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Resistance to the Third-Generation Cephalosporin Ceftazidime by a Deacylation-Deficient Mutant of the TEM β-Lactamase by the Uncommon Covalent-Trapping Mechanism

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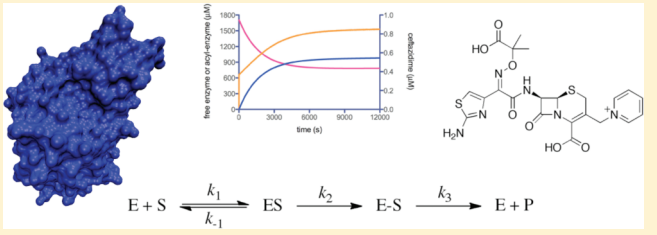
ABSTRACT: The Glu166Arg/Met182Thr mutant of *Escherichia coli* $TEM_{pTZ19-3}$ β-lactamase produces a 128-fold increase in the level of resistance to the antibiotic ceftazidime in comparison to that of the parental wild-type enzyme. The single Glu166Arg mutation resulted in a dramatic decrease in both the level of enzyme expression in bacteria and the resistance to penicillins, with a concomitant 4-fold increase in the resistance to ceftazidime, a third-generation cephalosporin. Introduction of the second amino acid substitution, Met182Thr, restored enzyme expression to a level comparable to that of the wild-type enzyme and resulted in an additional 32-fold increase in the minimal inhibitory concentration of ceftazidime to 64 μg/mL. The double mutant formed a stable covalent complex with ceftazidime that remained intact for the entire duration of the monitoring, which exceeded a time period of 40 bacterial generations. Compared to those of the wild-type enzyme, the affinity of the $TEM_{pTZ19-3}$ Glu166Arg/Met182Thr mutant for ceftazidime increased by at least 110-fold and the acylation rate constant was augmented by at least 16-fold. The collective experimental data and computer modeling indicate that the deacylation-deficient Glu166Arg/Met182Thr mutant of $TEM_{pTZ19-3}$ produces resistance to the third-generation cephalosporin ceftazidime by an uncommon covalent-trapping mechanism. This is the first documentation of such a mechanism by a class A β-lactamase in a manifestation of resistance.



β-Lactams are the most widely used antimicrobial agents.[1] They target the bacterial penicillin-binding proteins, enzymes responsible for the synthesis and maturation of the peptidoglycan, the major component of the bacterial cell wall.[2,3] There are several mechanisms of resistance to this class of antimicrobials,[4,5] but the most common is the enzymatic inactivation of the drug by β-lactamases, a group of enzymes capable of hydrolyzing the β-lactam ring of the antibiotics.[4,5] On the basis of similarities in their amino acid sequences, β-lactamases are divided into four classes. Members of classes A, C, and D possess a critical active-site serine residue involved in the turnover chemistry, whereas class B enzymes are zinc-dependent.[4,6]

The TEM-type β-lactamases are typical class A enzymes and some of the most studied and common in Gram-negative bacteria. The TEM-1 β-lactamase has excellent activity with penicillins and early-generation cephalosporins as substrates and is capable of inactivating some of these β-lactams with near catalytic perfection.[4,7] On the other hand, TEM-1 has little activity against third- and fourth-generation cephalosporins, such as ceftazidime and cefepime.[8] Mutations in several positions of the enzyme are responsible for increased catalytic activity against these antimicrobials and for resistance to β-lactamase inhibitors, turning the enzyme into an extended-spectrum or inhibitor-resistant β-lactamase.[2,8]

β-Lactamases cleave the β-lactam bond of the antibiotic, rendering it inactive, and do this by using a set of conserved amino acids within the active site of the enzyme. The catalytic mechanism proceeds with the formation of the noncovalent Michaelis complex, which leads to the formation of an acyl—enzyme species, which in turn undergoes hydrolysis.[2] While the details of the acylation mechanism are still the subject of debate,[9−14] there is consensus regarding the deacylation mechanism in which Glu166 (Ambler numbering[15]), a strictly conserved residue located within the so-called Ω-loop, plays the role of a catalytic base promoting a water molecule for the hydrolytic reaction.[16−18]

Increased resistance to third-generation cephalosporins by class A β-lactamases is typically achieved through increased affinity and improved hydrolytic activity of the mutant enzyme.[19,20] Several publications have reported on an unusual phenotype produced by mutants of TEM β-lactamases in which Glu166, the aforementioned residue critical in the deacylation step, has been mutated. Such mutations result in a loss of activity toward penicillins and early-generation cephalosporins but would appear to increase the activity against the clinically used third-generation cephalosporins, especially ceftazidime.[21,22] The discoveries of these mutant enzymes at the time were curiosities, and because of their marked instability, they have not been further characterized. Consequently, the mechanism of ceftazidime resistance in these cases remains unexplained.[21] Here we report our study of the deacylation-deficient $TEM_{pTZ19-3}$

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β-lactamase harboring Glu166Arg and Met182Thr substitutions that produces a significant 128-fold increase in the resistance to ceftazidime relative to that of the wild-type enzyme. We present evidence that this double mutant enzyme confers resistance to ceftazidime by the unusual covalent-trapping mechanism of the antibiotic.

MATERIALS AND METHODS

Strains and Plasmids. *Escherichia coli* JM83 was used to propagate plasmid DNA and to perform antibiotic susceptibility studies. Mutagenesis of the gene was performed using plasmid pTZ19-3, which has been described previously.[23] Induction of protein expression was performed in *E. coli* BL21(DE3) cells harboring a pET24a(+) vector (Invitrogen) with the indicated genes cloned between the *Nde*I and *Hin*dIII sites. For the assessment of enzyme expression, a vector for constitutive expression of C-terminally six-His-tagged proteins was constructed. This vector, pHF186, contains the pBR origin of replication, a kanamycin antibiotic selection marker, and a constitutive expression promoter. A synthetic DNA fragment encoding a hexahistidine tag and *Msc*I site was cloned into the unique *Nde*I and *Hin*dIII sites of pHF016[24] to generate plasmid pHF186. This vector allows the addition of a C-terminal hexahistidine tag to any protein of interest by cloning the gene between *Nde*I and *Msc*I. As digestion with *Msc*I would remove the first two base pairs of the hexahistidine tag, the PCR-amplified gene of interest must contain CA at the 3′ end to regenerate the first histidine codon.

Table 1. Primers Used in This Study

primer	sequence (5′−3′)[a]
166D	CTCGCCTTGATCGTTGG**NNN**CCGG-AGCTGAATGAAGCC[b]
166R	GGCTTCATTCAGCTCCGG**NNN**CCAAC-GATCAAGGCGAG[b]
oHF143	CTCGCCTTGATCGTTGG**GAA**CCGGAGCT-GAATGAAGCC
oHF144	GGCTTCATTCAGCTCCGG**TTC**CCAACGATC-AAGGCGAG
oNT1	AGGAAG**CA**TATGAGTATTCAACATTTC
oNT2	T**G**CCAATGCTTAATCAGTGAGG

[a] The introduced nucleotide substitutions are shown in bold. [b] NNN is a randomized sequence of the codon for Glu166.

PCR Mutagenesis of $TEM_{pTZ19-3}$. To randomly mutagenize the codon for Glu166 of the $TEM_{pTZ19-3}$ β-lactamase, we utilized primers 166D and 166R (Table 1). The 50 μL reaction mixture contained each dNTP at 0.5 mM, each primer at 200 pM, 4 ng of template DNA, 6% (v/v) Quik Solution (Stratagene), 1 μL of the buffer provided with the enzyme, and 2.5 units of PfuUltra High-Fidelity DNA Polymerase (Stratagene). The following program was used: 1 min at 95 °C and 30 cycles of 50 s at 95 °C, 50 s at 60 °C, and 4 min at 68 °C, followed one cycle of 7 min at 68 °C. The PCR product was digested for 1 h at 37 °C with *Dpn*I and directly used to transform chemically competent *E. coli* JM83 cells. Bacteria containing the desired plasmid were isolated by selection on LB agar supplemented with 60 μg/mL kanamycin. The DNA from 24 colonies was sequenced to identify mutants with various substitutions at the codon for Glu166.

To randomly introduce mutations into the gene for the $TEM_{pTZ19-3}$ β-lactamase with various substitutions at Glu166, we utilized a mixture of DNA from 10 mutants (see Table 2) and 30 cycles of PCR with the low-fidelity Taq polymerase, essentially as described previously.[23] Mutants with increased levels of resistance to ceftazidime were selected on LB agar supplemented with 8 μg/mL ceftazidime. We selected 60 colonies from the ceftazidime-supplemented agar and determined minimal inhibitory concentrations (MICs) of ceftazidime. Three of the selected clones produced the highest MIC of ceftazidime, 64 μg/mL. DNA sequencing of these mutants revealed that all contained two amino acid substitutions, Glu166Arg and Met182Thr.

The Met182Thr mutant was obtained by PCR mutagenesis, as described above, using DNA from the Glu166Arg/Met182Thr double mutant as a template and primers oHF143 and oHF144 (Table 1). The mutagenic primers allowed us to substitute arginine at position 166 of the mutant β-lactamase with the glutamic acid present in wild-type $TEM_{pTZ19-3}$. Bacteria containing the desired plasmid were isolated by selection on LB agar supplemented with 60 μg/mL kanamycin. The introduced mutation was verified by DNA sequencing.

Antibiotic Susceptibility. The MICs of several β-lactam antimicrobials were determined by the broth microdilution technique according to the Clinical and Laboratory Standards Institute

Table 2. Susceptibility of *E. coli* JM83 and *E. coli* JM83 Producing $TEM_{pTZ19-3}$, or Various Glu166 Mutants, to β-Lactams

	MIC (μg/mL)							
strain or variant	ampicillin	oxacillin	cephalothin	cefuroxime	ceftazidime	cefotaxime	cefepime	aztreonam
JM83[a]	2	256	4	2	0.250	0.03	0.015	0.06
Glu166	16000	>32000	128	4	0.5	0.06	0.25	0.25
Glu166Val	8	512	8	4	2	0.125	0.125	0.25
Glu166Phe	4	512	8	4	4	0.06	0.06	0.06
Glu166Leu	4	512	16	4	1	0.06	0.06	0.125
Glu166Ser	4	512	8	4	2	0.06	0.125	0.125
Glu166Gly	2	512	8	4	0.25	0.03	0.015	0.06
Glu166Pro	4	512	8	4	4	0.125	0.125	0.125
Glu166Asp	32	512	32	2	2	0.03	0.03	0.125
Glu166Arg	8	512	4	4	2	0.06	0.06	0.06
Glu166Asn	2	512	8	4	0.25	0.03	0.03	0.06
Glu166Gln	8	512	8	2	2	0.03	0.03	0.125

[a] Host strain that does not express any β-lactamase.

guidelines.[25] The different enzymes were expressed in *E. coli* JM83 using vector pTZ19-3. *E. coli* JM83 and *E. coli* JM83 harboring wild-type $TEM_{pTZ19-3}$ were used as controls. The MICs were determined in Mueller-Hinton II broth (Difco) using a bacterial inoculum of 5×10^5 cfu/mL. All plates were incubated at 37 °C for 20 h before results were interpreted. Each MIC determination was performed in triplicate.

Cloning of Genes into pHF186. The wild-type and three mutant enzymes used in this study were His-tagged to determine their expression level. To introduce a hexahistidine tag onto the C-terminus of these enzymes, we utilized the genes for the wild-type and mutant β-lactamases cloned into the pTZ19-3 vector as a template. Primers oNT1 and oNT2 (Table 1), which introduce a *Nde*I site at the 5′ end of the gene and restore the codon for histidine disrupted by digestion with *Msc*I, respectively, were used. The reaction mixture had a 50 μL volume, containing each dNTP at 0.4 mM, each primer at 200 pM, 4 ng of template DNA, 6% (v/v) Quik Solution (Stratagene), 1× buffer provided with the enzyme, and 0.5 unit of Deep $Vent_R$ DNA Polymerase (New England Biolabs). The following program was used: 1 min at 95 °C and 30 cycles of 50 s at 95 °C, 1 min at 60 °C, and 1 min at 68 °C, followed by one cycle of 7 min at 68 °C. The fragment was gel purified using the Wizard SV gel and PCR cleanup system (Promega) and digested with *Nde*I for 3 h at 37 °C. The digestion product was purified and ligated into the unique *Nde*I and *Msc*I sites of pHF186 and transformed into chemically competent *E. coli* JM83 cells. Bacteria containing the desired plasmid were isolated by selection on LB agar supplemented with 60 μg/mL kanamycin. The plasmid DNA was extracted and the nucleotide sequence of each gene verified by DNA sequencing.

Determination of Enzyme Expression Levels. Levels of expression of the wild-type and mutant enzymes were examined by immunoblotting. A 50 μL aliquot of an overnight culture of *E. coli* JM83 harboring the pHF186 plasmid encoding each gene of interest was used to inoculate 5 mL of LB broth supplemented with 60 μg/mL kanamycin. After cultures reached an OD_{600} of 0.6, following incubation at 37 °C, 1.5 mL of each culture was pelleted by centrifugation and the pellet was resuspended in 1× loading buffer followed by incubation for 5 min at 100 °C. Another 1.5 mL of culture was used to isolate proteins present in the periplasm by the osmotic shock procedure, and proteins were resuspended in a final volume of 1.5 mL.[26] A 30 μL aliquot of each sample was fractionated by SDS−PAGE and transferred to a Trans-Blot Transfer Medium membrane (Bio-Rad) by electroelution for 2 h at 200 mA. The membrane was blocked overnight with 3% BSA and 3% casein. After three 15 min washes in Tris-buffered saline with 0.1% Tween, the membrane was incubated with a 1:10000 dilution of 6× His tag antibody conjugated to horseradish peroxidase (Abcam) for 1 h. Following three more 15 min washes, as described above, the protein was detected using the Super Signal West Dura Extended Duration Substrate (Pierce) and Kodak BioMax Light Film (Kodak). Analysis of expression was performed using ImageJ (National Institutes of Health, Bethesda, MD).

Cloning $TEM_{pTZ19-3}$ Glu166Arg/Met182Thr into an Expression Vector. To facilitate secretion of the enzyme into the broth, the Glu166Arg/Met182Thr gene was fused with the leader sequence of the OmpA protein in the pSV106 vector as previously described.[23] After the sequence of the construct had been verified by DNA sequencing, the gene was recloned into the *Nde*I and *Hin*dIII sites of the pET24a(+) expression vector and transformed into chemically competent *E. coli* BL21(DE3) cells.

Purification of $TEM_{pTZ19-3}$ Glu166Arg/Met182Thr. For expression of $TEM_{pTZ19-3}$ Glu166Arg/Met182Thr, *E. coli* BL21(DE3) harboring the pET24a(+) vector containing the cloned gene was grown with shaking at 37 °C in 0.5 L of LB medium supplemented with 60 μg/mL kanamycin. When the optical density of the culture at 600 nm reached 0.6, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM. The culture was incubated overnight at 25 °C with shaking (180 rpm). The bacteria were pelleted by centrifugation at 20000*g* and 4 °C, and the supernatant was concentrated by centrifugal filtration at 3000*g* and 4 °C using a Centricon Plus 70 (Millipore) with a 10 kDa molecular mass cutoff filter. Following overnight dialysis against 10 mM Tris (pH 7.0), the dialyzed sample was loaded onto a DEAE anion-exchange column (Bio-Rad) equilibrated in the same buffer. The enzyme was eluted using a linear gradient from 10 to 100 mM Tris (pH 7.0). The fractions containing the desired enzyme were identified by SDS−PAGE, pooled, concentrated, and dialyzed against 10 mM Tris (pH 7.0). The enzyme concentration was determined using the predicted enzyme extinction coefficient at 280 nm ($\Delta\varepsilon$ = 28085 M^{-1} cm^{-1}).[27]

Determination of the Rate Constant Describing Acylation. All spectrophotometric data were recorded on a Cary 50 spectrophotometer (Varian) equipped with a stopped-flow apparatus (Hi-Tech Scientific, Salisbury, U.K.), using a mixing ratio of 1:1 at room temperature. Analyses were performed using the nonlinear regression program Prism 5 (GraphPad Software, Inc.) using data obtained from experiments performed in at least triplicate. Reactions of 50 mM NaP_i (pH 7.0) with varying concentrations of the β-lactam substrate were initiated by the addition of enzyme (final concentration of 4 μM) and opening of the β-lactam ring monitored at 260 nm ($\Delta\varepsilon = -10500$ M^{-1} cm^{-1}) for 1 h. The reaction was conducted under multiple-turnover conditions, where the substate is in excess of the enzyme. The time courses were fit with eq 1

$$A_t = A_\infty + (A_0 - A_\infty)e^{-k_2 t} \quad (1)$$

where A_t is the absorbance at time t, A_0 is the initial absorbance, A_∞ is the final absorbance, and k_2 is the first-order rate constant describing acylation.

Determination of the Steady-State Kinetic Parameters for Nitrocefin. Reactions of 50 mM NaP_i (pH 7.0) with varying concentrations of nitrocefin were initiated by the addition of 1 μM enzyme, and opening of the β-lactam ring was monitored at 500 nm ($\Delta\varepsilon$ = 15900 M^{-1} cm^{-1}) for 2 min. The observed rate constants were determined from the steady-state velocity during the linear phase of the reaction. They were plotted as a function of nitrocefin concentration and fit nonlinearly with the Michaelis−Menten equation, allowing the determination of a k_{cat} (1.9×10^{-3} s^{-1}) and a K_m (≤ 5 μM).

Determination of the Dissociation Constant for Ceftazidime. The dissociation constant for the noncovalent ceftazidime−enzyme complex was determined using nitrocefin as a reporter substrate. Nitrocefin is a poor substrate for the mutant enzyme, allowing us to treat ceftazidime as an inhibitor (ceftazidime is not a substrate). Reaction mixtures containing 50 mM NaP_i (pH 7.0), 10 μM enzyme, and 50 μM nitrocefin were incubated for 1 min at room temperature, followed by the addition of ceftazidime to a final concentration of 37.5, 75, 150, 300, 700, or 1000 μM. The reaction was followed for 2 min

by monitoring at a wavelength of 500 nm. The fractional activity (v_i/v_0) was plotted as a function of the ceftazidime concentration and fit with eq 2.

$$\frac{v_i}{v_0} = 1 - \frac{[E] + [I] + K_i^{app} - \sqrt{([E] + [I] + K_i^{app})^2 - 4[E][I]}}{2[E]} \quad (2)$$

where v_i is the steady-state velocity in the presence of ceftazidime, v_0 is the steady-state velocity in the absence of ceftazidime, [E] is the enzyme concentration, [I] is the ceftazidime concentration, and K_i^{app} is the apparent dissociation constant for the noncovalent ceftazidime−enzyme complex.

Evaluation of the Deacylation Rate of the $TEM_{pTZ19-3}$ Glu166Arg/Met182Thr−Ceftazidime Complex. Reactions of 10 mM Tris (pH 7.0) with 318 μM ceftazidime were initiated by the addition of 318 μM $TEM_{pTZ19-3}$ Glu166Arg/Met182Thr and the mixtures incubated at 25 °C. At various time points from 0 to 20 h, bocillin FL was added to a final concentration of 50 μM, and the reaction mixture was incubated for an additional 15 min at 25 °C. The reactions were quenched in Laemmli buffer and the mixtures fractionated by SDS−PAGE. Labeling of the deacylated enzyme with bocillin FL was visualized on a Storm 840 Fluorimager and the fluorescent signal analyzed using Image Quant 5.2.

Cloning of $TEM_{pTZ19-3}$ Glu166Arg/Met182Thr, with a Hexahistidine Tag, into an Expression Vector. The gene for $TEM_{pTZ19-3}$ Glu166Arg/Met182Thr, containing a C-terminal six-His tag, was recloned from the pHF016 plasmid into the *Nde*I and *Hin*dIII sites of the pET24a(+) expression vector and transformed into chemically competent *E. coli* BL21(DE3) cells. Bacteria containing the desired plasmid were isolated by selection on LB agar supplemented with 60 μg/mL kanamycin. The plasmid DNA was extracted and the nucleotide sequence of the gene verified by DNA sequencing.

Purification of $TEM_{pTZ19-3}$ Glu166Arg/Met182Thr with a Hexahistidine Tag. For expression of $TEM_{pTZ19-3}$ Glu166Arg/Met182Thr with a six-histidine tag, *E. coli* BL21(DE3) harboring the pET24a(+) vector containing the cloned gene was grown under the conditions described above for bacteria expressing the protein without the tag. The bacteria were pelleted by centrifugation at 20000*g* and 4 °C and resuspended in 80 mL of 10 mM Tris (pH 7.0) and 1 mM EDTA. After sonification, the sample was centrifuged at 20000*g* and 4 °C for 1 h and the supernatant was loaded onto a DEAE anion-exchange column (Bio-Rad) equilibrated with 10 mM Tris (pH 7.0). The protein was eluted using a linear gradient from 10 to 100 mM Tris (pH 7.0). The fractions containing the desired enzyme were identified by SDS−-PAGE, pooled, concentrated, and dialyzed against 10 mM Tris (pH 7.0). The dialyzed sample was loaded onto a HiTrap column (GE Healthcare) equilibrated in the same buffer. The enzyme was eluted using a stepwise gradient containing varying concentrations of imidazole in 10 mM Tris (pH 7.0). After identification of the fractions containing the enzyme by SDS−PAGE, they were pooled, concentrated, and dialyzed against 10 mM Tris (pH 7.0). The enzyme concentration was determined using the predicted enzyme extinction coefficient at 280 nm ($\Delta\varepsilon$ = 28085 M^{-1} cm^{-1}).[27]

Quantitative Immunoblot Analysis. The periplasmic content of 4×10^8 *E. coli* JM83 cells harboring the pHF016 plasmid encoding the $TEM_{pTZ19-3}$ Glu166Arg/Met182Thr six-histidine tag mutant was extracted as described above and analyzed by SDS−PAGE. Several amounts of the purified mutant protein carrying the histidine tag were used as calibration standards and fractionated on the same gel. The Western blot was made as described above. Image analysis and enzyme quantification were performed using ImageJ (National Institutes of Health) and Prism 5 (GraphPad Software, Inc.).

MATLAB Simulation of Periplasmic Contrations of Ceftazidime and Enzyme Species. The periplasmic concentrations of $TEM_{pTZ19-3}$ Glu166Arg/Met182Thr (E), ceftazidime (I_p), and their acyl−enzyme species (EI*) can be estimated using the non-steady-state model described by Frère et al.[28] The differential equations describing the change in concentration of E, I_p, and EI* with time were simulated by numerical integration using MATLAB (R2010b, The Mathworks). The following parameters were used during the simulation: the initial enzyme concentration, E_0 = 1.7 mM (determined in this report by quantitative immunoblot analysis); the initial periplasmic concentration, V_0 = 1; the first-order rate constant describing cellular growth, $k = 5.8 \times 10^{-4}$ s^{-1} (for a generation time of 20 min); the deacylation rate constant, $k_3 = 8.0 \times 10^{-6}$ s^{-1} (for a half-life of 1 day, which is an underestimation); the acylation rate constant, k_2 = 0.016 s^{-1} (determined in this report by stopped-flow kinetics); the dissociation constant of the noncovalent enzyme−ceftazidime complex, K = 18 μM (determined in this report as K_i^{app}); the first-order rate constant describing penetration of ceftazidime into the periplasm, $k_D = 4.8 \times 10^{-3}$ s^{-1};[29] the extracellular concentration of ceftazidime, I_e = 58 μM (32 μg/mL, 2-fold below the MIC value) or 117 μM (64 μg/mL, MIC value). The simulation was run over 12000 s, using a step size of 0.1 s.

RESULTS AND DISCUSSION

TEM-1 is a class A β-lactamase with good activity against penicillins and early-generation cephalosporins.[4,7] On the other hand, TEM-1 is not capable of efficiently hydrolyzing third- and fourth-generation cephalosporins because of bulky groups introduced into their structures. Over time, numerous extended-spectrum TEM β-lactamases have been selected both in vivo and in vitro. These enzymes contain from one to several amino acid substitutions that allow them to more efficiently turn over modern cephalosporins, including ceftazidime. Puzzling observations were reported in several earlier publications regarding TEM-1 derivatives with substitution of Glu166, a residue critical for deacylation of the covalent enzyme−β-lactam complex. It was observed that while these deacylation-deficient mutant enzymes lose their ability to hydrolyze penicillins, they gain the ability to confer resistance to cephalosporins, especially ceftazidime.[21,30] Whereas the effect of the Glu166 substitution alone on the MIC of ceftazidime was not significant in those cases, various combinations with other mutations resulted in higher levels of resistance.[21,30,31]

To elucidate the mechanism of ceftazidime resistance conferred by the mutants at position 166, presumed to be deacylation-deficient, of $TEM_{pTZ19-3}$ β-lactamase, we performed random site-directed mutagenesis of Glu166. Following mutagenesis and sequencing of the $TEM_{pTZ19-3}$ β-lactamase gene from 24 transformants, we were able to generate 10 different amino acid substitutions at position 166 of the enzyme. As expected, substitution of this critical catalytic residue resulted in a dramatic loss of resistance to penicillins (e.g., ampicillin and oxacillin) and to the first-generation cephalosporin cephalothin by bacteria producing any of the mutant enzymes (Table 2).

Table 3. Susceptibility of *E. coli* JM83 and *E. coli* JM83 Producing $TEM_{pTZ19-3}$, or Its Mutants, to β-Lactams

	MIC (μg/mL)				
β-lactam	JM83[a]	$TEM_{pTZ19-3}$	Glu166Arg/ Met182Thr	Glu166Arg	Met182Thr
ampicillin	2	16000	32	8	32000
benzylpenicillin	32	16000	32	32	16000
oxacillin	256	>32000	256	512	32000
piperacillin	4	>32000	128	4	>32000
ticarcillin	2	16000	64	16	16000
cephalothin	4	128	8	8	1024
cefuroxime	2	8	4	4	4
ceftazidime	0.25	0.5	64	2	0.5
ceftriaxone	0.06	0.06	2	0.03	0.06
cefotaxime	0.03	0.06	0.5	0.06	0.06
cefepime	0.015	0.25	0.5	0.06	0.25
cefoxitin	2	2	4	2	2
moxalactam	0.125	0.125	1	0.125	0.5
aztreonam	0.06	0.125	0.25	0.06	0.06

[a] Host strain that does not express any β-lactamase.

At the same time, resistance to the third- and fourth-generation cephalosporins (e.g., cefotaxime and cefepime) and the monobactam aztreonam remained at a level comparable to that of the parental enzyme. However, resistance to ceftazidime increased (with the exception of strains producing the Glu166Gly and Glu166Asn mutant enzymes) from 2- to 8-fold.

To further increase levels of resistance to ceftazidime produced by mutants of the $TEM_{pTZ19-3}$ β-lactamase with various substitutions of Glu166, we performed random PCR mutagenesis using a mixture of plasmid DNA from all 10 mutants as a template. Following one round of PCR mutagenesis and subsequent transformation, colonies were selected on agar supplemented with 8 μg/mL ceftazidime. Bacteria from three of these colonies were characterized by the highest MIC for ceftazidime (64 μg/mL). DNA sequencing established that the β-lactamases from all three strains were identical and harbored two amino acid substitutions, Glu166Arg and Met182Thr.

To evaluate the effect of individual mutations on the levels of resistance to β-lactam antibiotics, we determined the MICs of 14 β-lactams against strains producing the wild-type parental enzyme, or its Glu166Arg, Met182Thr, and Glu166Arg/Met182Thr mutants (Table 3). Substitution of the glutamic acid with arginine was responsible for significant alterations in the antibiotic susceptibility pattern. While the wild-type enzyme conferred high MICs for penicillins, the Glu166Arg mutation was responsible for a dramatic decrease in resistance, in some cases to the level of a background strain harboring no β-lactamase gene. This observation is consistent with the abrogation of activity in the enzyme upon mutation at position 166. However, this very amino acid substitution was also responsible for 8- and 4-fold decreases in the MICs for cephalothin and cefepime, respectively, and a 4-fold increase in the MIC for ceftazidime. The effect of substitutions at position 166 of class A enzymes is well-known.[21,30] Several reports have associated mutations at this position with an evident loss of activity toward penicillins and early generations of cephalosporins, but increases in activity against the later-generation cephalosporins, especially ceftazidime.[21,30−32]

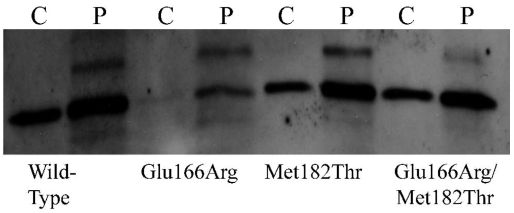

Figure 1. Steady-state levels of wild-type, Glu166Arg, Met182Thr, and Glu166Arg/Met182Thr $TEM_{pTZ19-3}$ evaluated by Western blotting, using an anti-histidine tag antibody. An equal number of bacteria, or an equal volume of the periplasmic fraction, was loaded for analysis. P denotes the periplasmic fraction and C the whole bacteria.

In contrast to the effect of the Glu166Arg mutation, the effect of the Met182Thr substitution on resistance was insignificant, and a maximal 4-fold difference was observed between the levels of resistance produced by this mutant and the wild-type β-lactamase. The Met182Thr substitution is common in mutant TEM β-lactamases producing an expanded-spectrum or inhibitor-resistance phenotype.[33−35] It was demonstrated that although the Met182Thr substitution does not directly affect TEM β-lactamase activity, it helps in stabilizing the enzyme, offsetting the deleterious effects introduced by other mutations.[34,36] Consistent with the proposed stabilizing role of threonine at position 182, introduction of the Met182Thr mutation into $TEM_{pTZ19-3}$ Glu166Arg resulted in an 8-fold increase in the resistance to cefotaxime, cefepime, and moxalactam and 32- and 64-fold increases in the MIC of ceftazidime and ceftriaxone, respectively (Table 3). Although the antibiotic susceptibility profile of the double mutant seems much less impressive than that seen with the wild-type enzyme, when compared to the control strain lacking the β-lactamase, the presence of the enzyme elevates the MIC value of most β-lactam antibiotics tested. The greatest effect was seen with cephalosporins, with the most dramatic being a 256-fold increase in the MIC for ceftazidime.

Some of these effects might be due to differential levels of expression of these enzymes. To evaluate expression levels of the mutant enzymes in comparison to the wild-type β-lactamase, we added a hexahistidine tag to the C-terminus of each enzyme. Introduction of the tag did not affect MIC values for all antibiotics tested (data not shown). Protein expression levels in both the periplasm and whole bacteria were screened using an anti-histidine tag antibody (Figure 1). In the Western blot, it was possible to observe two bands: an upper band, corresponding to the cytoplasmic unprocessed form of the enzyme, and a lower band, corresponding to the periplasmic processed protein from which the leader sequence had been removed. The Glu166Arg mutation was responsible for a significant decrease in the level of expression of the enzyme. Its presence in the periplasm was barely detectable, indicative of the deleterious effect of the Glu166Arg substitution, likely based on the reduced stability of the protein and/or weakened expression of the protein. Our result is consistent with previous observations that the Ω-loop region, where residue 166 resides, does not easily tolerate substitutions,[33] and amino acid substitutions at Glu166 are usually associated with the formation of periplasmic inclusion bodies, suggesting a role for Glu166 in protein folding during secretion to the periplasm.[12,21,30,37] While the introduction of the Met182Thr substitution alone had little effect on protein expression, its introduction into the Glu166Arg mutant resulted in a significant increase in the level of expression, similar to that of

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E\text{-}S \xrightarrow{k_3} E + P$$

Figure 2. General model for enzymatic hydrolysis of β-lactams by β-lactamases. E represents the enzyme. S the substrate, ES the noncovalent enzyme−substrate complex, E-S the acyl−enzyme complex, and P the product.

the wild-type β-lactamase. Our results confirm previous observations that the Met182Thr substitution increases the stability of TEM β-lactamases.[38] Using quantitative immunoblot analysis, we evaluated the periplasmic concentration of this double mutant enzyme, and it was found to be ~1.7 mM or ~68000 molecules per bacterium.

As periplasmic expression of $TEM_{pTZ19\text{-}3}$ Glu166Arg/Met182Thr is comparable to that of wild-type $TEM_{pTZ19\text{-}3}$ (Figure 1), the increase in MIC values of ceftazidime and other β-lactams conferred by the Glu166Arg/Met182Thr mutant cannot be attributed to higher concentrations of the enzyme. Furthermore, as all MIC experiments were performed using the same *E. coli* JM83 strain, differences in MIC values produced by the wild-type and Glu166Arg/Met182Thr mutant enzymes cannot be explained by changes in the sensitivity of bacterial PBPs or the rate of penetration of the antibiotic into the periplasm. Thus, the observed differences in the conferred β-lactam resistance could be caused only by differential mechanistic manifestations by the mutant enzymes.

Enzymatic hydrolysis of β-lactam antibiotics by β-lactamases follows the general scheme presented in Figure 2. The first step in catalysis is binding of the antibiotic to the enzyme, resulting in the Michaelis complex. To evaluate whether the Glu166Arg and Met182Thr mutations altered the affinity of the enzyme for ceftazidime, we determined the affinity of $TEM_{pTZ19\text{-}3}$ Glu166Arg/Met182Thr for this β-lactam using a competition assay with nitrocefin. We were unable to determine a true K_i as the Michaelis constant for the competing substrate was too small to be evaluated accurately; however, we were able to determine the apparent K_i [18 ± 2 μM (Table 2)]. This value indicates that there is an at least 110-fold increase in the affinity of $TEM_{pTZ19\text{-}3}$ Glu166Arg/Met182Thr for ceftazidime, compared to that of the wild-type enzyme.[22]

It is known that mutations at position 166 of class A enzymes are responsible for significant changes in the microscopic rate constants describing acylation (k_2) and deacylation (k_3). Whether acylation (k_2), deacylation (k_3), or both are affected, as well as the extent of the change, varies with the identity of the amino acid at position 166 and the β-lactam used as the substrate.[12,37,39]

We determined the acylation rate constant for ceftazidime using a stopped-flow apparatus. Formation of the acyl−enzyme species followed single-exponential growth with an amplitude equivalent to the concentration of the enzyme used in the experiment (Figure 3). The experiment was performed under multiple-turnover conditions, because there was no detectable deacylation over the time frame of the experiment, as will be explained below. Two concentrations of ceftazidime, 50 and 100 μM, were used and gave rate constants of 0.016 ± 0.001 and 0.015 ± 0.001 s^{-1}, respectively. These rate constants measured at two antibiotic concentrations are identical, indicating that the enzyme was saturated and that this rate constant is equivalent to the acylation rate constant, k_2. The value of k_2 showed an increase of at least 16-fold, compared to that of the wild-type enzyme with

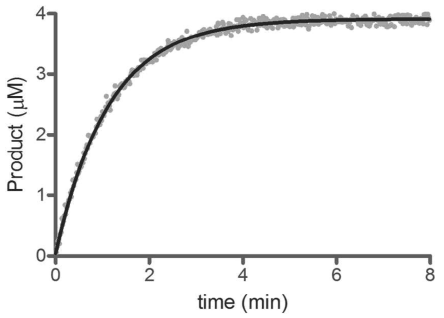

Figure 3. Representative time course for acylation of $TEM_{pTZ19\text{-}3}$ Glu166Arg/Met182Thr (4 μM) by ceftazidime (50 μM), with the line representing the best fit for eq 1. Reactions were initiated by the addition of enzyme, and the acylation event was monitored at 260 nm.

Table 4. Kinetic Parameters for Ceftazidime Hydrolysis by TEM Enzymes

	k_2 (s^{-1})	k_3 (s^{-1})	K_i (μM)	K_i^{app} (μM)
wild type[a]	$<1 \times 10^{-3}$	$<1 \times 10^{-3}$	2000	
Glu166Tyr[a]	1.7×10^{-2}	D[b]	19	
Glu166Arg/Met182Thr	$(1.6 \pm 0.1) \times 10^{-2}$	ND[c]		18 ± 2

[a] Data from ref 22. [b] Detected but not measurable. [c] Not detected.

the same substrate (Table 4). This agrees with reports from several other deacylation-deficient β-lactamases in which rapid formation of the acyl−enzyme complex was observed.[40,41]

The $TEM_{pTZ19\text{-}3}$ β-lactamase containing a Glu166Arg substitution was expected to be deacylation-deficient, as indicated earlier. We evaluated the ability of the covalent complex between the Glu166Arg/Met182Thr mutant and ceftazidime to deacylate using a chase experiment with the fluorescent β-lactam bocillin. The Glu166Arg/Met182Thr mutant was incubated with ceftazidime, giving rise to the acyl−enzyme complex. A control in which no ceftazidime was added was used to document that the activity of enzyme was not affected over the time course of the experiment. The extent of enzyme deacylation was monitored over a period of 20 h by collecting aliquots at several time points and adding bocillin, the reporter fluorescent substrate. The deacylated enzyme would form a new acyl−enzyme complex with bocillin, which could be detected and quantified by fluorescence. Over the course of the experiment, no differences in the intensity of fluorescence were observed (data not shown), indicating that the covalent enzyme−ceftazidime complex was stable for at least 20 h. After this 20 h incubation, the signal from the control to which no ceftazidime had been added matched the one seen at time zero, indicating that the conditions of the assay did not affect the enzyme activity. Again, the dramatic attenuation of the deacylation rate for this mutant enzyme was expected, as the glutamic acid at position 166 of class A β-lactamases is known to be crucial for deacylation of the enzyme. Similar results have been reported with various TEM β-lactamase mutants harboring amino acid substitutions at this position.[12,22,37]

The inability of the Glu166Arg/Met182Thr−ceftazidime complex to undergo deacylation, while still conferring ceftazidime resistance to strains that express it, is reminiscent of a similar observation with class C β-lactamases. It was reported in

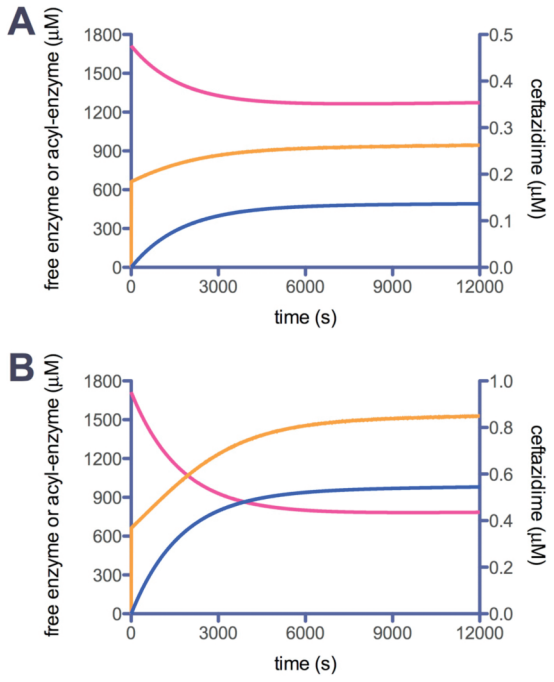

Figure 4. Simulated time course for the periplasmic concentrations of $TEM_{pTZ19\text{-}3}$ Glu166Arg/Met182Thr (free enzyme, pink), ceftazidime (yellow), and their acyl−enzyme complex (blue) under conditions where the extracellular concentration of ceftazidime is 58 (A) or 117 μM (B). Once steady state has been reached, the concentrations of free enzyme are 1236 (A) and 761 μM (B), those of ceftazidime are 0.25 (A) and 0.82 μM (B), and those of the acyl−enzyme complex are 477 (A) and 951 μM (B).

the early 1980s that strains with derepressed expression of class C β-lactamases were resistant to later-generation cephalosporins and some other β-lactams.[42,43] On the basis of these observations, the concept of resistance due to nonhydrolytic or noncovalent trapping was introduced for class C β-lactamases.[42−46] It was proposed that the elevated MIC values were the result of noncovalent trapping of the incoming antibiotic by the enzyme present in the bacterial periplasm at high concentrations. Later it was shown that class C β-lactamases actually form a stable covalent complex with β-lactam antibiotics and are capable of undergoing deacylation but only at a very low rate.[46−48] This mechanism of resistance to β-lactams, in which the strongly expressed class C β-lactamase forms a stable acyl−enzyme species with the antibiotic, was termed "covalent trapping". In two studies of the covalent-trapping mechanism, the half-life for the covalent complex was estimated to be 19 and 210 min.[49,50] This mechanism has also been proposed for certain PBPs but has not been experimentally verified.[51] In the case of the $TEM_{pTZ19\text{-}3}$ Glu166Arg/Met182Thr mutant, we were unable to detect any deacylation of the enzyme−ceftazidime complex over a period of 20 h. Considering that the doubling time of exponentially growing *E. coli* is 30 min, this would mean that the covalent complex would be stable over at least 40 bacterial generations.

The relationship between the sensitivity to β-lactam antibiotics and the production of β-lactamases has previously been established.[28,52] The non-steady-state model requires a slow deacylation rate, slow penetration of the β-lactam, and a high β-lactamase concentration to observe trapping via formation of a covalent acyl−enzyme species. To evaluate whether the amount of enzyme expressed and its kinetic properties would account for the observed increase in the MIC for ceftazidime, we simulated our system using the model of Frère et al.[28] The simulation was performed 2-fold below the MIC and at the MIC value of ceftazidime against *E. coli* JM83 harboring $TEM_{pTZ19\text{-}3}$ Glu166Arg/Met182Thr, which correspond to 58 and 117 μM, respectively (Figure 4). Our results show that with extracellular ceftazidime concentrations of 58 and 117 μM, the periplasmic concentrations of the antibiotic stabilize at 0.26 and 0.82 μM, respectively. The MIC for ceftazidime indicates that its periplasmic concentration must be less than 0.46 μM (Table 3; MIC = 0.25 μg/mL) for *E. coli* JM83 to survive. Thus, our simulation predicts that with the level of $TEM_{pTZ19\text{-}3}$ Glu166Arg/Met182Thr expression that we detect (~1.7 mM), the bacteria would survive at 0.26 μM, but not at 0.82 μM, which corresponds to an MIC value of 64 μg/mL. This simulation is in agreement with our experimental determination of the MIC (Table 3).

Although our experiments were conducted in *E. coli* with ceftazidime, it is likely that a covalent-trapping mechanism could operate in other Gram-negative organisms and/or with other antimicrobials. The level of resistance that can be achieved by this mechanism is dependent upon many parameters, such as β-lactamase expression level, its kinetic parameters, and the permeability of the outer membrane to the antibiotic. The lack of the outer membrane in Gram-positive bacteria would likely preclude this mechanism from occurring, but this is not certain, awaiting the obsevation of such an outcome in nature.

CONCLUSION

The Glu166Arg/Met182Thr double mutant of the $TEM_{pTZ19\text{-}3}$ β-lactamase produces a 128-fold increase in the resistance to ceftazidime in comparison to that of the parental wild-type enzyme. We have demonstrated that the covalent enzyme−ceftazidime complex remains stable over a period corresponding to at least 40 bacterial generations, indicating that the double mutant produces resistance to ceftazidime via a covalent-trapping mechanism. The Glu166Arg/Met182Thr mutant β-lactamase operates essentially as a PBP that is capable of forming a covalent acyl−enzyme complex with an antibiotic but lacks the ability to deacylate, yet to be able to confer resistance, the mutant β-lactamase has to protect the bacterial PBPs from binding by the incoming antibiotic. We have demonstrated that the increase in resistance to ceftazidime is not the result of an increased level of protein expression. The detectable improvement in the affinity of the $TEM_{pTZ19\text{-}3}$ Glu166Arg/Met182Thr mutant for ceftazidime alone cannot account for the ability of the mutant β-lactamase to protect the bacterial PBPs from acylation by the antibiotic, as it still has an affinity lower than that reported for PBP-3, for example, which is the major target of ceftazidime in *E. coli* K12.[53] On the other hand, significant improvement in the acylation rate constant for ceftazidime in the mutant enzyme could play a crucial role in efficient depletion of the incoming antibiotic, leading to protection of PBPs and culminating in increased resistance to ceftazidime. We also demonstrated that the amount of protein present in the periplasm is enough to protect bacteria from ceftazidime.

Although it has never been detected in clinical bacterial pathogens, covalent trapping represents a novel resistance mechanism for class A β-lactamases and is yet another example of the amazing plasticity of these enzymes, allowing them to expand their substrate profile by utilizing various, even noncanonical, strategies.

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ABBREVIATIONS

dNTP, deoxyribonucleotide triphosphate; MIC, minimal inhibitory concentration; NaP_i, inorganic sodium phosphate; PBP, penicillin binding protein; PCR, polymerase chain reaction; SDS—PAGE, sodium dodecyl sulfate—polyacrylamide gel electrophoresis.

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