Characterization of the β-Lactam Antibiotic Sensor Domain of the MecR1 Signal Sensor/Transducer Protein from Methicillin-Resistant *Staphylococcus aureus*†

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ABSTRACT: Methicillin-resistant *Staphylococcus aureus* (MRSA) has evolved two mechanisms for resistance to β-lactam antibiotics. One is production of a β-lactamase, and the other is that of penicillin-binding protein 2a (PBP 2a). The expression of these two proteins is regulated by the *bla* and *mec* operons, respectively. BlaR1 and MecR1 are β-lactam sensor/signal transducer proteins, which experience acylation by β-lactam antibiotics on the cell surface and transduce the signal into the cytoplasm. The C-terminal surface domain of MecR1 ($MecR^S$) has been cloned, expressed, and purified to homogeneity. This protein has been characterized by documenting that it has a critical and unusual N^{ζ}-carboxylated lysine at position 394. Furthermore, the kinetics of interactions with β-lactam antibiotics were evaluated, a process that entails conformational changes for the protein that might be critical for the signal transduction event. Kinetics of acylation of $MecR^S$ are suggestive that signal sensing may be the step where the two systems are substantially different from one another.

Staphylococci are important human pathogens in community-acquired and nosocomial infections (*1*). Resistance to antibiotics among the staphylococci, including to β-lactams (penicillins and cephalosporins), is common. Methicillin-resistant *Staphylococcus aureus* (MRSA) is resistant to all available β-lactam antibiotics, and the recently emerged vancomycin-resistant *S. aureus* (VRSA) is among the most serious pathogens (*2*−*4*).

The mechanisms of resistance of *S. aureus* to β-lactams are complex but largely due to the functions of the *blaZ* and *mecA* genes. The former encodes the β-lactamase that hydrolytically destroys the β-lactam antibiotics, and the latter encoded penicillin-binding protein 2a (PBP 2a). PBP 2a is not inhibited well by β-lactam antibiotics and, hence, is able to perform its physiological role of cross-linking the cell wall in the face of the challenge by the antibiotic (*5*−*8*). These two genes are regulated by the β-lactam sensor/signal transducer proteins BlaR1 and MecR1 for β-lactamase and PBP 2a, respectively. Each β-lactam sensor/signal transducer and its repressor gene (*blaI* and *mecI*, respectively) are located immediately upstream of the structural gene (*blaZ* or *mecA*) and are transcribed in the opposite directions (*9*). Repressor binds to the palindromic sites within the *bla* and *mec* promoter region as a homodimer, repressing expression of both the regulatory and structural genes (*10*, *11*). Though the nucleotide sequence identity of the *mecA*−*mecR1* and *blaZ*−*blaR1* intergenic regions is only 57−60%, the binding sites for both repressors are almost identical, explaining the fact that BlaI and MecI are virtually interchangeable as repressors of *blaZ* and *mecA* transcription (*12*).

When *S. aureus* is exposed to β-lactam antibiotics, the C-terminal domain of β-lactam sensor/signal transducer proteins (BlaR1 and MecR1) experiences acylation at a specific serine by the drug (Figure 1). This protein modification by the antibiotic is known to cause conformational changes in BlaR1, which are likely involved in signal transduction across the membrane (*13*, *14*). The cytoplasmic domain, which possesses a sequence motif of a zinc-dependent protease, is proposed to experience autocleavage. This autocleavage event is believed to activate the protease domain for its hydrolytic processing of the repressor proteins BlaI and MecI, which leads to derepression of transcription of *blaZ* and *mecA* (*12*, *15*, *16*). As indicated above, the functions of the repressors BlaI and MecI are interchangeable for both the *bla* and *mec* promoter regions, but when it comes to the β-lactam sensor/signal transducers, things are quite different. First, the identity of the amino acids between BlaR1 and MecR1 is only about 30%, and the identity between the C-terminal domains of the corresponding proteins is 48%. Second, the in vivo time courses for the manifestation of the phenotype between the two are quite distinct. BlaR1 induces β-lactamase formation in minutes, whereas MecR1 induces expression of PBP 2a in hours (*17*). Furthermore, BlaR1 can take over the control of expression of PBP 2a when *mecR1* is deleted from the genome of the strain (*6*, *17*).

To explain some of these observations, we have cloned, expressed, and purified to homogeneity the surface sensor domain of MecR1 (i.e., $MecR^S$, 235 amino acids). As will be outlined, this protein has substantially poorer kinetics in its interactions with β-lactam antibiotics compared to the sensor domain of BlaR1. However, we document that the two sensor domains share a number of features such as the unusual posttranslational modification that leads to an N^{ζ}-carboxylated lysine in the active site and a conformational

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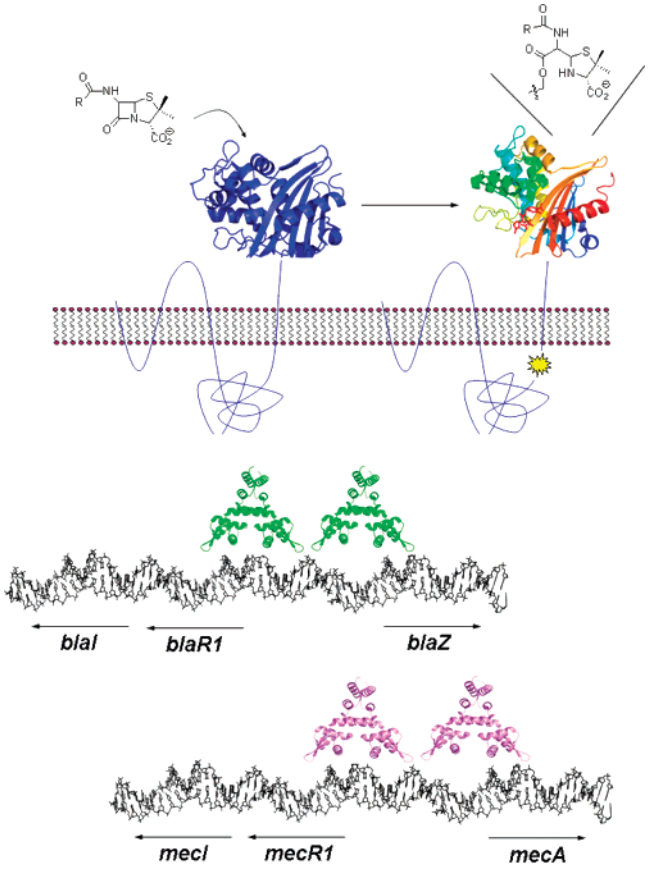

FIGURE 1: Signal transduction system for resistance to β-lactam antibiotics in staphylococci. The cytoplasmic membrane displays BlaR1 or MecR1 on its external surface. Acylation of the sensor domain by the antibiotic leads to transduction of the information to the cytoplasmic side, where genes experience derepression. The repressor proteins BlaI and MecI are shown bound to the DNA.

change on protein acylation. As will be discussed, the involvement of the N^{ζ}-carboxylated lysine is critical for the function of the protein.

EXPERIMENTAL PROCEDURES

Materials. *Pfu* Turbo DNA polymerase was purchased from Stratagene. The custom-designed primers were synthesized by MWG. The restriction endonucleases, polynucleotide kinase, and calf intestine phosphatase were purchased from New England Biolabs. The pET24a(+) and *Escherichia coli* Origami B(DE3) were purchased from Novagen. IPTG was purchased from Fisher Scientific.

Cloning of the C-Terminal Domain of MecR1 (MecR^S) and Lys394Ala Mutagenesis. We used the genomic DNA from *S. aureus* subsp. *aureus* (ATCC 700699) as the source of the *mecR1* gene. The portion of this gene encoding amino acids 351−585 of the C-terminal surface domain was PCR-amplified (gene product for this fragment is referred to as MecR^S hereafter) by *Pfu* Turbo DNA polymerase and two custom-designed primers, MecR^S-D (5′-ATCCTCATATGTTAAATCAACTAGCTCCGT-3′, *Nde*I site underlined; this site includes the ATG start codon for translation) and MecR^S-R (5′-GAGCCGAATTCTTATATTAACTCCATTTCT-3′, *Eco*RI site underlined). The PCR product was purified with a gel extraction kit (QIAGEN Sciences) and phosphorylated with polynucleotide kinase. The pUC19 vector was digested with *Sma*I and dephosphorylated with calf intestine alkaline phosphatase to prevent self-ligation of the vector. The PCR fragment was then ligated into the *Sma*I site of pUC19, and the product of ligation was used to transform *E. coli* JM83. After selection on an LB agar plate supplemented with ampicillin (100 μg/mL), the sequence of the insert was confirmed by sequencing of the gene in both directions. The resulting recombinant plasmid (pUC19:*mecR*^{S}) was digested with *Nde*I and *Eco*RI restriction endonucleases, and the gene was introduced to the corresponding sites of the pET24a(+) vector. The ligation mixture was used to transform *E. coli* BL21(DE3) and *E. coli* Origami B(DE3) (Novagen, Madison, WI) strains.

A His tag was also introduced at the 3′-end of the *mecR*^{S} gene. The plasmid pET24a(+):*mecR*^{S} was amplified by *Pfu* Turbo DNA polymerase with two primers, MecR^S-D (see above) and MecR^{S}His-R (5′-AGACCCTCGAGTATTAACTCCATTTCTTTT-3′, *Xho*I site underlined). Primer MecR^S-His-R includes the *Xho*I restriction endonuclease recognition sequence and lacks the stop codon for the *mecR*^{S} gene. This allows to clone the *mecR*^{S} gene in frame with the His tag of the pET24a(+) vector. Following the ligation of the PCR fragment into the *Nde*I and *Xho*I sites of pET24a(+), the resulting construct [pET24a(+):*mecR*^{S}His] was used to transform *E. coli* BL21(DE3). The sequence was confirmed by DNA sequencing in both directions.

Plasmid pUC19:*mecR*^{S} was utilized to create the Lys394Ala mutant variant of MecR^S. The plasmid was amplified by *Pfu* Turbo DNA polymerase with the two mutagenic primers: MecR^{S}Lys394Ala-D, 5′-CACCTAATTCTACTTAC*GCA*ATTTATTTAGCGTTAATGGC-3′, and MecR^{S}Lys394Ala-R, 5′-GCCATTAACGCTAAATAAAT*TGC*GTAAGTAGAATTAGGTG-3′ (mutation sites are italicized in each primer). The PCR product was cleaned with a gel extraction kit (QIAGEN Sciences) and treated with the restriction enzyme *Dpn*I to remove the methylated template plasmid. The amplified plasmid was used to transform *E. coli* JM83. The nucleotide sequence was verified for the entire gene in both directions. The mutated gene (*mecR*^{S}Lys394Ala) was excised from the recombinant plasmid, pUC19:*mecR*^{S}Lys394Ala, with *Nde*I and *Eco*RI restriction endonucleases and recloned into the corresponding sites of the pET24a(+) vector. The resulting construct [pET24a(+):*mecR*^{S}Lys394Ala] was used to transform *E. coli* BL21(DE3) and *E. coli* Origami B(DE3).

Purification of Soluble MecR^S. The soluble form of MecR^S was purified from the *E. coli* Origami B(DE3) strain. First, a single colony of *E. coli* Origami B(DE3) harboring pET24a(+):*mecR*^{S} was used to inoculate 5 mL of the LB medium containing 30 μg/mL kanamycin A, and bacteria were allowed to grow overnight at 37 °C. The cell culture was diluted 100-fold into fresh Terrific Broth, supplemented with 30 μg/mL kanamycin A. The culture was grown at 37 °C to an OD_{600} of ~0.8 and then was cooled to 25 °C. Expression of the MecR^S protein was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside (IPTG). The induced cultures were shaken at 25 °C for 24 h.

The cells were pelleted by centrifugation. The pellet was resuspended in 10 mM Tris base buffer, pH 7.0, and the cytoplasmic content was liberated by sonication. The suspension was centrifuged at 21000*g* for 1 h, and the supernatant was loaded onto a Macro-Prep DEAE column (2.5 × 20 cm), equilibrated with 10 mM Tris buffer, pH 7.0. The MecR^S

protein eluted at 98% of a linear gradient of 10−100 mM Tris (850 mL), pH 7.0. The fractions containing the protein were concentrated and loaded onto Sephacryl S-100 columns (2.5 × 75 cm; Amersham Biosciences) equilibrated with 10 mM sodium phosphate buffer, pH 7.0. Subsequent to the elution of the protein from the column by the same buffer, the protein-containing fractions were analyzed by SDS−PAGE, and the protein was concentrated to 3 mg/mL and was kept at 4 °C for further use. At this point, the protein was pure to apparent homogeneity. The above operations were all carried out at 4 °C.

A soluble form of $MecR^S$His was also purified from the *E. coli* Origami B(DE3) strain. Bacteria were grown, harvested, and sonicated as described above, and the protein was purified using the Hi-Trap chelating HP column. The column was charged with Ni^{2+} according to the manufacturer's instruction (Amersham Biosciences) and was equilibrated with the binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4). The sample was applied by syringe, and the column was washed with 10 column volumes of the binding buffer. The target protein was eluted by a stepwise gradient of the binding buffer mixed with the elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). A total of 10 such steps were used, each of 10 mL total. The amount of elution buffer was increased in increments of 10% for each step.

Refolding of $MecR^S$ Inclusion Bodies. Bacteria were grown essentially as described above except the induction was performed for 18 h in TB instead of LB medium. The cells were pelleted by centrifugation at 5300*g* for 30 min. The pellet was suspended in wash buffer (20 mM Tris-HCl buffer, 10 mM EDTA, 1% Triton X-100, pH 7.5) and sonicated. The sonicated suspension was centrifuged at 10000*g* for 10 min, and the supernatant was discarded. The washing step was repeated twice more, and the resulting inclusion bodies were used in the next step.

A total of 10 ProFoldin refolding columns and several propriety solutions manufactured by ProFoldin (Westborough, MA) were used according to manufacturer's recommendations to determine optimal conditions for refolding of $MecR^S$ from inclusion bodies. The $MecR^S$ inclusion bodies were prepared as described in the previous paragraph. A portion of wet inclusion bodies (1.5 mg) was dissolved in 100 mM Tris-HCl, 50 mM EDTA, and 8 M urea, pH 7.5 (150 μL), and was incubated at room temperature for 2 h. At this point the solution was mixed with 150 μL of solution A. After 5 min, 25 μL of the sample was transferred to a ProFoldin column (all 10 columns were tried individually), and the solution was spun down in a refrigerated bench top centrifuge at 1100*g* for 4 min. The eluent was incubated at 4 °C for 4 h. A 50 μL portion of solution B was mixed, and the solution was allowed to sit at 4 °C overnight. The solution was centrifuged at 18000*g* for 5 min, and the supernatant was used for the acylation assays with nitrocefin, a chromogenic cephalosporin. We compared the kinetic parameters for acylation of the refolded proteins with that of the nonrefolded soluble $MecR^S$ protein. This analysis revealed that ProFoldin column 4 was useful in correct refolding of the protein to the native state. This column was used in the following experiment.

For a large-scale refolding procedure we used column 4 (ProFoldin). Experiments were performed at 4 °C. A 2 mg/mL solution of the $MecR^S$ (1.3 mL) was mixed with 1.3 mL of solution A (ProFoldin), and the mixture was incubated for 5 min. At this point the solution was loaded onto the column, and it was allowed to run by gravity. The flow-through solution was discarded. A 3.5 mL portion of solution C was loaded on top of the column. The column was eluted by gravity until there were no more eluents from the column. The solution was incubated for 4 h. Another portion of solution B (2 mL) was mixed, and the solution was allowed to incubate overnight. The refolded protein was dialyzed against 50 mM Tris-HCl and 12.5% glycerol, pH 7.2. The refolding of $MecR^S$Lys394Ala was performed with the above method as well.

Kinetic Studies with β-Lactam Antibiotics. The initial experiments were carried out on a Cary 50 UV/vis spectrophotometer (Varian Inc.) equipped with an SFA-20 stopped-flow apparatus (Hi-Tech Scientific, Salisbury, U.K.) at 25 °C. Rapid protein acylation by nitrocefin ($\Delta\epsilon_{500} = +15900\ cm^{-1}\ M^{-1}$) was monitored from 20 ms to 10−15 min. Solutions of nitrocefin (2−50 μM) were used in the reaction with 1 μM $MecR^S$ in 500 μL of reaction buffer (100 mM sodium phosphate, 50 mM sodium bicarbonate, pH 7.0).

Nitrocefin was used as a reporter molecule to determine the apparent first-order rate constants for acylation by other nonchromogenic (or poorly chromogenic) β-lactams in competitive reactions (*13*, *18*). The progress curves, obtained by monitoring the formation of acyl-enzyme species between nitrocefin and $MecR^S$ protein at different concentrations of the β-lactam, were fit to the equation:

$$A_t = A_{max}[1 - \exp(-k'_{obs}t)]$$

where $k'_{obs} = k_n + k_i$, and k_n and k_i are the observed first-order rate constants at 200 μM nitrocefin in the absence of the competing β-lactam and at different concentrations of the competing β-lactam, respectively. The k_i values were plotted against the β-lactam concentrations, and the data obtained for each β-lactam were processed using nonlinear regression analysis (Grafit 4; Erithacus Software, Middlesex, U.K.) to calculate the k_2 and K_s values.

The deacylation rate constants with various β-lactams were determined with the fluorescent penicillin BOCILLIN FL (*19*). A typical reaction mixture (60 μL) contained 4.5 μM $MecR^S$ and a β-lactam antibiotic at a concentration at least 2-fold higher than its K_s value. The mixture was incubated at 25 °C for 15 min in the reaction buffer (described above). The excess of β-lactam antibiotic was removed by passing the mixture through a Micro Bio-Spin6 column (Bio-Rad). Each aliquot (4.5 μL) of the mixture was diluted 4.5-fold with reaction buffer to a total of 20 μL. Several reactions were diluted as above and incubated for different time intervals at 37 °C. The amount of the free protein, liberated from the acyl-protein species, was detected by the addition of BOCILLIN FL to a final concentration of 20 μM and incubating for an additional 5 min at 37 °C. A total of 15 μL of the SDS sample buffer (125 mM Tris, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, pH 6.8) was added, and the mixtures were boiled for 4 min. The samples were loaded onto 15% SDS−PAGE, and the gel was developed and scanned using a Storm 840 Fluorimager. The fluorescent bands were analyzed by Image Quant 5.2 software. A control sample was also prepared, where the $MecR^S$ protein was

incubated with BOCILLIN FL under the same condition as with other β-lactam antibiotics, with the exception that the SDS sample buffer was added immediately after the 4.5-fold dilution of the reaction mixture. This control sample was used to determine the amount of the acylated protein at time zero. The data were fit to the equation:

$$-k_3t = \ln[\text{B-I}]_t/[\text{B-I}]_0$$

where $[\text{B-I}]_0$ and $[\text{B-I}]_t$ are the concentrations of the acylated MecR^S at time zero and at time *t*, respectively, calculated as $[\text{B-I}]_t = [\text{B-I}]_0 - [\text{B-I}^B]_t$, where $[\text{B-I}^B]_t$ is the concentration of the acylated protein with BOCILLIN FL at time *t*.

Circular Dichroism Spectroscopy. The CD spectrum of the wild-type MecR^S (10 μM protein in 50 mM Tris-HCl and 50 mM sodium bicarbonate, pH 7.2) was recorded on a stopped-flow circular dichroism spectrometer (Aviv Instruments Inc., 202 SF, 2 mm path length) in the absence and presence of ampicillin and ceftazidime. The contribution of substrate was subtracted from the CD spectrum of the protein with substrate. The concentrations of the β-lactam were generally 2-fold higher than the respective K_s values. Prior to recording the spectra of the proteins with a β-lactam antibiotic, the proteins were incubated with the β-lactam antibiotics for 15 min at 25 °C.

Determination of the Dissociation Constant of Carbon Dioxide. Fluorometric measurements were performed in a Cary Eclipse fluorescence spectrophotometer (Varian Inc.). The wild-type MecR^S and the Lys394Ala mutant variant of MecR^S (2 μM each) were incubated in 100 mM sodium phosphate buffer (pH 7.5). Aliquots of concentrated $NaHCO_3$, prepared in the same buffer, were added to the enzyme solution to provide the desired final carbon dioxide concentration. The carbon dioxide concentration in solution was calculated as described previously (*20*). The quenching of fluorescence after binding of carbon dioxide to Lys394 was observed by the emission scan at 330 nm after excitation at 290 nm. The experimental data were fit to a single binding site model using Grafit 4 software and the quadratic equation:

$$\Delta F/F_0 = (\Delta F_{max}/2F_0[\text{E}]_t)\{(K_d + [\text{E}]_t + [\text{L}]_t) - ((K_d + [\text{E}]_t + [\text{L}]_t)^2 - 4[\text{E}]_t[\text{L}]_t)^{1/2}\}$$

F_0 is the initial intrinsic fluorescence of the protein, ΔF is the change in fluorescence, ΔF_{max} is the maximum change in fluorescence after saturation by carbon dioxide, $[\text{L}]_t$ is the concentration of total carbon dioxide, and $[\text{E}]_t$ is the enzyme concentration in the assay. The experiment was carried out three times, and analysis of the data was according to the literature methods of Olson et al. (*21*, *22*).

^{13}C NMR Measurement. Each buffer for this experiment was degassed and purged with nitrogen prior to use. A total of 1.5 mg of each of the wild-type MecR^S and the Lys394Ala mutant variant of MecR^S was incubated in an Amicon ultracentrifugal filter device (Millipore) with several changes of buffer (25 mM sodium acetate, 10% glycerol, pH 4.5) to release CO_2 from the active site. Then, the sample was incubated with several changes of 10 mM TES, 0.1 mM EDTA, and 10% glycerol, pH 7.5. Finally, the sample was incubated with 10 mM TES, 0.1 mM EDTA, 10% glycerol, 5 mM $NaH^{13}CO_3$, and 10% D_2O, pH 7.5, and concentrated up to 150 μM just prior to the experiment on a Bruker Avance 800 equipped with cryoprobe (0.68 s/AQ). Data were acquired over 23 h.

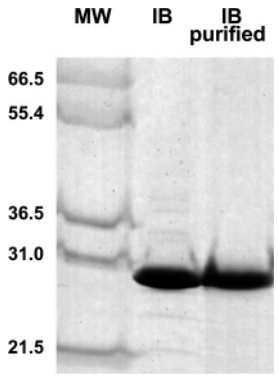

FIGURE 2: SDS−PAGE of MecR^S. The preparation of the inclusion bodies was refolded by the use of a ProFoldin column (far right column). Abbreviations: MW, molecular weight marker; IB, inclusion body.

RESULTS AND DISCUSSION

The *mecR^S* gene from *S. aureus* was cloned and expressed in *E. coli*. The expressed protein was present largely as inclusion bodies. The isolated inclusion bodies were resolubilized in a buffer containing 8 M urea, and the protein was subsequently refolded. Initially, we investigated a traditional method for refolding of the protein by a stepwise dialysis with decreasing concentrations of urea from 8 to 0 M. Unfortunately, the refolded protein could not be prepared by this method consistently. We resorted next to the use of a ProFoldin column, which gave consistency from one preparation to another.

We also prepared the soluble MecR^S from the cytoplasmic fraction for comparison with the refolded version. For this purpose, we transformed pET24a+:*mecR^S* into the so-called Origami strain [Origami B(DE3)]. Whereas this strain is largely useful in expression of proteins that possess disulfide bonds (none is present in MecR^S), it gave more soluble protein than other strains of *E. coli*. The kinetic properties of the two samples, the refolded protein from the inclusion bodies (by the use of the ProFoldin column) and the samples from the cytoplasmic fraction of the Origami strain, were the same. The k_2 and K_s parameters were determined with nitrocefin to compare the activity of the soluble form and the refolded form of MecR^S. The values for the soluble form were 0.021 ± 0.003 s^{-1}, 163 ± 37 μM and those for the refolded form were 0.019 ± 0.001 s^{-1}, 167 ± 25 μM, respectively.

These experiments indicated that the refolding process generated a functional protein. The yield of the active soluble form of MecR^S was not sufficient for our studies (less than 0.5 mg of pure protein/L of LB media), but we could readily obtain about 30 mg/L of the desired protein by the refolding method. Since we confirmed that the refolded purified protein was as functional as the protein purified from the soluble fraction of the cytoplasm, we used the refolded protein for all of the subsequent experiments (Figure 2).

N^{ζ}-Carboxylation of Lys394 in the MecR^S Protein. The class D OXA β-lactamases and the surface β-lactam sensor domains of BlaR1 and MecR1 are similar in sequence and in three-dimensional structures (*23*−*26*). It was initially observed in the structure of the OXA-10 β-lactamase that

the active site lysine, whose side chain promotes the serine residue for acylation by substrates, was N^{ζ}-carboxylated, the product of addition of carbon dioxide to the free base form of the lysine (*27*−*29*). The side chain of the lysine of the OXA-10 β-lactamase sits in a hydrophobic environment comprised of residues Phe69, Val117, Phe120, Trp154, and Leu155, which reduces the pK_a of the lysine predisposing it to N^{ζ}-carboxylation by the ubiquitous carbon dioxide. The environment of the active site stabilizes the carbamate group on the lysine side chain (Figure 3A,B). Interestingly, the X-ray structures of the sensor domains of BlaR1 (*23*, *24*, *26*) and MecR1 (*25*) do not show the presence of the carbamate group (Figure 3C, D). We note that this was also true for the initial reports of the crystal structures of the OXA-10 β-lactamase by two different groups (*30*, *31*), but subsequent crystallography revealed the presence of the modified lysine (*27*−*29*). The signature of the cluster of the residues comprising the hydrophobic pocket, where the side chain of the active site lysine is ensconced, is preserved in both sensor domains. Indeed, as revealed in the panels of Figure 3, the three active sites are extremely similar. Therefore, we had assumed that, despite the crystallographic reports to the contrary, the lysine carbamates were likely to exist in both BlaR1 and MecR1. We assert here that the N^{ζ}-carboxylated lysine forms of BlaR1 and MecR1 were not amenable to crystallization under the reported conditions, but the N^{ζ}-decarboxylated versions were.

Unequivocal evidence for N^{ζ}-carboxylation of the surface domain of BlaR1 is now in hand by several methods. The evidence comes from fluorescence measurements of titration of the enzyme active site by carbon dioxide in a saturable manner and from both ^{13}C (NMR experiments) and ^{14}C labeling (scintillation quantification) of the carbonyl of the N^{ζ}-carboxylation protein (*13*). More recently, an FT-IR signature resonance for the N^{ζ}-carboxylated protein was also documented by differential ^{12}C and ^{13}C spectral measurements (*14*), all clearly pointing to the existence of the critical N^{ζ}-carboxylated Lys392 in the sensor domain of BlaR1.

We have now extended these studies to MecR1. N^{ζ}-Carboxylation of lysine is reversible and can in principle give a mixture of N^{ζ}-carboxylated and N^{ζ}-decarboxylated versions on purification of MecR^S. Supplementation of the solution with sodium bicarbonate generates a sufficient concentration of carbon dioxide in solution that it carboxylates the free base form of the active site lysine. This supplementation increased the acylation rate of MecR^S by β-lactam antibiotics by 3-fold, suggestive that the protein at the end of the purification had lost some of the N^{ζ}-carboxylation. This observation also revealed that MecR1 is likely to be N^{ζ}-carboxylated, just as are BlaR1 and the class D β-lactamases.

N^{ζ}-Carboxylated lysine in proteins exhibits a ^{13}C NMR signature resonance at δ 164. Detection of this signal is considerably enhanced if the functional group is enriched in ^{13}C. N^{ζ}-Carboxylated lysine could be decarboxylated by incubation at pH 4.5. The pH was then raised to 7.5, and [^{13}C]sodium bicarbonate, as the source of labeled carbon dioxide, was added to the solution. By doing so, the fully ^{13}C-labeled protein is reconstituted and ready for the NMR measurement. The ^{13}C NMR experiment showed that lysine N^{ζ}-carboxylation is observed in the MecR^S protein (164 ppm, Figure 4) just as we have documented it in BlaR^S and OXA-10 β-lactamase. The same experiment was performed with the Lys394Ala mutant of MecR^S, and we did not observe the carboxylated lysine resonance (Figure 4B). This experiment documents the presence of N^{ζ}-carboxylated Lys394 in MecR^S.

The presence of Trp477 (Figure 3D) in the active site was used as a fluorescence tool in quantification of N^{ζ}-carboxylation of Lys394. The fluorescence quenching study revealed that as the concentration of carbon dioxide increased, the intrinsic fluorescence quenching increased in a saturable manner (Figure 5). The dissociation constant (K_d) for carbon dioxide for the protein was evaluated at 4.3 ± 0.8 μM. When we performed the same experiment with the Lys394Ala mutant protein, we did not see any tryptophan fluorescence quenching, which indicates that the dissociation constant that was evaluated for the wild-type protein was for N^{ζ}-carboxylation of residue 394, the spatial counterpart to the N^{ζ}-carboxylated lysines in BlaR1 and the OXA-10 β-lactamase. We hasten to add that a K_d of 4.3 ± 0.8 μM for the reversible N^{ζ}-carboxylation of Lys394 indicates that the MecR1 is N^{ζ}-carboxylated at all times in vivo, in light of the concentration of 1.3 mM for carbon dioxide in bacteria (*32*). The corresponding K_d values reported for N^{ζ}-carboxylation of the OXA-10 β-lactamase and for the BlaR1 protein are 0.23 ± 0.05 μM (*27*) and 0.6 ± 0.2 μM (*13*), respectively.

Kinetics of Interactions of MecR^S with β-Lactam Antibiotic. In light of the documentation that MecR^S is N^{ζ}-carboxylated at the active site lysine, we supplemented the buffer with sodium bicarbonate (as a source of carbon dioxide) in our kinetic experiments. This supplementation would result in fully active purified MecR^S.

Kinetics of acylation of MecR^S by two penicillins, three cephalosporins, and one carbapenem were investigated in competition with nitrocefin, as a reporter substrate (Table 1) (*18*). Stopped-flow kinetics were attempted at first to observe any potential rapid formation of the acyl-protein species, which incidentally was necessary for the studies of BlaR^S (*13*). The rate constants for acylation (k_2) of MecR^S were significantly smaller than those of BlaR^S that the need for stopped-flow experiments was obviated (Table 1). More intuitive than k_2 values are half-times ($t_{1/2}$) for the event, which turned out to be in the range of 8−99 s for MecR^S (this study) and 27−690 ms for BlaR^S (*13*). The deacylation rate constants for MecR^S were extremely small and comparable to those of BlaR^S and other penicillin-binding proteins, with $t_{1/2}$ in the range of 72−825 min (Table 1). These values are consistent with unassisted deacylation of the acyl-protein species. The deacylation rate constants for the cases of oxacillin, ceftazidime, and imipenem could actually not be determined due to the extremely slow nature of the process that became a technical impediment to the determination. We also note that the dissociation constants (K_s) for the reversible binding of the antibiotics to the MecR^S protein are elevated compared to the case of BlaR^S. As such, the preacylation complex is less readily formed with MecR1, and when formed, the forward direction leading to the acyl-protein species does not proceed as rapidly as in the case of BlaR^S. As a consequence, it is interesting to observe that the second-order rate constants for the encounter between β-lactam antibiotics and the MecR^S protein (k_2/K_s) are typically about 10^2 M^{-1} s^{-1}, which are

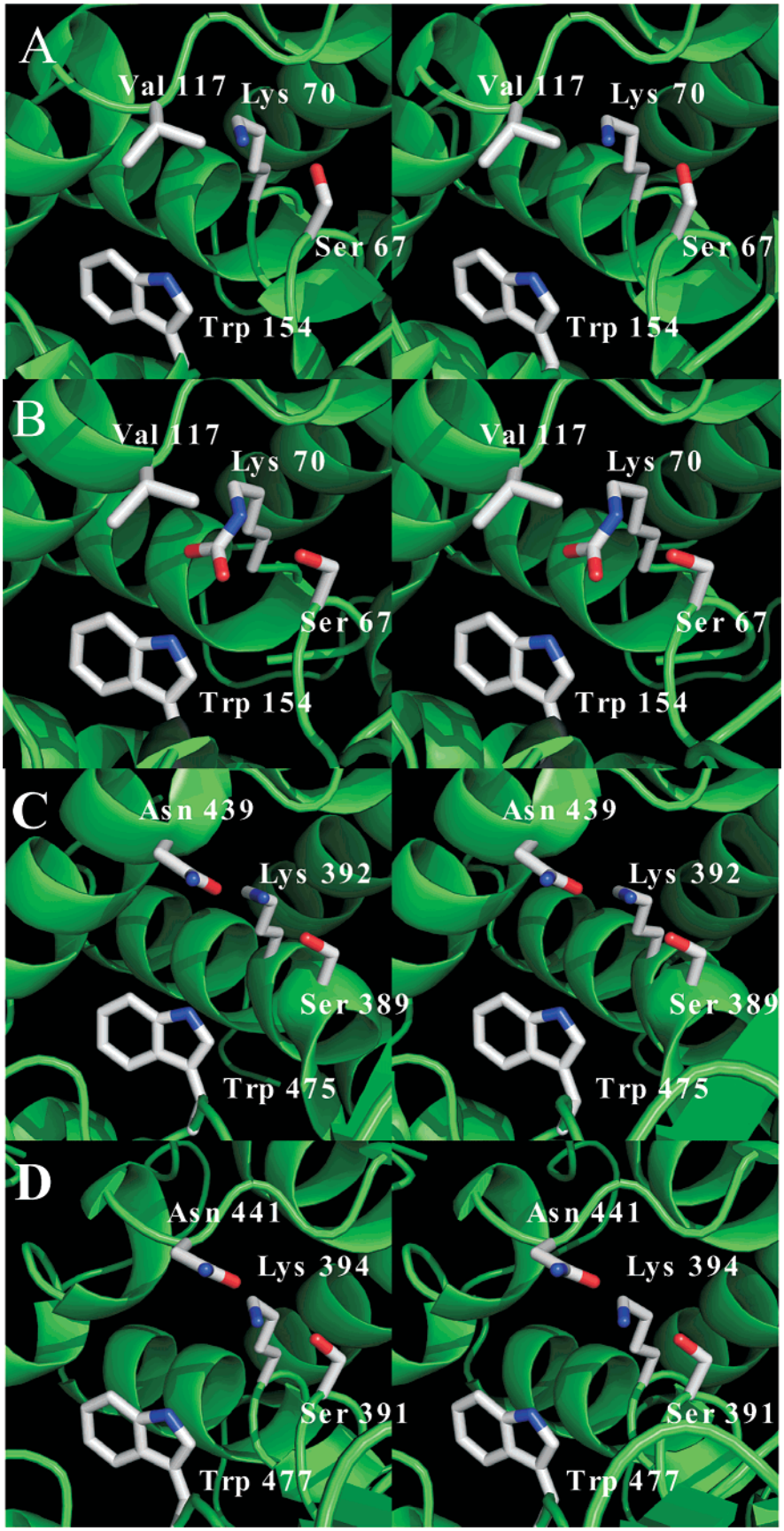

FIGURE 3: Stereoview of the active sites from X-ray structures of (A) the class D OXA-10 β-lactamase N^{ζ}-uncarboxylated at the lysine (PDB code 1EWZ), (B) the class D OXA-10 β-lactamase N^{ζ}-carboxylated at the lysine (PDB code 1K57), (C) the C-terminal domain of BlaR1 N^{ζ}-uncarboxylated at the lysine (PDB code 1XA1), and (D) the C-terminal domain of MecR1 N^{ζ}-uncarboxylated at the lysine (PDB code 2IWB). Key active site residues are depicted as capped sticks, colored according to atom type (C, N, and O as white, blue, and red), and are numbered according to the numbering system for the given protein. The protein backbone is drawn as the ribbon representation in green.

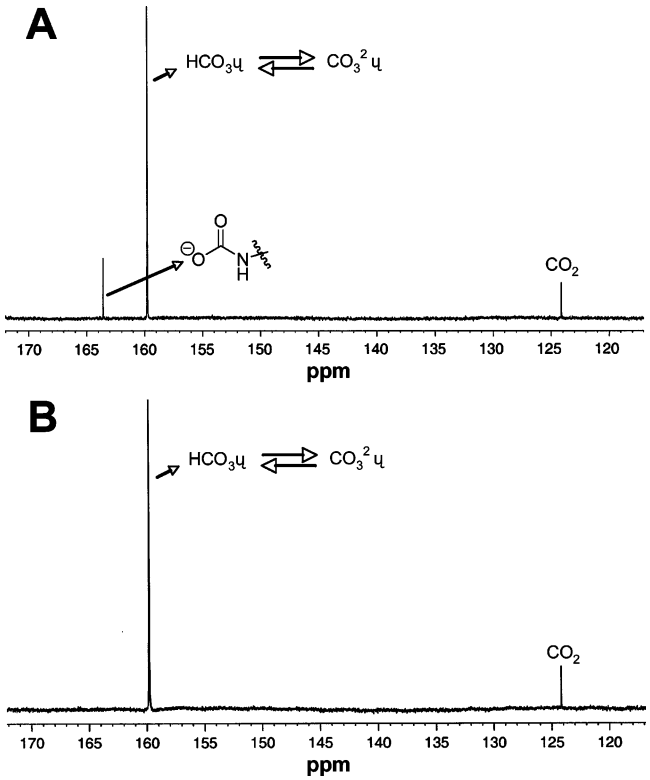

FIGURE 4: ^{13}C NMR spectra (201.2 MHz) of 150 μM each of (A) the wild-type MecR^S and (B) the Lys394Ala mutant variant of MecR^S. Experiments were performed in 10 mM TES, 0.1 mM EDTA, 10% glycerol, and 10% D_2O, pH 7.5, supplemented with 5 mM $NaH^{13}CO_3$.

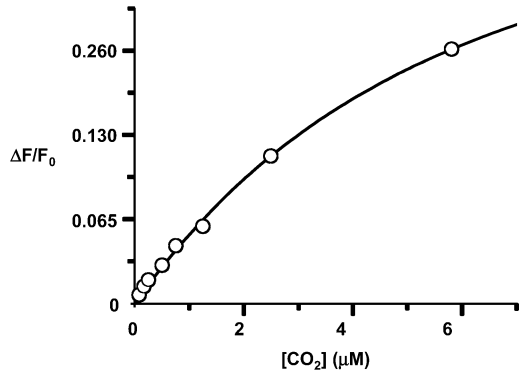

FIGURE 5: Relative quenching of the intrinsic tryptophan fluorescence of MecR^S protein (2 μM) vs total concentration of carbon dioxide (μM). Data were fit to a single binding site model.

as much as 2−4 orders of magnitude smaller than the corresponding parameters for BlaR^S (*13*, *33*).

According to the crystal structures, the overall fold of the C-terminal domains of MecR1 and BlaR1 is the same. Yet, it is known that MecR1 takes hours to induce the production of PBP 2a, in contrast to BlaR1, which takes only minutes to induce the β-lactamase production (*12*). Because of the complexity of the events that are mediated by BlaR1 and MecR1, the difference in phenotypic outcome could come about in any number of steps from detection of β-lactams, to transduction of the signal, to the events on the cytoplasmic side of the bacterium. Analyses that are presented here indicate that sensing of the antibiotic is at least partly responsible for the difference. We have documented here that MecR1 exhibits smaller k_2 and larger K_s values compared to BlaR1. The attendant attenuation on k_2/K_s, the second-order rate constant for acylation, is as much as 2−4 orders of magnitude. Whereas it is not possible to take the value for a kinetic parameter for any process and predict an outcome for the phenotypic consequence, the observation here is that sensing of the signal (i.e., the β-lactam antibiotic) by MecR1 is significantly worse than that by BlaR1.

We also performed kinetics with the Lys394Ala mutant variant of MecR^S. The protein exhibited impairment of acylation by BOCILLIN FL. The mutant protein showed approximately 200-fold less binding in the gel assay. The residual activity was not sufficient to evaluate k_2 and K_s reliably. These results are not surprising, considering that the N^{ζ}-carboxylated Lys394 is the residue that promotes Ser391 for acylation. Similarly, the Lys392Ala mutant variant of BlaR^S was severely impaired in acylation of the active site serine by oxacillin (by a factor of 6730-fold) as evaluated earlier (*13*). A clarification for the levels of attenuation of the rate constants with the two mutant proteins is relevant here. The k_2 value for the wild-type BlaR^S was larger than that for the wild-type MecR^S, so on mutation at the corresponding lysine the k_2 value attenuated more for the former than for the latter. The basal levels of activity for the two Lys to Ala mutants were roughly the same.

Signal Transduction Subsequent to β-Lactam Sensing by the Surface Domain. As described earlier, the presence of β-lactam antibiotics is sensed by MRSA by acylation of the active site serines of BlaR1 and MecR1 proteins. The transduction of information from the surface of the membrane to the cytoplasmic side of the protein by necessity requires conformational changes. In the case of the BlaR1 protein conformational changes have been implicated for this process, either as an inherent property of the surface domain by itself after acylation in *S. aureus* (*13*, *14*) or via interactions of the L2 loop with the receptor active site in *Bacillus licheniformis* (*34*).

We document here that on interactions of the antibiotics with the MecR^S protein as well one sees conformational changes (Figure 6). Upon incubation with a 2−3-fold excess of ampicillin or ceftazidime over the corresponding K_s values, MecR^S undergoes conformational changes, as discerned by circular dichroic spectra (Figure 6A,B). As indicated earlier, the Lys394Ala mutant of the MecR^S protein is impaired in the acylation step. This mutant variant shows small changes in the CD spectrum on interaction with ceftazidime, indicative of the possibility that the conformational changes may be set in motion at the preacylation stage and reach the full extent on the formation of the acyl-protein species (Figure 6C). The same general observations were made with the BlaR^S (*13*). It is also worth noting that during the data acquisition time for the CD spectra (15 min) with the wild-type MecR^S the protein was fully acylated ($t_{1/2}$ is 8−99 s). The CD spectra reveal that in the cases of both BlaR^S and MecR^S the conformational changes observed during interaction with the antibiotics are quite comparable, and they take place within similar time frames. As such, transduction of the signal might not be the step that sets apart the two proteins in the phenotypic response time, at least at the level of the sensor domain. We acknowledge that our analysis cannot take into account the events that would take place within the membrane spanning domain.

Concluding Remarks. When penicillin was introduced to clinical use in the 1940s, more than 90% of *S. aureus* strains

Table 1: Kinetic Parameters for Interactions of $MecR^S$ and $BlaR^S$ with β-Lactam Antibiotics[a]

	$MecR^S$				$BlaR^S$			
substrate	k_2 (s^{-1})	k_3 (s^{-1}) $\times 10^5$	K_s (μM)	k_2/K_s ($M^{-1}\,s^{-1}$) $\times 10^{-2}$	k_2 (s^{-1})	k_3 (s^{-1}) $\times 10^5$	K_s (μM)	k_2/K_s ($M^{-1}\,s^{-1}$) $\times 10^{-5}$
nitrocefin	0.021 ± 0.003	16 ± 3	163 ± 37	1.3 ± 0.3	26 ± 6	92 ± 10	24 ± 9	11 ± 4
ampicillin	0.009 ± 0.001	8.4 ± 0.3	50 ± 11	1.8 ± 0.4	1.0 ± 0.1	9 ± 1	23 ± 2	0.4 ± 0.1
oxacillin	0.010 ± 0.002	ND	103 ± 26	1.0 ± 0.3	18 ± 1	4.8 ± 0.6	49 ± 5	3.7 ± 0.4
cefepime	0.007 ± 0.001	1.4 ± 0.3	262 ± 29	0.3 ± 0.0	1.4 ± 0.1	12.6 ± 0.3	25 ± 3	0.6 ± 0.1
ceftazidime	0.084 ± 0.011	ND	148 ± 27	5.7 ± 1.3	4.1 ± 0.5	9.1 ± 0.4	68 ± 16	0.6 ± 0.2
imipenem	0.030 ± 0.001	ND	154 ± 7	2.0 ± 0.1	6 ± 1	10 ± 1	183 ± 66	0.3 ± 0.1

[a] The data for $BlaR^S$ are from Golemi-Kotra et al. (*13*). The kinetics were performed in 100 mM sodium phosphate buffer (pH 7.0), supplemented with 50 mM sodium bicarbonate. ND: not determined.

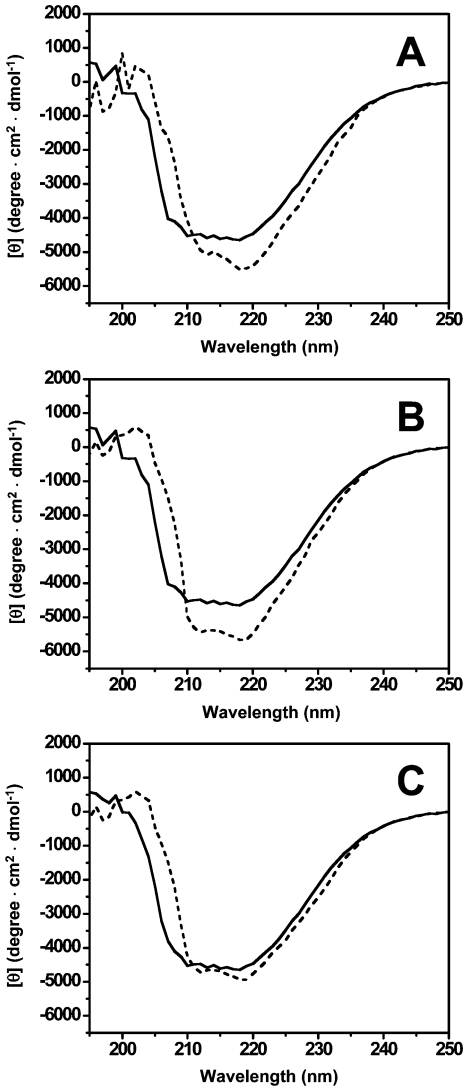

FIGURE 6: Circular dichroic spectra of (A) the wild-type $MecR^S$ (10 μM, solid line) and the wild-type $MecR^S$ incubated with ampicillin (200 μM, broken line), (B) the wild-type $MecR^S$ (10 μM, solid line) and the wild-type $MecR^S$ incubated with ceftazidime (450 μM, broken line), and (C) the Lys394Ala mutant variant of $MecR^S$ (10 μM, solid line) and the Lys394Ala mutant variant of $MecR^S$ incubated with ceftazidime (450 μM, broken line). All of the spectra were corrected for the minor contribution from the antibiotic in the mixture.

were susceptible to it. Resistance to penicillin emerged rapidly in the clinic and by the late 1950s almost 50% of *S. aureus* strains were resistant. This resistance to penicillin exceeded 90% of all strains in the 1990s (*35*). Plasticity of the genome of *S. aureus* in expanding its resistance profile to virtually any known antibiotic is indeed notable, making it a formidable pathogen (*36*−*39*).

The report of the β-lactamase activity of *S. aureus* actually appeared in the literature before any widespread use of the antibiotic clinically (*40*). This indicates that the *bla* machinery was in place by the early 1940s. The breadth of activity of the class A β-lactamase from *S. aureus* is limited to the early penicillins. While it was sufficient for the survival needs of the organism in the early 1940s into the 1950s, it proved not good enough when second- and third-generation β-lactams were introduced. The class A β-lactamases in a number of Gram-negative bacteria have undergone a most impressive selection for mutant variants that expand their breadth of activity against the newer β-lactam antibiotics (*41*−*44*). For example, there are 157 known variants of the TEM-1 β-lactamase from *E. coli* (http://www.lahey.org/studies/webt.htm). For reasons that are not understood, this mutational expansion of the breadth of activity is not seen in the β-lactamase from *S. aureus*. Some organisms actually harbor more than one β-lactamase to expand their resistance profile (*45*−*47*). This has not been seen in *S. aureus* either.

S. aureus responded to the advent of the newer semisynthetic β-lactam antibiotics in a unique way: it acquired the *mec* system from a non-native source (*48*). The function of the *mecA* gene product as a penicillin-binding protein that resisted inhibition by all commercially available β-lactams provided complete resistance to the entire class of β-lactam antibiotics (*5*, *18*). This event marked the emergence of what came to be known as methicillin-resistant *S. aureus* (MRSA), which became a clinical scourge. The first MRSA was reported in 1961 in the U.K., but the organism was identified in other countries shortly thereafter (*49*). Methicillin had been introduced clinically in 1959; hence emergence of MRSA can directly be linked to the development of penicillanase-resistant penicillins approximately 2 decades after the report of resistance due to the production of the β-lactamase.

The *mec* and *bla* machineries worked together in affording complete resistance to all commercially available β-lactam antibiotics. Characterization of these systems at the protein level has commenced only recently. We have described in this report the nature of the C-terminal β-lactam sensor domain of the MecR1 protein of MRSA. It shares a substantial similarity and identity in amino acid sequence, and indeed similarity in three-dimensional structure, with $BlaR^S$, yet the two proteins have different activities in vivo. We identified the unusual N^{ξ}-carboxylation in the active site lysine and conformational changes upon binding with β-lactam antibiotics in $MecR^S$, as also were observed for $BlaR^S$ (*13*, *14*, *23*). The nature and the time frame of the confor-

mational changes are comparable between the two antibiotic sensors. However, kinetics of acylation of $MecR^S$ by the antibiotics indicate that this protein is substantially less effective in sensing of β-lactam antibiotics compared to $BlaR^S$. We also documented that the dissociation constant for carbon dioxide is 7-fold larger than that of $BlaR^S$, but this difference is likely to be insignificant since the in vivo concentration for carbon dioxide is 1.3 mM and both proteins will be fully carboxylated under this condition.

It has previously been demonstrated that MecI is a stronger repressor of *mecA* transcription than the BlaI. A poorly reversible repression of *mecA* whose regulation is directed by the MecI/MecR1 system could result in a survival disadvantage for bacteria in the presence of β-lactam antibiotics (*12*). Consistent with this observation, our findings in this report demonstrate that MecR1 is less efficient than BlaR1 in sensing β-lactam antibiotics. Thus it is reasonable to surmise that a selection pressure exists in elimination of the MecR1/MecI machinery. It is known that some of the highly resistant MRSA strains do not possess or possess defective MecR1/MecI machineries (*1*). In these strains BlaR1 takes over the entire process; it might have been possible at some point that production of PBP 2a was liberated from the control of the MecR1/MecI system and fell under the control of the more efficient *bla* machinery. The results in this report provide a mechanistic basis for selection against MecR1/MecI and explain in part the move toward reliance on the *bla* machinery for expression of both the β-lactamase and PBP 2a in MRSA.

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REFERENCES

1. Berger-Bachi, B., and Rohrer, S. (2002) Factors influencing methicillin resistance in staphylococci, *Arch. Microbiol. 178*, 165−171.
2. Chang, S., Sievert, D. M., Hageman, J. C., Boulton, M. L., Tenover, F. C., Downes, F. P., Shah, S., Rudrik, J. T., Pupp, G. R., Brown, W. J., Cardo, D., and Fridkin, S. K. (2003) Infection with vancomycin-resistant *Staphylococcus aureus* containing the vanA resistance gene, *N. Engl. J. Med. 348*, 1342−1347.
3. Gonzalez-Zorn, B., and Courvalin, P. (2003) *vanA*-mediated high level glycopeptide resistance in MRSA, *Lancet Infect. Dis. 3*, 67−68.
4. Severin, A., Tabei, K., Tenover, F., Chung, M., Clarke, N., and Tomasz, A. (2004) High level oxacillin and vancomycin resistance and altered cell wall composition in *Staphylococcus aureus* carrying the staphylococcal *mecA* and the enterococcal *vanA* gene complex, *J. Biol. Chem. 279*, 3398−3407.
5. Fuda, C., Suvorov, M., Vakulenko, S. B., and Mobashery, S. (2004) The basis for resistance to beta-lactam antibiotics by penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*, *J. Biol. Chem. 279*, 40802−40806.
6. Chambers, H. F. (1997) Methicillin resistance in staphylococci: Molecular and biochemical basis and clinical implications, *Clin. Microbiol. Rev. 10*, 781.
7. Katayama, Y., Zhang, H. Z., and Chambers, H. F. (2004) PBP 2a mutations producing very-high-level resistance to β-lactams, *Antimicrob. Agents Chemother. 48*, 453−459.
8. 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*, 10886−10891.
9. Zhu, Y. F., Curran, I. H. A., Joris, B., Ghuysen, J. M., and Lampen, J. O. (1990) Identification of blar, the signal transducer for β-lactamase production in *Bacillus licheniformis*, as a penicillin-binding protein with strong homology to the OXA-2 β-lactamase (class-D) of *Salmonella typhimurium*, *J. Bacteriol. 172*, 1137−1141.
10. Garcia-Castellanos, R., Mallorqui-Fernandez, G., Marrero, A., Potempa, J., Coll, M., and Gomis-Ruth, F. X. (2004) On the transcriptional regulation of methicillin resistance−MecI repressor in complex with its operator, *J. Biol. Chem. 279*, 17888−17896.
11. Van Melckebeke, H., Vreuls, C., Gans, P., Filee, P., Llabres, G., Joris, B., and Simorre, J. P. (2003) Solution structural study of BlaI: Implications for the repression of genes involved in β-lactam antibiotic resistance, *J. Mol. Biol. 333*, 711−720.
12. McKinney, T. K., Sharma, V. K., Craig, W. A., and Archer, G. L. (2001) Transcription of the gene mediating methicillin resistance in *Staphylococcus aureus* (*mecA*) is corepressed but not coinduced by cognate *mecA* and β-lactamase regulators, *J. Bacteriol. 183*, 6862−6868.
13. Golemi-Kotra, D., Cha, J. Y., Meroueh, S. O., Vakulenko, S. B., and Mobashery, S. (2003) Resistance to β-lactam antibiotics and its mediation by the sensor domain of the transmembrane BlaR signaling pathway in *Staphylococcus aureus*, *J. Biol. Chem. 278*, 18419−18425.
14. Thumanu, K., Cha, J., Fisher, J. F., Perrins, R., Mobashery, S., and Wharton, C. (2006) Discrete steps in sensing of β-lactam antibiotics by the BlaR1 protein of the methicillin-resistant *Staphylococcus aureus* bacterium, *Proc. Natl. Acad. Sci. U.S.A. 103*, 10630−10635.
15. Lewis, R. A., Curnock, S. P., and Dyke, K. G. H. (1999) Proteolytic cleavage of the repressor (BlaI) of β-lactamase synthesis in *Staphylococcus aureus*, *FEMS Microbiol. Lett. 178*, 271−275.
16. 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.
17. Ryffel, C., Kayser, F. H., and Bergerbachi, B. (1992) Correlation between regulation of *mecA* transcription and expression of methicillin resistance in staphylococci, *Antimicrob. Agents Chemother. 36*, 25−31.
18. Graves-Woodward, K., and Pratt, R. F. (1998) Reaction of soluble penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus* with beta-lactams and acyclic substrates: kinetics in homogeneous solution, *Biochem. J. 332*, 755−761.
19. Zhao, G. S., Meier, T. I., Kahl, S. D., Gee, K. R., and Blaszczak, L. C. (1999) BOCILLIN FL, a sensitive and commercially available reagent for detection of penicillin-binding proteins, *Antimicrob. Agents Chemother. 43*, 1124−1128.
20. Butter, N. J. (1982) *Carbon Dioxide Equilibria and Their Applications*, Addison-Wesley, Reading, MA.
21. Bernat, B. A., and Armstrong, R. N. (2001) Elementary steps in the acquisition of Mn^{2+} by the fosfomycin resistace protein (FosA), *Biochemistry 40*, 12712−12718.
22. Olson, S. T., and Shore, J. D. (1981) Binding of high affinity heparin to antithrombin III. Characterization of the protein fluorescence enhancement, *J. Biol. Chem. 256*, 11065−11072.
23. Birck, C., Cha, J. Y., Cross, J., Schulze-Briese, C., Meroueh, S. O., Schlegel, 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. Am. Chem. Soc. 126*, 13945−13947.
24. Kerff, F., Charlier, P., Colombo, M. L., Sauvage, E., Brans, A., Frere, 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.
25. Marrero, A., Mallorqui-Fernandez, G., Guevara, T., Garcia-Castellanos, R., and Gomis-Ruth, 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.
26. 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-acylated forms of the BlaR1 β-lactam sensor of *Staphylococcus aureus*, *J. Biol. Chem. 279*, 47278−47287.
27. Golemi, D., Maveyraud, L., Vakulenko, S. B., Samama, J. P., and Mobashery, S. (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.
28. Maveyraud, L., Golemi, D., Kotra, L. P., Tranier, S., Vakulenko, S. B., Mobashery, S., and Samama, J. P. (2000) Insights into class

D β-lactamases are revealed by the crystal structure of the OXA-10 enzyme from *Pseudomonas aeruginosa*, *Structure 8*, 1289−1298.
29. 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.
30. Paetzel, M., Danel, F., de Castro, L., Mosimann, S. C., Page, M. G. P., and Strynadka, N. C. J. (2000) Crystal structure of the class D beta-lactamase OXA-10, *Nat. Struct. Biol. 7*, 918−925.
31. Golemi, D., Maveyraud, L., Vakulenko, S., Tranier, S., Ishiwata, A., Kotra, L. P., Samama, J. P., and Mobashery, S. (2000) The first structural and mechanistic insights for class D β-lactamases: Evidence for a novel catalytic process for turnover of β-lactam antibiotics, *J. Am. Chem. Soc. 122*, 6132−6133.
32. Tien, M., Berlett, B. S., Levine, R. L., Chock, P. B., and Stadtman, E. R. (1999) Peroxynitrite-mediated modification of proteins at physiological carbon dioxide concentration: pH dependence of carbonyl formation, tyrosine nitration, and methionine oxidation, *Proc. Natl. Acad. Sci. U.S.A. 96*, 7809−7814.
33. Duval, V., Swinnen, M., Lepage, S., Brans, A., Granier, B., Franssen, C., Frere, J. M., and Joris, B. (2003) The kinetic properties of the carboxy terminal domain of the *Bacillus licheniformis* 749/I BlaR penicillin-receptor shed a new light on the derepression of β-lactamase synthesis, *Mol. Microbiol. 48*, 1553−1564.
34. Hanique, S., Colombo, M. L., Goormaghtigh, E., Soumillion, P., Frere, J. M., and Joris, B. (2004) Evidence of an intramolecular interaction between the two domains of the BlaR1 penicillin receptor during the signal transduction, *J. Biol. Chem. 279*, 14264−14272.
35. Jones, R. N. (1996) Impact of changing pathogens and antimicrobial susceptibility patterns in the treatment of serious infections in hospitalized patients, *Am. J. Med. 100*, 3S−12S.
36. Coates, A., Hu, Y. M., Bax, R., and Page, C. (2002) The future challenges facing the development of new antimicrobial drugs, *Nat. Rev. Drug Discov. 1*, 895−910.
37. Fuda, C. C., Fisher, J. F., and Mobashery, S. (2005) β-lactam resistance in *Staphylococcus aureus*: the adaptive resistance of a plastic genome, *Cell Mol. Life Sci. 62*, 2617−2633.
38. Hiramatsu, K., Cui, L., Kuroda, M., and Ito, T. (2001) The emergence and evolution of methicillin-resistant *Staphylococcus aureus*, *Trends Microbiol. 9*, 486−493.
39. Sievert, D. M., Boulton, M. L., Stoltman, G., Johnson, D., Stobierski, M. G., Downes, F. P., Somsel, P. A., Rudrik, J. T., Brown, W., Hafeez, W., Lundstrom, T., Flanagan, E., Johnson, R., and Mitchell, J. (2002) *Staphyloccocus aureus* resistant to vancomycin—United States 2002, *Morbid. Mortal. Weekly Rep. 51*, 565−567.
40. Kirby, W. M. M. (1944) Extraction of a highly potent penicillin inactivator from penicillin resistant staphylococci, *Science 99*, 452−453.
41. Canica, M. M. M., Lu, C. Y., Krishnamoorthy, R., and Paul, G. C. (1997) Molecular diversity and evolution of bla(TEM) genes encoding β-lactamases resistant to clavulanic acid in clinical *E. coli*, *J. Mol. Evol. 44*, 57−65.
42. Nordmann, P., Mariotte, S., Naas, T., Labia, R., and Nicolas, M. H. (1993) Biochemical properties of a carbapenem-hydrolyzing β-lactamase from *Enterobacter cloacae* and cloning of the gene into *Escherichia coli*, *Antimicrob. Agents Chemother. 37*, 939−946.
43. Rasmussen, B. A., and Bush, K. (1997) Carbapenem-hydrolyzing β-lactamases, *Antimicrob. Agents Chemother. 41*, 223−232.
44. Yang, Y. J., Bhachech, N., Bradford, P. A., Jett, B. D., Sahm, D. F., and Bush, K. (1998) Ceftazidime-resistant *Klebsiella pneumoniae* and *Escherichia coli* isolates producing TEM-10 and TEM-43 beta-lactamases from St. Louis, Missouri, *Antimicrob. Agents Chemother. 42*, 1671−1676.
45. Soge, O. O., Adeniyi, B. A., and Roberts, M. C. (2006) New antibiotic resistance genes associated with CTX-M plasmids from uropathogenic Nigerian *Klebsiella pneumoniae*, *J. Antimicrob. Chemother. 58*, 1048−1053.
46. Bradford, P. A., Bratu, S., Urban, C., Visalli, M., Mariano, N., Landman, D., Rahal, J. J., Brooks, S., Cebular, S., and Quale, J. (2004) Emergence of carbapenem-resistant *Klebsiella* species possessing the class A carbapenem-hydrolyzing KPC-2 and inhibitor-resistant TEM-30 beta-lactamases in New York City, *Clin. Infect. Dis. 39*, 55−60.
47. Naas, T., Bogaerts, P., Bauraing, C., Degheldre, Y., Glupczynski, Y., and Nordmanni, P. (2006) Emergence of PER and VEB extended-spectrum β-lactamases in *Acinetobacter baumannii* in Belgium, *J. Antimicrob. Chemother. 58*, 178−182.
48. Oliveira, D. C., Tomasz, A., and de Lencastre, H. (2002) Secrets of success of a human pathogen: molecular evolution of pandemic clones of meticillin-resistant *Staphylocloccus aureus*, *Lancet Infect. Dis. 2*, 180−189.
49. Jevons, M. P. (1961) "Celbenin"-resistant staphylococci, *Br. Med. J. 1*, 124−125.

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