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The Tipper-Strominger Hypothesis and Triggering of Allostery in Penicillin-Binding Protein 2a of Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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Abstract

The transpeptidases involved in the synthesis of the bacterial cell wall (also known as penicillin-binding proteins, PBPs) have evolved to bind the acyl-D-Ala-D-Ala segment of the stem peptide of the nascent peptidoglycan for the physiologically important crosslinking of the cell wall. The Tipper-Strominger hypothesis stipulates that β -lactam antibiotics mimic the acyl-D-Ala-D-Ala moiety of the stem, and thus are recognized by the PBPs with bactericidal consequences. We document that this mimicry exists also at the allosteric site of PBP2a of methicillin-resistant *Staphylococcus aureus* (MRSA). Interactions of different classes of β -lactam antibiotics, as mimics of the acyl-D-Ala-D-Ala moiety at the allosteric site, lead to a conformational change, across a distance of 60 Å to the active site. We directly visualize this change using an environmentally sensitive fluorescent probe affixed to the protein loops that frame the active site. This conformational mobility, documented in real time, allows antibiotic access to the active site of PBP2a. Furthermore, we document that this allosteric trigger enables synergy between two different β -lactam antibiotics, wherein occupancy at the allosteric site by one facilitates occupancy by a second at the transpeptidase catalytic site, thus lowering the minimal-inhibitory concentration. This synergy has important implications for the mitigation of facile emergence of resistance to these antibiotics by MRSA.

The cell wall is a crosslinked polymer that encases the entire bacterium. Its integrity is critical for the survival of the bacterium. The cell wall is a peptidoglycan polymer comprised of a repeating *N*-acetylglucosamine (NAG)-*N*-acetylmuramic acid (NAM) disaccharide, having a stem peptide on each NAM unit. The full-length stem peptide (“pentapeptide stem”) in the Gram-positive bacterium *Staphylococcus aureus* is L-Ala- γ -D-Gln-L-Lys(X)-D-Ala-D-Ala, where (X) is a pentaglycyl extension attached to the ϵ -amine of the L-lysine.¹ Polymerization of Lipid II by the bacterial transglycosylases produces the linear (NAG-

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ASSOCIATED CONTENT

Supporting Information

Supporting figures, monitoring the fluorescent PBP2a emission, antisense MRSA synergy study. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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NAM)_n glycan strand of the peptidoglycan. These strands are subsequently crosslinked to each other, using the stem peptides, through transpeptidase catalysis. These transpeptidases are called penicillin-binding proteins (PBPs) due to their inactivation by covalent bond formation with β -lactam antibiotics (penicillins, cephalosporins, carbapenems). The consequence of failed cell-wall crosslinking is bacterial death.²⁻³

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a variant of *S. aureus* that first emerged in the UK in 1961, and was disseminated globally within two years.⁴ This organism acquired the *mecA* gene encoding a unique transpeptidase, called PBP2a, from a non-*S. aureus* source.⁵⁻⁷ PBP2a resists inhibition by the family of β -lactam antibiotics, and hence it confers broad resistance to MRSA against these antibiotics.^{5,8} The molecular basis for resistance of MRSA to β -lactams is a closed conformation⁹ for the active site of PBP2a that discriminates against the β -lactam inhibitor but permits access to the peptidoglycan substrate.¹⁰ We recently disclosed that the transpeptidase active site opens in response to binding by the nascent peptidoglycan at a peptidoglycan-binding allosteric domain that is 60 Å distant from the active site.¹⁰ The nascent peptidoglycan, synthesized by a partner transglycosylase, engages the allosteric site to initiate a conformational change cascade that opens the active site so as to enable the physiological crosslinking. We showed previously that ceftaroline (Figure 1), a recently approved cephalosporin with activity against MRSA,¹¹ acts as a peptidoglycan mimetic to bind to this allosteric site and to trigger the opening of the active site.^{10,12} This triggering leaves the open active site vulnerable to inhibition by a second molecule of ceftaroline. The X-ray structure for PBP2a confirmed that ceftaroline binds at both sites.¹⁰

Tipper and Strominger argued that the β -lactam antibiotics, and especially the penicillins, mimic the acyl-D-Ala-D-Ala terminus of the pentapeptide stem (Figure 1).¹³ We reasoned that this same mimicry would explain also the recognition of ceftaroline at the allosteric site. Indeed, ceftaroline binds where the acyl-D-Ala-D-Ala segment of the nascent peptidoglycan is predicted to locate (Figure S1).¹⁰ Here we confirm this hypothesis. Moreover, we show that interactions at the allosteric site lead to a conformational change of the two loops that guard the (60 Å distant) active site. We demonstrate that triggering of allostery by ceftaroline allows inhibition of the active site of PBP2a—the first transpeptidase identified as regulated by allostery—by other β -lactam antibiotics, which are otherwise ineffective. The structure of the β -lactam antibiotic mimics a particular conformation of the acyl-D-Ala-D-Ala segment of the nascent peptidoglycan (Figure 1 and Figure S1). Nascent peptidoglycan is generated at sites of active cell-wall biosynthesis. Maturation of the cell wall by crosslinking generates a tetrapeptide stem peptide by acyl-transfer (by loss of the terminal D-Ala) and a tripeptide stem (by loss of both terminal D-Ala residues) as the most abundant forms of the cell wall.^{14,15} The presence of the stem pentapeptide in the mature cell wall is rare. Hence, the β -lactam has evolved to mimic the full-length stem peptide and to subvert catalysis by the PBP.¹⁶ Whereas it has been noted that β -lactams might better mimic the transition state for active-site acylation of PBPs,¹⁷⁻¹⁹ the mimicry that we document here for binding at the allosteric site does not involve covalency. PBP2a uses the presence of nascent peptidoglycan (containing the pentapeptide) to trigger the allosteric opening of the active site to allow transpeptidase catalysis.¹⁰ We had shown previously that the presence of a synthetic

peptidoglycan fragment enhances the acylation rate of PBP2a, as the first evidence of allosteric control of catalysis by a PBP.²⁰ The mimicry of this process by ceftaroline accounts for its recognition at the allosteric site. The question is whether ceftaroline recognition at the allosteric site is unique to ceftaroline, or if it is a general principle applicable to all β -lactam antibiotics. This question is addressed in the present report and would appear to be a general principle.

Results and Discussion

Determination of K_d for β -lactam binding at allosteric site

To test our hypothesis that the allosteric site of PBP2a has evolved to recognize β -lactams as mimics of the peptidoglycan, we investigated binding by β -lactam antibiotics other than ceftaroline to the allosteric site of PBP2a. We reported recently a methodology for this analysis using PBP2a inactivated by the penicillin oxacillin (as a result of acylation of serine-403 at the transpeptidase active site).²¹ Analysis of X-ray structures of PBP2a acylated at the active site by β -lactams (nitrocefin, penicillin G, methicillin, ceftaroline and ceftobiprole) shows that this acylation does not perturb the allosteric site.^{9,10,22} The intrinsic fluorescence of the protein within the allosteric site of this modified PBP2a is modulated by the presence of other β -lactam antibiotics. A saturable decrease in protein fluorescence upon binding of the β -lactam to the allosteric site was seen in all cases. Previously we determined that the K_d value for ceftaroline binding to the allosteric site of the wild-type PBP2a was $20 \pm 4 \mu\text{M}$.²¹ All of the other β -lactams we now tested—cefepime (a cephalosporin), piperacillin (a penicillin), imipenem (a carbapenem), and ceftazidime (another cephalosporin)—bind to the allosteric site in a saturable manner, for which the K_d values are 130 ± 50 , 400 ± 70 , 420 ± 80 and $170 \pm 20 \mu\text{M}$, respectively. These K_d values are ~ 7 - to 21-fold larger than the value for ceftaroline. The fact that binding of all these β -lactams to the allosteric site of PBP2a is saturable indicates that the allosteric site has evolved to bind nascent peptidoglycan, which these antibiotics mimic. Yet cefepime, piperacillin, imipenem, and ceftazidime are ineffective as antibiotics to treat MRSA infections, because concentrations above their respective dissociation constants for the allosteric site cannot be attained *in vivo*.^{23–26} This outcome contrasts with the example of ceftaroline.^{11,27,28} Hence, many typical β -lactams are unable to trigger allostery, notwithstanding their structural mimicry for recognition at the allosteric site.

Real-time monitoring of conformational change of fluorescently labeled PBP2a

Among the tested β -lactams, ceftaroline binds best to the allosteric site ($K_d = 20 \pm 4 \mu\text{M}$ versus values in the range of 130–420 μM for others). This ability explains the exceptional utility of ceftaroline as an anti-MRSA agent, an ability vested in the unique structural features of ceftaroline. We documented earlier by X-ray analysis that binding of ceftaroline at the allosteric site of PBP2a propagated a conformational change cascade across the 60 Å breadth of distance between the allosteric and the active sites.¹⁰ This conformational change was likened to dominoes falling so as to ultimately open the active site.¹⁰ The active site is framed by two loops, comprised of amino acids 417–454 and 603–613 (Figure 2A). We prepared two fluorescent versions of PBP2a, by separate tagging of these loops, in order to explore whether binding of ceftaroline (or of other antibiotics) to the allosteric site would

affect conformational change at the active site in solution. We individually mutated an amino acid in the middle of each loop to a cysteine (Y446C and Q607C). The two mutant proteins were purified, and the thiol of their respective cysteines was modified with an environmentally sensitive fluorescent probe, 7-diethylamino-3-(((2-maleimidyl)ethyl)amino)carbonyl)coumarin (MDCC, Figure 2B,C). We monitored the change in fluorescent emission ($\lambda_{\text{ex}} = 419 \text{ nm}$ and $\lambda_{\text{em}} = 480 \text{ nm}$) over time in the absence and in the presence of β -lactam antibiotics (ceftaroline, imipenem, ceftazidime, and oxacillin). Because MDCC is an environmentally sensitive fluorophore, movement of the loops changes the environment around the fluorophore, which results in an increase or decrease in the fluorescent emission. An increased fluorescence emission for both modified PBP2a proteins followed incubation with imipenem, ceftazidime, and oxacillin (Figures S2–S3). With ceftaroline, however, we saw an increase in fluorescence of the modified Q607C mutant, but a decrease in fluorescence of the modified Y446C mutant (Figure 2D). These results indicate that the protein conformational change with ceftaroline is different than with the other β -lactams. Moreover—and congruent with our documentation of higher affinity of PBP2a for ceftaroline at the allosteric site—the changes in fluorescence were seen at lower concentrations of ceftaroline, and in a shorter duration. The shorter duration seen for ceftaroline, as compared to the other β -lactams tested, is in the time frame for the allosteric response. The process observed here by fluorescent spectroscopy is complicated in that it is triggered by a non-covalent binding of one antibiotic at the allosteric site, followed by a first-order conformational change, which leads to binding of another identical antibiotic molecule in a covalent manner at the active site. These events could not be fit to a simple first-order process. These results are also consistent with the effectiveness of ceftaroline both as an antibiotic and as an allosteric-triggering ligand. MRSA strains that express PBP2a are susceptible to ceftaroline, but are resistant to the other β -lactams.

Synergy of ceftaroline and a second β -lactam as antimicrobial agents

Having documented that ceftaroline is the best allosteric ligand (among those β -lactams tested) and that the binding of ceftaroline entails observable conformational changes at the active-site, we wondered whether the ceftaroline-induced conformational change rendered the active site more vulnerable to inhibition by other β -lactams. If so, ceftaroline might synergize the antibacterial activity of these otherwise ineffective antibiotics. Using the checkerboard synergy assay²⁹ we determined the MIC values of each antibiotic (cefepime, ceftazidime, oxacillin, piperacillin, imipenem, meropenem) alone and in the presence of ceftaroline (Table 1) with three MRSA strains (NRS70, NRS100, and NRS123), all of which express a PBP2a having the identical sequence as our recombinant PBP2a. We calculated the fractional-inhibitory concentration index (FICI) for the antibiotics in combination with one-fourth the MIC of ceftaroline (0.25 $\mu\text{g/mL}$). The results were interpreted as synergy (S), indifference (I), or antagonism (A).²⁹ We saw either synergy between ceftaroline and the other β -lactams, or indifference. Antagonism was not seen. The synergistic effect, which was a 4- to 16-fold decrease of the MIC of the second antibiotic, was seen in thirteen out of the eighteen possible permutations. The greatest synergistic effect exerted by ceftaroline was seen for imipenem and meropenem (with all three strains), cefepime (NRS70 and NRS123), and oxacillin (NRS100) (Table 1).

The molecular basis for this synergy was investigated using a methodology reported by Roemer and colleagues.^{30,31} The background strain MRSA COL was transformed with anti-sense interference plasmids that upon induction with xylose tuned down expression of various PBPs individually. When ceftaroline was used in combination with meropenem, imipenem, or oxacillin, the synergistic effect was seen (Figure S4) in the absence of xylose induction, as documented also by the checkerboard method. The attendant diminution of expression of PBP2a in the presence of xylose induction caused sensitization to the individual antibiotics with little to no synergy for the antibiotic combinations. This observation supports PBP2a as the instigator of the synergistic effect due to its allosteric regulation. A similar result was seen upon decreased expression of PBP2. This result can be rationalized by the fact that PBP2 and PBP2a work cooperatively in MRSA in the presence of β -lactam antibiotic challenge.³² However, attenuated expression of either PBP1 or PBP3 did not alter the observation of the degree of synergism seen in the absence of induction, indicating that neither enzyme is involved in manifestation of synergism.

Does this synergy result from communication between the allosteric and active sites of PBP2a? Analysis by mass spectrometry supports a positive answer. At 100 μ M imipenem, a concentration approximately four-fold lower than the dissociation constant for the allosteric site (as evaluated in the present report), imipenem was unable to acylate the Ser-403 of the active site. However, in the presence of 20 μ M ceftaroline (a value equal to its dissociation constant for the allosteric site) and at the same concentration of imipenem (100 μ M), acylation of the active site occurred by both ceftaroline and imipenem. Ceftaroline serves as the allosteric trigger enabling access to the active site by both antibiotics (Figure 3). This experiment also rules out the possibility that there exist two populations of PBP2a at all times, one with an open and another with a closed active site. If co-existing populations were present, then some acylation of the active site by imipenem should have been observed in the absence of ceftaroline. This result is not seen. A further advantage of the combination of a second β -lactam with ceftaroline might be complementary inhibition of the other PBPs in MRSA, as previously noted in a different context.³³

Whereas emergence of resistance for any antibiotic is inevitable, our observation of synergy has the important implication of suggesting that the facile emergence of resistance might be forestalled by combination β -lactam chemotherapy. Recently, mutations within the allosteric site were discovered in clinical isolates with elevated MICs (2- to 32-fold) for ceftaroline.^{34–37} This elevation is likely due to the mutations interfering with the propagation of the allosteric response.²¹ The use of a synergistic mixture of ceftaroline and another β -lactam antibiotic in MRSA infections by such mutant variants could be treatable, as the MICs would remain below the break points for resistance. Such mutant strains would not be isolated as *bona fide* resistance mutants.

Conclusion

The findings presented here emerged from the discovery of allosteric regulation of PBP2a in MRSA. The triggering of allostery by ceftaroline sheds light on the prospect of both non-traditional structure-activity development for β -lactams targeting PBPs subject to allosteric control,³⁸ and to non- β -lactam compounds that could bind to the allosteric site and elicit the

same potentiation of β -lactam efficacy against MRSA infection. There is considerable interest in potentiators of antibiotics, in light of the profound clinical problems with resistance.^{31,39–41} Our studies also indicate that binding to the acyl-D-Ala-D-Ala terminus of the pentapeptide of the nascent peptidoglycan has driven not only evolution of the transpeptidase active site for its substrate, but also that of the allosteric site. The existence of the allosteric domain is presently documented only in PBP2a, but no doubt that other PBP examples in pathogenic bacteria await discovery.

The literature on PBP2a is replete with the assertion that its active site has “low affinity” for β -lactam antibiotics. Our results indicate that this assertion is an oversimplification. The Tipper-Strominger hypothesis is as valid for the allosteric site as it is for the active site of PBP2a. The high *in vitro* concentration of a given β -lactam antibiotic (500 μ M oxacillin, for example, as used here) to achieve acylation of the active site does not indicate low affinity for the active site, and also reflects allosteric control of the active-site affinity. Each of the very few β -lactams that have progressed to clinical approval for MRSA chemotherapy, or have been considered for the purpose, has the particular ability to effectively inhibit PBP2a. Their structures have arisen from empirical structure-activity study. Although it is premature to generalize that each of these β -lactams has acquired this ability by usurping the allosteric site, as we demonstrate here for ceftaroline, the fluorescent methodology we show in Figure 2 has the potential to answer this question.

Currently, there does not exist an experimental method for monitoring the effect of the allosteric regulation on the turnover kinetics of the PBP2a enzyme. Nonetheless, our results support a sequential model for allostery,^{42–45} wherein non-covalent ligand binding at the allosteric site initiates a conformational change that opens the active site to either substrate, or to the β -lactam inhibitor.

As a result of the successful development of agents such as ceftaroline, clinical control of *mecA* (PBP2a) MRSA infection is possible. Bacterial resistance is not static, however, as emphatically underscored by the recent emergence of a *mecA* variant (*mecC*, PBP2a') MRSA.^{46,47} We anticipate that our experimental approach likewise will clarify the basis for allosteric control in PBP2a resistance mutants, as well as guide drug discovery whether as single agent or as dual potentiation agents strategies.

Experimental Procedures

Cloning of the Y446C and Q607C mutant versions of PBP2a

In order to probe the conformational change that takes place around the active site, one residue on the α 2– α 3 loop (Tyr446) and one on the β 3– β 4 loop (Gln607) were mutated to cysteine, so that the residue could be labeled with a fluorescent probe. We chose the environmentally sensitive, thiol-reactive fluorescent MDCC (7-diethylamino-3-(((2-maleimidyl)-ethyl)amino)carbonyl)coumarin) for this purpose. Site-directed mutagenesis was done to produce the Q607C mutant using the wild-type *mecA* gene in pET24d(+) as template and primers Q607Cfwd (5'-CAGAACTCAAAATGAAATGCGGAGAACTGGCAGAC-3') and Q607Crev (5'-GTCTGCCAGTTTCTCCGCATTTTATTTGAGTTCTG-3'). To introduce the Y446C

mutation, the following procedure was performed: the 5' half of *mecA* was amplified using forward primer that introduced an NcoI site (5'-TACCATGGCTTCAAAAGATAAAGAAATTAATAATAC-3') and reverse primer that introduced the Y446C mutation and a HincII site by silent mutagenesis (5'-ATTACCGTTAACCACTTCACATCTTGTAACGTTG-3') to make PCR product #1. The 3' half of *mecA* was amplified using a forward primer to introduce a HincII site by silent mutagenesis (5'-GAAGTGGTTAACGGTAATATCGAC-3') and a reverse primer with the stop codon and HindIII site (5'-CCGCAAGCTTTTATTCATCTATATCG-3') to make PCR product #2. The resulting PCR products were digested with NcoI/HpaI or HpaI/HindIII and then ligated into pET24d(+) that had been digested with NcoI and HindIII. The desired mutation was confirmed by DNA sequencing of the entire gene on both strands and *Escherichia coli* DH5a and BL21 Star (DE3) were transformed by the plasmid for expression. Mutant proteins were expressed and purified as previously described for the wild-type PBP2a.²¹

Fluorescent labeling of Y446C and Q607C mutant PBP2a

The mutations Y446C or Q607C introduced the only cysteine residue in the protein. To prepare the Y446C and Q607C PBP2a mutants for fluorescent labeling, the Cys was reduced by extensive dialysis against 25 mM HEPES (pH 7.0), 1 M NaCl, containing 1 mM TCEP (tris(2-carboxyethyl)phosphine). The reduced protein was then incubated with an excess of MDCC overnight at 4 °C, protected from light. Excess MDCC was removed by a desalting spin column (Thermo Scientific) and labeling was confirmed by fluorescent imaging of an SDS-PAGE gel (Figure 2C) and by mass spectrometry. The fluorescently labeled proteins, Y446C-MDCC and Q607C-MDCC, were quantified by A_{280} (ϵ_{280} Y446C = 96835 M⁻¹cm⁻¹, ϵ_{280} Q607C = 98325 M⁻¹cm⁻¹) and were used immediately in experiments.

Fluorescent monitoring of active-site conformational changes

The fluorescence of the Y446C-MDCC and Q607C-MDCC enzymes in the absence and presence of β -lactam antibiotics was monitored by excitation of the MDCC moiety at 419 nm using a Cary Eclipse Fluorometer. Emission scans were taken every minute for 45 min at room temperature of 500 nM protein in 25 mM HEPES (pH 7.0), 1 M NaCl. The fluorescent emission was stable for protein alone for the 45 minutes of the experiments. Various amounts of ceftaroline, oxacillin, ceftazidime, and imipenem were added to the protein prior to the emission scans.

Dissociation constants for β -lactam antibiotics (cefepime, piperacillin, imipenem, and ceftazidime) at the allosteric site of the wild-type PBP2a were determined as previously described for ceftaroline.²¹

Antibiotic-susceptibility tests

Minimal-inhibitory concentrations (MICs) of ceftaroline, cefepime, ceftazidime, oxacillin, piperacillin, imipenem, and meropenem against methicillin-resistant *Staphylococcus aureus* strains NRS70 (N315), NRS100 (COL), and NRS123 (MW2, USA400) were determined according to Clinical and Laboratory Standards Institute guidelines using serial dilutions in a 96-well microplate.⁴⁸ The wells were inoculated to a final concentration of 5×10^5

CFU/mL. The effect of combination of other β -lactam antibiotics with ceftaroline was determined using a checkerboard assay.²⁹ All experiments were done at least in triplicate. The fractional-inhibitory-concentration index (FICI) values for combinations of ceftaroline with cefepime, ceftazidime, oxacillin, piperacillin, imipenem, and meropenem were determined by equation 1:

$$FICI = FIC_{CFT} + FIC_{AB} = \frac{[CFT]}{MIC_{CFT}} + \frac{[AB]}{MIC_{AB}} \quad (1)$$

where FIC is the fractional-inhibitory concentration, [CFT] is the concentration of ceftaroline, MIC_{CFT} is the minimal-inhibitory concentration of ceftaroline alone, [AB] is the concentration of the second antibiotic, and MIC_{AB} is the MIC of the second antibiotic alone.

Synergy study using antisense MRSA strains

The MRSA COL strains that were transformed with plasmids that contain xylose-inducible antisense interference fragments for *pbpA* (which encodes for PBP1), *pbp2* (PBP2), *mecA* (PBP2a), or *pbp3* (PBP3) were developed by Roemer et al.^{30,31} and were a generous gift to us. LB/agar plates containing 34 μ g/mL chloramphenicol, with and without 50 mM xylose, were seeded with 1×10^7 CFU/mL of each strain and allowed to dry. Ceftaroline (0.01 μ g), meropenem (6.25 μ g), imipenem (12.5 μ g), and oxacillin (100 μ g) were added by spotting the plate with 10 μ L of the antibiotic alone and in combination. The plates were incubated at 36 °C overnight. Zones of inhibition were visualized and provided the basis to compare combinations of antibiotics (Figure S4). When compared to the inhibition by antibiotic alone, the three β -lactams (meropenem, imipenem, and oxacillin) in combination with ceftaroline each gave larger zones of inhibition in the absence of xylose, indicating a synergistic effect. On plates containing xylose, in which PBP2 or PBP2a expression was decreased, synergy was seen to a lesser extent due to hypersensitization of the organism. There was no change seen for the PBP1 or PBP3 strains, indicating their lack of involvement in the synergistic effect.

LC/MS analysis of β -lactam-acylated PBP2a

Wild-type PBP2a was incubated with a sub- K_d concentration of imipenem (100 μ M) in the absence or presence of 20 μ M ceftaroline (at K_d for the allosteric site) at room temperature for 45 min. The sample was then zip-tipped, diluted, and injected into a Dionex RSLC ultra-high pressure liquid chromatograph equipped with a Phenomenex Aeris Widepore C4 column (150 $\times$ 2.1 mm; 3.4 μ m particle size) coupled to a Bruker microTOF-QII electrospray mass spectrometer. The data were analyzed using Compass Data Analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CFT	ceftaroline
CPM	cefepime
PIP	piperacillin
IPM	imipenem
CAZ	ceftazidime
FICI	fractional-inhibitory concentration index
OXA	oxacillin
MER	meropenem
MIC	minimal-inhibitory concentration

Alternative name for the *S. aureus* strain NRS70 is N315, for strain NRS100 is COL, and for strain NRS123 are MW2 and USA400.

REFERENCES

1. Figueiredo TA, Sobral RG, Ludovice AM, Almeida JM, Bui NK, Vollmer W, Lencastre H, Tomasz A. PLoS Pathog. 2012; 8:e1002508. [PubMed: 22303291]
2. Leemans, E.; Fisher, J.; Mobashery, S. The β -Lactam Antibiotics: Their Future in the Face of Resistance. In: Marinelli, F.; Genilloud, O., editors. Antimicrobials. Springer Berlin Heidelberg: 2014. p. 59-84.
3. Yao Z, Kahne D, Kishony R. Mol. Cell. 2012; 48:705–712. [PubMed: 23103254]
4. Jevons MP. BMJ. 1961; 1(5219):124–125.
5. Fuda C, Suvorov M, Vakulenko SB, Mobashery S. J. Biol. Chem. 2004; 279(39):40802–40806. [PubMed: 15226303]
6. Fuda C, Suvorov M, Shi Q, Hesek D, Lee M, Mobashery S. Biochemistry. 2007; 46(27):8050–8057. [PubMed: 17567045]
7. de Lencastre H, Oliveira D, Tomasz A. Curr. Opin. Microbiol. 2007; 10:428–435. [PubMed: 17921044]
8. Llarrull LI, Fisher JF, Mobashery S. Antimicrob. Agents Chemother. 2009; 53:4051–4063. [PubMed: 19470504]
9. Lim D, Strynadka NC. Nat. Struct. Biol. 2002; 9(11):870–876. [PubMed: 12389036]
10. Otero LH, Rojas-Altuve A, Llarrull LI, Carrasco-Lopez C, Kumarasiri M, Lastochkin E, Fishovitz J, Dawley M, Hesek D, Lee M, Johnson JW, Fisher JF, Chang M, Mobashery S, Hermoso JA. Proc. Natl. Acad. Sci. U.S.A. 2013; 110(42):16808–16813. [PubMed: 24085846]
11. Saravolatz L, Stein G, Johnson L. Clin. Infect. Dis. 2011; 52(9):1156–1163. [PubMed: 21467022]
12. Villegas-Estrada A, Lee M, Hesek D, Vakulenko SB, Mobashery S. J. Am. Chem. Soc. 2008; 130(29):9212–9213. [PubMed: 18582062]
13. Tipper DJ, Strominger JL. Proc. Natl. Acad. Sci. U.S.A. 1965; 54(4):1133–1141. [PubMed: 5219821]
14. Lee M, Hesek D, Llarrull LI, Lastochkin E, Pi H, Boggess B, Mobashery S. J. Am. Chem. Soc. 2013; 135(9):3311–3314. [PubMed: 23421439]
15. Reith J, Mayer C. Appl. Microbiol. Biotechnol. 2011; 92(1):1–11. [PubMed: 21796380]

16. de Jonge BLM, Gage D, Xu N. *Antimicrob. Agents Chemother.* 2002; 46(10):3151–3155. [PubMed: 12234837]
17. Lee B. J. *Mol. Biol.* 1971; 61:463–469. [PubMed: 4257008]
18. Boyd DB. *Proc. Natl. Acad. Sci. U.S.A.* 1977; 74(12):5239–5243. [PubMed: 271949]
19. Silvaggi NR, Josephine HR, Kuzin AP, Nagarajan R, Pratt RF, Kelly JA. *J. Mol. Biol.* 2005; 345:521–533. [PubMed: 15581896]
20. Fuda C, Heseck D, Lee M, Morio K, Nowak T, Mobashery S. *J. Am. Chem. Soc.* 2005; 127:2056–2057. [PubMed: 15713078]
21. Fishovitz J, Rojas-Altuve A, Otero L, Dawley M, Carrasco-López C, Chang M, Hermoso J, Mobashery S. *J. Am. Chem. Soc.* 2014; 136:9814–9817. [PubMed: 24955778]
22. Lovering AL, Gretes MC, Safadi SS, Danel F, de Castro L, Page MG, Strynadka NC. *J. Biol. Chem.* 2012; 287(38):32096–32102. [PubMed: 22815485]
23. Asín-Prieto E, Rodríguez-Gascón A, Trocóniz IF, Soralue A, Maynar J, Sánchez-Izquierdo JA, Isla A. *J. Antimicrob. Chemother.* 2014; 69(1):180–189. [PubMed: 23908259]
24. Bernabeu-Wittel M, Pichardo C, García-Curiel A, Pachón-Ibáñez ME, Ibáñez-Martínez J, Jiménez-Mejías ME, Pachón J. *Clin. Microbiol. Infect.* 2005; 11(4):319–325. [PubMed: 15760430]
25. Conil J-M, Georges B, Ravat F, Ruiz S, Seguin T, Metsu D, Fourcade O, Saivin S. *Clin. Ther.* 2013; 35(10):1603–1612. [PubMed: 24094465]
26. Walker P, Neuhauser MN, Tam VH, Willey JS, Palmer JL, Bruera E, Prince RA. *J. Pain Symptom Manage.* 2005; 30(2):170–174. [PubMed: 16125032]
27. Kaushik D, Rathi S, Jain A. *Int. J. Antimicrob. Agents.* 2011; 37(5):389–395. [PubMed: 21420284]
28. MacGowan AP, Noel AR, Tomaselli S, Bowker KE. *Antimicrob. Agents Chemother.* 2013; 57(6):2451–2456. [PubMed: 23459495]
29. Eliopoulos, GM.; Moellering, RC, Jr. *Antimicrobial Combinations.* In: Lorian, V., editor. *Antibiotics in Laboratory Medicine.* 3rd ed.. Baltimore, MD: Williams & Wilkins; 1991. p. 432–492.
30. Lee SH, Jarantow LW, Wang H, Sillaots S, Cheng H, Meredith TC, Thompson J, Roemer T. *Chem. Biol.* 2011; 18:1379–1389. [PubMed: 22118672]
31. Tan CM, Therien AG, Lu J, Lee SH, Caron A, Gill CJ, Lebeau-Jacob C, Benton-Perdomo L, Monteiro JM, Pereira PM, Elsen NL, Wu J, Deschamps K, Petcu M, Wong S, Daigneault E, Kramer S, Liang L, Maxwell E, Claveau D, Vaillancourt J, Skorey K, Tam J, Wang H, Meredith TC, Sillaots S, Wang-Jarantow L, Ramtohul Y, Langlois E, Landry F, Reid JC, Parthasarathy G, Sharma S, Baryshnikova A, Lumb KJ, Pinho MG, Soisson SM, Roemer T. *Sci Transl Med.* 2012; 4:126–135.
32. Pinho MG, de Lencastre H, Tomasz A. *Proc. Natl. Acad. Sci. U.S.A.* 2001; 98(19):10886–10891. [PubMed: 11517340]
33. Memmi G, Filipe SR, Pinho MG, Fu Z, Cheung A. *Antimicrob. Agents Chemother.* 2008; 52:3955–3966. [PubMed: 18725435]
34. Mendes RE, Tsakris A, Sader HS, Jones RN, Biek D, McGhee P, Appelbaum PC, Kosowska-Shick K. *J. Antimicrob. Chemother.* 2012; 67(6):1321–1324. [PubMed: 22398650]
35. Alm RA, McLaughlin RE, Kos VN, Sader HS, Iaconis JP, Lahiri SD. *J. Antimicrob. Chemother.* 2014; 69(8):2065–2075. [PubMed: 24777906]
36. Long SW, Olsen RJ, Mehta SC, Palzkill T, Cernoch PL, Perez KK, Musick WL, Rosato AE, Musser JM. *Antimicrob. Agents Chemother.* 2014; 58:6668–6674. [PubMed: 25155594]
37. Kelley WL, Jousset A, Barras C, Lelong E, Renzoni A. *Antimicrob. Agents Chemother.* 2015; 59(4):1922–1930. [PubMed: 25583724]
38. Dave K, Palzkill T, Pratt RF. *ACS Med. Chem. Lett.* 2014; 5:154–157. [PubMed: 24900789]
39. Cox G, Wright GD. *Int. J. Med. Microbiol.* 2013; 303(6–7):287–292. [PubMed: 23499305]
40. Roemer T, Boone C. *Nat. Chem. Biol.* 2013; 9(4):222–231. [PubMed: 23508188]
41. Podoll JD, Liu Y, Chang L, Walls S, Wang W, Wang X. *Proc. Natl. Acad. Sci. U.S.A.* 2013; 110:15573–15578. [PubMed: 24019472]
42. Nussinov R, Tsai C-J. *Cell.* 2013; 153(2):293–305. [PubMed: 23582321]

43. Whitley MJ, Lee AL. Curr. Protein Pept. Sci. 2009; 10(2):116–127. [PubMed: 19355979]
44. Cui Q, Karplus M. Protein Sci. 2008; 17(8):1295–1307. [PubMed: 18560010]
45. Goodey NM, Benkovic SJ. Nat Chem Biol. 2008; 4(8):474–482. [PubMed: 18641628]
46. Kim C, Milheirico C, Gardete S, Holmes MA, Holden MT, de Lencastre H, Tomasz A. J. Biol. Chem. 2012; 287:36854–36863. [PubMed: 22977239]
47. Paterson GK, Harrison EM, Holmes MA. Trends Microbiol. 2014; 22:42–47. [PubMed: 24331435]
48. CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. CLSI document M100-S22. Wayne, PA: Clinical and Laboratory Standards Institute;

Figure 1.

The chemical structure of ceftaroline (as a representative cephalosporin) and its mimicry of the acyl-D-Ala-D-Ala terminus of the peptidoglycan pentapeptide (colored in blue) are shown. Chemical structures of a penicillin and a carbapenem are also shown. The mimicry is preserved across these three templates of the β -lactam family of antibiotics.

Figure 2.

(A) The closed conformation of PBP2a with the side chains of Y446 and Q607 shown as capped sticks in black, indicated by arrows at 1 o'clock. (B) Reaction of the fluorescent label MDCC with the free thiol of Y446C or Q607C. (C) Coomassie stain and fluorescent scan of PBP2a-Y446C mutant before and after labeling with MDCC, followed by a desalting column to remove excess MDCC. Labeling of Q607C was confirmed in the same way. (D) Fluorescent emission scans of PBP2a-Y446C-MDCC or PBP2a-Q607C-MDCC in

the absence (black) and in the presence of 20 μM ceftaroline after 5 (red), 10 (blue), and 15 (green) minutes.

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Figure 3.

Deconvoluted mass spectra from LC/MS analysis of the wild-type PBP2a in the presence of 100 μ M imipenem (IPM) and 20 μ M ceftaroline (CFT) (top) or no ceftaroline (CFT) (bottom). In the absence of ceftaroline, only the molecular ion for the apoenzyme is seen.

Table 1

Susceptibility (MIC, $\mu\text{g/mL}$) of three *mecA*-dependent MRSA strains to β -lactam antibiotics alone and in combination with one-fourth the MIC of ceftaroline (0.25 $\mu\text{g/mL}$).

NRS70				NRS100				NRS123			
MIC ($\mu\text{g/mL}$)				MIC ($\mu\text{g/mL}$)				MIC ($\mu\text{g/mL}$)			
-CFT	+CFT	FICI ^a		-CFT	+CFT	FICI ^a		-CFT	+CFT	FICI ^a	
CFT	1	-	-	-	1	-	-	-	1	-	-
CPM	64	8	0.38	S	256	128	0.75	I	64	8	0.38
CAZ	256	128	0.75	I	512	512	1.25	I	128	8	0.31
OXA	32	8	0.5	S	256	32	0.38	S	32	8	0.5
PIP	32	32	1.25	I	128	32	0.5	S	128	64	0.75
IPM	1	0.06	0.5	S	64	8	0.38	S	1	0.03	0.28
MER	4	1	0.5	S	32	2	0.31	S	4	0.13	0.28

^aFICI = [CFT]/MIC_{CFT} + [drug]/MIC_{drug}

CFT, ceftaroline; CPM, ceftipime; CAZ, ceftazidime; OXA, oxacillin; PIP, piperacillin; IPM, imipenem; MER, meropenem

S synergy, FICI 0.5; I indifference, 0.5 < FICI 4