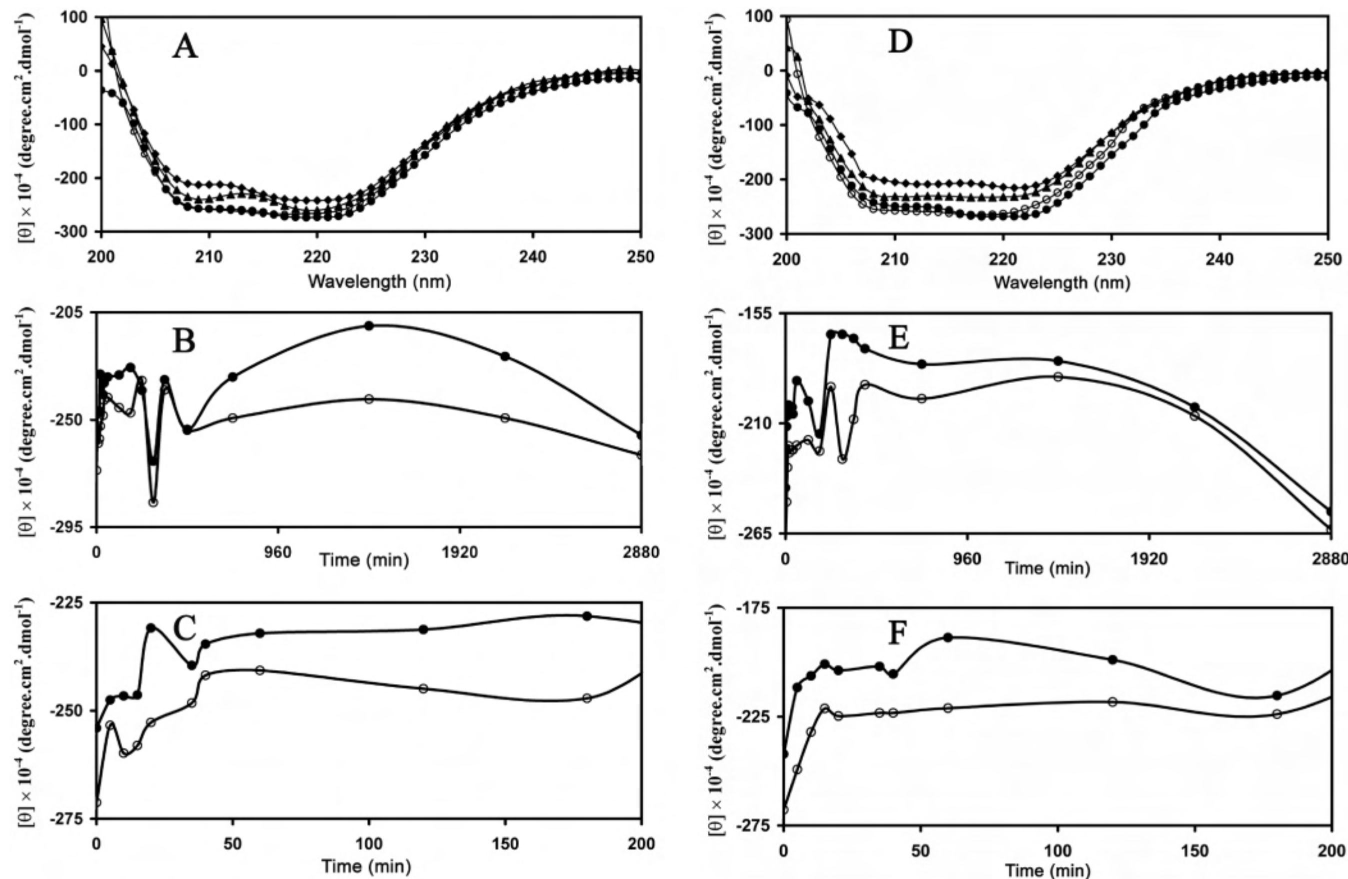


FIGURE 4: (A) Far-UV circular dichroic spectrum of MecA of *S. sciuri* [9 μM (●)], during interaction with oxacillin at 1 (▲), 24 (◆), and 48 h (○). The lines simply connect the data points and were not fit to any specific model. (B) Change in the molar ellipticity of MecA of *S. sciuri* at 208 (●) and 222 nm (○) as a function of time (2880 min) in the presence of oxacillin (30 μM). (C) Change in the molar ellipticity of MecA of *S. sciuri* at 208 (●) and 222 nm (○) over the shorter duration (200 min) for oxacillin (30 μM). (D) Far-UV circular dichroic spectrum of MecA of *S. sciuri* [9 μM (●)], during interactions with ceftazidime at 1 (▲), 24 (◆), and 48 h (○). The lines simply connect the data points and were not fit to any specific model. (E) Change in the molar ellipticity of MecA of *S. sciuri* at 208 (●) and 222 nm (○) as a function of time (2880 min) in the presence of ceftazidime (30 μM). (F) Change in the molar ellipticity of MecA of *S. sciuri* at 208 (●) and 222 nm (○) over the shorter duration (200 min) for ceftazidime (30 μM).

1−19 M^{-1} s^{-1} for PBP 2a. It is important to note that evolution of PBP 2a of *S. aureus* (presumably from that of *S. scuiri*) would appear to have gone in the direction of poorer interaction with these clinically used antibiotics, as would be intuitively reasonable. The acylation rate constants (k_2) and the second-order rate constants for inhibition (k_2/K_d) are overall poorer for PBP 2a of *S. aureus*. Again, like the case for PBP 2a of MRSA, the rate constants for deacylation (k_3) of the acyl−enzyme species were exceedingly poor, with $t_{1/2}$ values in the range of 26−48 h, compared to 26−77 h for PBP 2a. The side-by-side comparison of the kinetic parameters for MecA of *S. sciuri* and PBP 2a of MRSA in Table 1 reveals both enzymes behaving remarkably similarly in the presence of β-lactams. The dual causes of failure of effective inhibition of these proteins by the β-lactam antibiotics are the depressed k_2 values and elevated dissociation constants (K_d). The basis for both these manifestations is the closed nature of the active sites. For either the peptidoglycan substrate or the β-lactam inhibitor to gain access to the active site, the protein should open up the active site by a conformational change.

We have argued that the closed conformation of PBP 2a was advantageous to the organism in the face of the challenge by β-lactam antibiotics, yet the enzyme still had to perform its catalytic function with the peptidoglycan substrate or would be of no use to the organism. To rationalize this apparent anomaly, we explored the possibility of the existence of an allosteric site outside the active site of PBP 2a of *S. aureus* that could be triggered by the peptidoglycan to open up the active site (*16*). A synthetic peptidoglycan fragment prepared in our laboratory, the structure of which (compound **1**) is shown in Figure 3, proved of use (*26*, *27*). We documented that binding of the peptidoglycan at the allosteric site of the *S. aureus* protein triggered a conformational change that made the active site more accessible to the β-lactam inhibitors. The allosteric site would appear to be present also in the MecA protein from *S. sciuri*.

Compound **1** (Figure 3) is an analogue of a fragment of the *S. sciuri* cell wall, which possesses the *N*-acetylglucosamine (NAG)−*N*-acetylmuramic acid (NAM) disaccharide backbone of the cell wall. To the NAM residue is appended the pentapeptide (NAM-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala) linker unit that is typical of *S. sciuri*. While *S. aureus* has a pentaglycyl moiety appended to the L-Lys side chain that participates in the PBP 2a-catalyzed cross-linking reaction, *S. sciuri* has a pentapeptide chain comprised of one alanine and four glycine residues (*14*). The lysine-appended pentapeptide was intentionally left out of the structure, as we did not want the cell wall fragment to serve as a substrate.

The kinetic parameters for interactions of the MecA protein from *S. scuiri* with nitrocefin in the presence of increasing concentrations of the cell wall surrogate are given in Table 2. Several trends were established in the presence of the cell wall surrogate that are comparable to those observed with PBP 2a of MRSA (*16*). Similar to PBP 2a, the MecA homologue of *S. sciuri* is activated by the cell wall surrogate. As the concentration of the cell wall surrogate increased, the rate constant for acylation by nitrocefin (k_2) became larger and the corresponding dissociation constant (K_d) became smaller (Table 2). Both parameters contributed to the increase in k_2/K_d values for nitrocefin in the presence of increasing concentrations of the cell wall surrogate. Furthermore, a 4-fold enhancement in the rate constants for deacylation (k_3) was also observed in the presence of compound **1**.

We had previously argued that β-lactam antibiotics gain greater accessibility to the active site of PBP 2a in the presence of increasing concentrations of the cell wall surrogate, a consequence of the allosteric binding interaction mentioned earlier (*16*, *17*). The implication is that the allosteric modulator (cell wall) converts the structure of the enzyme from the closed form to the active and open one. The open and closed forms for another PBP have also been documented by crystallography recently (*28*). The MecA protein of *S. sciuri* demonstrates the same behavior, manifested in the enhanced facility of enzyme acylation and the improved stability of the noncovalent preacylation complex observed for the protein and antibiotic.

Although the exact role of the MecA protein in *S. sciuri* remains unknown, the protein's ability to interact with and process peptidoglycan was demonstrated in recent studies, wherein the *mecA* homologue from a methicillin-resistant strain of *S. sciuri* was introduced into a susceptible strain of *S. aureus*, increasing drug resistance and allowing continued growth and cell wall synthesis of bacteria in the presence of high concentrations of antibiotic (*14*). We determined the K_d^P (dissociation constant for peptidoglycan) for interaction of the minimalist cell wall surrogate outside the transpeptidase active site of the MecA protein to be 1.0 ± 0.2 mM, compared to a value of 2.0 ± 0.8 mM for the same compound with PBP 2a of MRSA (*16*). These low millimolar values might be consistent with our previous assertion that PBPs of the bacterial cytoplasmic membrane are exposed to high local concentrations of the cell wall components (an entropic effect) (*27*, *29*).

In addition, we had previously shown conformational changes underlie the interactions of PBP 2a with both β-lactams and cell wall surrogates, which can be detected by circular dichroism (*15*−*17*). We carried out a series of similar studies for the MecA protein of *S. sciuri* to complement our kinetic data. We first incubated the MecA protein with either oxacillin (a penicillin) or ceftazidime (a cephalosporin), monitoring the ensuing conformational changes in the protein at the helical 208 and 222 nm minima (Figure 4A,D). We observed an overall decrease in the α-helix content upon exposure to antibiotic, characterized by a series of conformational changes within the first four $t_{1/2}$ values for acylation. These conformational changes continued for the duration of the monitoring, whereupon the protein returned to the native state after 2 days (Figure 4B,C,E,F). The changes in ellipticity were not as dramatic as those observed with PBP 2a of MRSA, indicating that some qualitative differences between the two proteins exist.

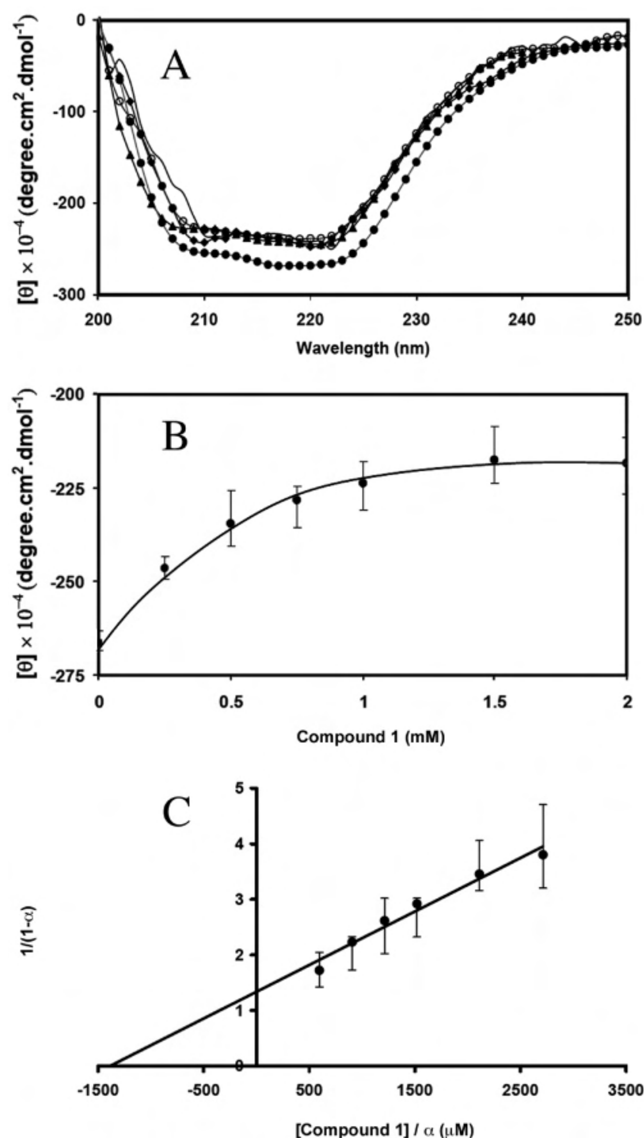


FIGURE 5: (A) Far-UV circular dichroic spectra of MecA of *S. sciuri* [9 μM (●)] in the presence of compound **1** at 0.5 (◆), 1.0 (▲), 1.5 (○), and 2.0 mM (−). The lines connect the data points and were not fit to any specific model. (B) Change in the molar ellipticity of MecA of *S. sciuri* at 222 nm (●) as a function of the concentration of compound **1**. (C) Dissociation constant for peptidoglycan (K_d^P) determined by plotting the change in ellipticity of MecA at 222 nm upon saturation with compound **1**, where $\alpha = \Delta\Theta/\Delta\Theta_{max}$.

We also detected conformational changes for the protein in the presence of the cell wall surrogate (compound **1**) by itself, which mirrored our study with PBP 2a (*16*). The MecA protein undergoes a change in conformation consistent with a decrease in the degree of helicity upon addition of compound **1** (Figure 5A). The process is saturable (Figure 5B) with respect to the cell wall fragment, indicative of the existence of a specific site for its binding. This binding interaction likely initiates the catalytic events in cell wall cross-linking. The dissociation constant for compound **1** was determined to be 1.0 ± 0.4 mM using circular dichroism (Figure 5C), a value identical to the determination from enhancement of the rate constants for acylation (i.e., 1.0 ± 0.2 mM), as described above. We have now shown that the

enzyme has to undergo a conformational change to accommodate the binding of both the β-lactam antibiotic and the peptidoglycan fragment. The changes in the conformation are likely not limited to a mere decrease in helicity, but a more definitive analysis of the structural consequences of binding of the peptidoglycan fragments to MecA should await X-ray analyses of these complexes.

We have documented herein that biochemical properties of the MecA proteins from both *S. aureus* and *S. sciuri* are uncannily similar. These characteristics start with sequence similarity but extend to biochemical behavior in inhibition by β-lactam antibiotics, to the existence of the allosteric site for binding of peptidoglycan, to the issues of the sheltered active site, and to the need for conformational change to make the active site accessible to the substrate and the inhibitors. These behaviors are too similar to be coincidental, and we are of the opinion that the evolutionary kinship for the protein that was surmised by epidemiologic and genetic analyses should be valid. These observations are revealing in that *S. sciuri*, an animal pathogen, could ultimately have shared genetic determinants with *S. aureus* in the evolutionary development of MRSA, a human pathogen that has become a clinical scourge.

REFERENCES

1. Jevons, M., Rolinson, G., and Knox, R. (1961) "Celbenin" resistant staphylococci, *Br. Med. J.*, 124−125.
2. Centers for Disease Control and Prevention (2002) *Staphylococcus aureus* resistant to vancomycin, in *Morbidity and Mortality Weekly Report*, pp 824−825, Centers for Disease Control and Prevention, New York.
3. Centers for Disease Control and Prevention (2004) Vancomycin-resistant *Staphylococcus aureus*, in *Morbidity and Mortality Weekly Report*, pp 322−323, Centers for Disease Control and Prevention, New York.
4. Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H., and Spratt, B. G. (2002) The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA), *Proc. Natl. Acad. Sci. U.S.A. 99*, 7687−7692.
5. Couto, I., Wu, S. W., Tomasz, A., and de Lencastre, H. (2003) Development of methicillin resistance in clinical isolates of *Staphylococcus sciuri* by transcriptional activation of the mecA homologue native to s, *J. Bacteriol. 185*, 645−653.
6. Couto, I., de Lencastre, H., Severina, E., Kloos, W., Webster, J. A., Hubner, R. J., Sanches, I. S., and Tomasz, A. (1996) Ubiquitous presence of a mecA homologue in natural isolates of *Staphylococcus sciuri*, *Microb. Drug Resist. 2*, 377−391.
7. Hauschild, T., and Schwarz, S. (2003) Differentiation of *Staphylococcus sciuri* strains isolated from free-living rodents and insectivores, *J. Vet. Med., Ser. B 50*, 241−246.
8. Kloos, W. E. (1980) Natural populations of the genus *Staphylococcus*, *Annu. Rev. Microbiol. 34*, 559−592.
9. Wu, S., de Lencastre, H., and Tomasz, A. (1998) Genetic organization of the mecA region in methicillin-susceptible and methicillin-resistant strains of *Staphylococcus sciuri*, *J. Bacteriol. 180*, 236−242.
10. Marsou, R., Bes, M., Boudouma, M., Brun, Y., Meugnier, H., Freney, J., Vandenesch, F., and Etienne, J. (1999) Distribution of *Staphylococcus sciuri* subspecies among human clinical specimens, and profile of antibiotic resistance, *Res. Microbiol. 150*, 531−541.
11. Couto, I., Sanches, I. S., Sa-Leao, R., and de Lencastre, H. (2000) Molecular characterization of *Staphylococcus sciuri* strains isolated from humans, *J. Clin. Microbiol. 38*, 1136−1143.
12. Goffin, C., and Ghuysen, J. M. (1998) Multimodular penicillin-binding proteins: An enigmatic family of orthologs and paralogs, *Microbiol. Mol. Biol. Rev. 62*, 1079−1093.
13. Wu, S. W., de Lencastre, H., and Tomasz, A. (2001) Recruitment of the mecA gene homologue of *Staphylococcus sciuri* into a resistance determinant and expression of the resistant phenotype in *Staphylococcus aureus*, *J. Bacteriol. 183*, 2417−2424.
14. Severin, A., Wu, S. W., Tabei, K., and Tomasz, A. (2005) High-level β-lactam resistance and cell wall synthesis catalyzed by the mecA homologue of *Staphylococcus sciuri* introduced into *Staphylococcus aureus*, *J. Bacteriol. 187*, 6651−6658.
15. Fuda, C., Suvorov, M., Vakulenko, S. B., and Mobashery, S. (2004) The basis for resistance to β-lactam antibiotics by penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*, *J. Biol. Chem. 279*, 40802−40806.
16. Fuda, C., Hesek, D., Lee, M., Morio, K., Nowak, T., and Mobashery, S. (2005) Activation for Catalysis of Penicillin-Binding Protein 2a from Methicillin-Resistant *Staphylococcus aureus* by Bacterial Cell Wall, *J. Am. Chem. Soc. 127*, 2056−2057.
17. Fuda, C., Hesek, D., Lee, M., Heilmayer, W., Novak, R., Vakulenko, S., and Mobashery, S. (2006) Mechanistic Basis for the Action of New Cephalosporin Antibiotics Effective against Methicillin- and Vancomycin-resistant *Staphylococcus aureus*, *J. Biol. Chem. 281*, 10035−10041.
18. Graves-Woodward, K., and Pratt, R. F. (1998) Reaction of soluble penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus* with β-lactams and acyclic substrates: Kinetics in homogeneous solution, *Biochem. J. 332*, 755−761.
19. Leskovac, V. (2003) Enzyme Activation, in *Comprehensive Enzyme Kinetics*, pp 111−116, Plenum Publishers, New York.
20. Schwede, T., Kopp, J., Guex, N., and Peitsch, M. C. (2003) SWISS-MODEL: An automated protein homology-modeling server, *Nucleic Acids Res. 31*, 3381−3385.
21. Maveyraud, L., Golemi-Kotra, D., Ishiwata, A., Meroueh, O., Mobashery, S., and Samama, J. P. (2002) High-resolution X-ray structure of an acyl-enzyme species for the class D OXA-10 β-lactamase, *J. Am. Chem. Soc. 124*, 2461−2465.
22. Wu, S., Piscitelli, C., de Lencastre, H., and Tomasz, A. (1996) Tracking the evolutionary origin of the methicillin resistance gene: Cloning and sequencing of a homologue of mecA from a methicillin susceptible strain of *Staphylococcus sciuri*, *Microb. Drug Resist. 2*, 435−441.
23. Pinho, M. G., de Lencastre, H., and Tomasz, A. (2001) An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci, *Proc. Natl. Acad. Sci. U.S.A. 98*, 10885−10891.
24. Pinho, M. G., Filipe, S. R., de Lencastre, H., and Tomasz, A. (2001) Complementation of the essential peptidoglycan transpeptidase function of penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in *Staphylococcus aureus*, *J. Bacteriol. 183*, 6525−6531.
25. Lim, D., and Strynadka, N. C. (2002) Structural basis for the β-lactam resistance of PBP2a from methicillin-resistant *Staphylococcus aureus*, *Nat. Struct. Biol. 9*, 870−876.
26. Hesek, D., Lee, M., Morio, K., and Mobashery, S. (2004) Synthesis of a fragment of bacterial cell wall, *J. Org. Chem. 69*, 2137−2146.
27. Hesek, D., Suvorov, M., Morio, K., Lee, M., Brown, S., Vakulenko, S. B., and Mobashery, S. (2004) Synthetic peptidoglycan substrates for penicillin-binding protein 5 of Gram-negative bacteria, *J. Org. Chem. 69*, 778−784.
28. Lovering, A. L., De Castro, L., Lim, D., and Strynadka, N. C. (2006) Structural analysis of an "open" form of PBP1B from *Streptococcus pneumoniae*, *Protein Sci. 15*, 1701−1709.
29. Lee, M., Hesek, D., Suvorov, M., Lee, W., Vakulenko, S., and Mobashery, S. (2003) A mechanism-based inhibitor targeting the DD-transpeptidase activity of bacterial penicillin-binding proteins, *J. Am. Chem. Soc. 125*, 16322−16326.

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