Mechanistic Basis for the Emergence of Catalytic Competence against Carbapenem Antibiotics by the GES Family of β-Lactamases

Hilary Frase, Qicun Shi, Sebastian A. Testero, Shahriar Mobashery, and Sergei B. Vakulenko

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

Received for publication, April 21, 2009, and in revised form, August 4, 2009 Published, JBC Papers in Press, August 5, 2009, DOI 10.1074/jbc.M109.011262

A major mechanism of bacterial resistance to β-lactam antibiotics (penicillins, cephalosporins, carbapenems, etc.) is the production of β-lactamases. A handful of class A β-lactamases have been discovered that have acquired the ability to turn over carbapenem antibiotics. This is a disconcerting development, as carbapenems are often considered last resort antibiotics in the treatment of difficult infections. The GES family of β-lactamases constitutes a group of extended spectrum resistance enzymes that hydrolyze penicillins and cephalosporins avidly. A single amino acid substitution at position 170 has expanded the breadth of activity to include carbapenems. The basis for this expansion of activity is investigated in this first report of detailed steady-state and pre-steady-state kinetics of carbapenem hydrolysis, performed with a class A carbapenemase. Monitoring the turnover of imipenem (a carbapenem) by GES-1 (Gly-170) revealed the acylation step as rate-limiting. GES-2 (Asn-170) has an enhanced rate of acylation, compared with GES-1, and no longer has a single rate-determining step. Both the acylation and deacylation steps are of equal magnitude. GES-5 (Ser-170) exhibits an enhancement of the rate constant for acylation by a remarkable 5000-fold, whereby the enzyme acylation event is no longer rate-limiting. This carbapenemase exhibits $k_{cat}/K_m$ of $3 \times 10^5$ $\text{m}^{-1}\text{s}^{-1}$, which is sufficient for manifestation of resistance against imipenem.

Bacterial production of β-lactamases is a primary mechanism of resistance to β-lactam antibiotics (1). These enzymes hydrolytically process the β-lactam bond of the antibiotic, and by so doing, inactivate them. Four classes of β-lactamases, A, B, C, and D, are known, of which the class A enzymes are most prevalent (1, 2). Enzymes belonging to classes A, C, and D are serine-dependent. A critical active site serine in these enzymes experiences acylation by the antibiotic followed by deacylation. The mechanistic details of the deacylation steps vary for each class (1). Class B enzymes are zinc-dependent, and their mechanistic details are distinct. Random mutations in the genes for these enzymes have allowed for selection of novel variants within the clinic having an increased breadth for substrate preference (3). Variants of β-lactamases with the ability to turn over both penicillins and cephalosporins are referred to as extended spectrum β-lactamases (4). Extended spectrum β-lactamases typically do not have the ability to hydrolyze carbapenems; however, exceptions to this rule are emerging among members of classes A, B, and D and are called carbapenemases (5, 6). Within class A, members of the KPC and GES families are becoming increasingly problematic within the clinic (3, 5, 7). The GES type class A β-lactamases were identified for the first time only 10 years ago, but they have been increasingly detected worldwide among Gram-negative bacteria (3). The first variants detected were plasmid borne and isolated from Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, and Enterobacter cloacae (8–14); however, newer variants have been identified in the chromosome of P. aeruginosa (15, 16). A distinguishing characteristic of this family of enzymes is its ability to develop resistance to all classes of β-lactam antibiotics, including third-generation cephalosporins, cephamycins, monobactams, and/or carbapenems. This is in stark contrast to “classical” β-lactamases, such as the TEM family of enzymes, which also rapidly evolved to produce numerous extended spectrum and inhibitor-resistant variants, yet have failed to evolve activity against carbapenem antibiotics. This combination of resistance to both extended spectrum β-lactams and carbapenem antibiotics makes the organisms that harbor the genes for these enzymes dangerous in a clinical setting as treatment options dwindle.

GES-1, the first member of the GES family to be identified, has no significant ability to hydrolyze carbapenems, leading to its classification as only an extended spectrum β-lactamase (10). Other GES-type enzymes were later discovered with increased resistance to carbapenems. Two of these variants, GES-2 and -5, contain only a single amino acid substitution compared with the sequence of GES-1, both at position 170 (Ambler numbering is used) (17). This position is located within an Ω-loop forming one of the walls of the active site (1). The canonical residue at this position of class A β-lactamases is asparagine, which is found in GES-2. However, GES-1 contains a glycine, and GES-5 contains a serine (17). We recently solved the x-ray crystal structure of the GES-1 enzyme; however, it was not clear from the structural data why such a substitution would convey resistance in GES-2 and -5, in contrast to the case of GES-1, which does not (18). The mechanistic basis for the extended profile for resistance is not known, and it is the subject of study in this report. Previous kinetic analyses of class A car-
bapenemases, including GES enzymes, have been limited to steady-state kinetic parameters (8, 10, 11). In this report, we have performed an in-depth analysis of the GES family, including both steady-state and the first pre-steady-state analyses (for any class A carbapenemase) to elucidate the nature of the microscopic steps (binding, acylation, deacylation, and product release) in the turnover process by these clinically important enzymes.

EXPERIMENTAL PROCEDURES

Minimal Inhibitory Concentration Determinations—The minimal inhibitory concentrations (MICs)\(^2\) of \(\beta\)-lactam antibiotics were determined using the broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute (19). GES-1, -2, and -5 were expressed in E. coli JM83 using the plasmids pHF:GES-1, pHF:GES-2, and pHF:GES-5, respectively. E. coli JM83 harboring pHF016 was used as a control. MICs were determined in Mueller–Hinton II broth (Difco) using a bacterial inoculum of \(5 \times 10^5\) colony-forming units/ml. All plates were incubated at 37°C for 16–20 h before results were interpreted.

Data Collection and Analysis—All spectrophotometric data were collected on a Cary 50 spectrophotometer (Varian) at 22°C. Analyses were performed using the nonlinear regression program Prism5 (GraphPad Software, Inc.) using data obtained from at least three independent experiments.

Determination of Steady-state Kinetic Parameters—Reactions containing 50 mM NaPi (pH 7.0), 100 mM NaCl, and varying concentrations of the \(\beta\)-lactam substrate were initiated by the addition of enzyme. The absorbance was monitored at 500 nm, and the steady-state absorbance was monitored at 500 nm, and the steady-state velocities were determined from the linear phase of the reaction time courses. The steady-state velocity data were plotted as a function of \(\beta\)-lactam concentration and fit non-linearly with the Michaelis-Menten equation to obtain the steady-state kinetic parameters (8, 10, 11). In this report, we present the dissociation constants for acylation and deacylation (k\(_a\) and k\(_d\)). The parameters for nitrocefin hydrolysis (GES-1, -2, and -5) were determined using the broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute (20). Reactions containing 50 mM NaPi (pH 7.0), 100 mM NaCl, 160 \(\mu\)M (GES-1, 50 \(\mu\)M (GES-2), or 120 \(\mu\)M (GES-5) of nitrocefin, and varying concentrations of GES-1, -2, and -5 were initiated by the addition of the enzyme (200 \(\mu\)M final for GES-1 and GES-5 or 10 \(\mu\)M final for GES-2). The absorbance was monitored at 500 nm, and the steady-state velocities were determined from the linear phase of the reaction time courses. The steady-state velocity data were plotted as a function of imipenem concentration and fit with Equation 1.

\[
\nu = \nu_o \left(1 - \frac{E + I + \left(K_1 + \frac{S}{K_p}\right)}{2E + I + \left(K_1 + \frac{S}{K_p}\right)}\right) + \sqrt{\frac{E + I + \left(K_1 + \frac{S}{K_p}\right)}{2E}} - 4EI
\]

(Eq. 1)

where \(v\) is the steady-state velocity, \(v_o\) is the steady-state velocity in the absence of imipenem, \(I\) is the enzyme concentration, \(K_1\) is the dissociation constant for imipenem, \(S\) is the nitrocefin concentration, and \(K_p\) is the Michaelis constant for nitrocefin hydrolysis.

The dissociation constants for the product of imipenem hydrolysis (K\(_{p}^E\)) were determined as follows. Reactions containing 50 mM NaPi (pH 7.0), 100 mM NaCl, 160 \(\mu\)M (GES-1), 5 \(\mu\)M (GES-2), or 200 \(\mu\)M (GES-5) nitrocefin, and varying concentrations of hydrolyzed imipenem (up to 4 \(\mu\)M) were initiated by the addition of the enzyme (200 \(\mu\)M GES-1 and -5 or 10 \(\mu\)M GES-2 final). The absorbance was monitored at 500 nm, and the steady-state velocities were determined from the linear phase of the reaction time courses.

Determination of the Microscopic Rate Constants for Acylation and Deacylation—The first-order rate constants describing acylation (k\(_a\)) of GES-1, -2, and -5 by imipenem were determined under single turnover conditions. Reaction mixtures contained 50 mM NaPi (pH 7.0), 100 mM NaCl, 75 \(\mu\)M enzyme, and 15 \(\mu\)M imipenem. Acylation was detected by monitoring the opening of the \(\beta\)-lactam ring of imipenem at 297 nm (\(\Delta A_{297} = -10,930\) cm\(^{-1}\) m\(^{-1}\)) with the aid of an SFA-20 stopped-flow apparatus (Hi-Tech Scientific, Salisbury, UK), and the time courses were fit with Equation 2.

\[
A_t = (A_0 - A_\infty) e^{-kt} + A_\infty
\]

(Eq. 2)

where \(A_t\) is the absorbance at time \(t\), \(A_0\) is the initial absorbance, \(A_\infty\) is the final absorbance, and \(k_t\) is the first-order rate constant for enzyme acylation. Acylation of GES-5 by imipenem under single turnover conditions resulted in two distinct exponential phases, which was fit with Equation 3.

\[
A_t = (A_0 - A_\infty)(F_{fast}) e^{-k_{fast} t} + (A_0 - A_\infty)(1 - F_{fast}) e^{-k_{slow} t} + A_\infty
\]

(Eq. 3)

where \(A_0\), \(A_\infty\), and \(A_\infty\) are as defined in Equation 2, \(F_{fast}\) is the fraction of the reaction corresponding to the fast phase, \(k_{fast}\) is the observed first-order rate constant for the fast phase, and \(k_{slow}\) is the observed first-order rate constant for the slow phase.

The rate at which imipenem was displaced from the active site of GES-1, -2, and -5 was determined as described previously (21). Briefly, a reaction containing 50 mM NaPi (pH 7.0), 100 mM NaCl, and 1 \(\mu\)M GES-1 and -2 or 750 mM GES-5 in the absence and presence of 20 \(\mu\)M (in the case of GES-1), 5 \(\mu\)M (in the case of GES-2), or 15 \(\mu\)M (in the case of GES-5) imipenem (10 \(\times\) \(K_{p}^E\)) was incubated at room temperature for 2 min. This reaction was diluted 1:100 and/or 1:1,000 into a reaction mixture containing 50 mM NaPi (pH 7.0), 100 mM NaCl, and 800 \(\mu\)M (in the case of GES-1), 50 \(\mu\)M (in the case of GES-2), or 600 \(\mu\)M (in the case of GES-5) of nitrocefin, and nitrocefin hydrolysis was monitored at 500 nm. The rate constant describing recovery of enzymatic activity (k\(_s\)) was determined by fitting the absorbance versus time data with Equation 4.

\[
A_t = A_0 + \nu_e t - \frac{\nu_i}{k_s}(1 - e^{-k_{slow} t})
\]

(Eq. 4)

where \(A_t\) is the absorbance at time \(t\), \(A_0\) is the initial absorbance, \(\nu_e\) is the steady-state velocity, and \(k_s\) is the rate constant for the recovery of enzymatic activity (i.e. deacylation).

\(^2\)The abbreviation used is: MIC, minimal inhibitory concentration.
Calculation of the Microscopic Rate Constants Describing Imipenem Hydrolysis—Hydrolysis of imipenem by GES-1, -2, and -5 is considered in Scheme 1.

\[
E + S \rightleftharpoons ES \rightarrow E - S \rightarrow E + P
\]

SCHEME 1

The following kinetic parameters can be derived for Scheme 1.

\[
k_{\text{cat}} = \frac{k_1 k_3}{k_2 + k_3}
\]

(Eq. 5)

\[
K_m = \frac{k_2 k_3'}{k_2 + k_3'}
\]

(Eq. 6)

\[
K_s' = \frac{k_1 + k_2}{k_1'}
\]

(Eq. 7)

\[
K_s = \frac{k_1'}{k_1'}
\]

(Eq. 8)

The values for \(k_1\) and \(k_{-1}\) for GES-1, -2, and -5 and the value for \(k_3\) for GES-5 were calculated using Equations 5–8 and the experimentally determined values for \(k_{\text{cat}}, K_m, K_s, k_2,\) and/or \(k_3\).

RESULTS AND DISCUSSION

\(\beta\)-Lactam Resistance Profile—We constructed a constitutive expression vector to directly compare the MICs of \(\beta\)-lactam antibiotics conferred by individual GES enzymes under an identical promoter in \(E. coli\) JM83. As the results of Table 1 (and supplemental Table 2) reveal, the breadth of activity for all three enzymes is broad. All are resistant to penicillins, including the penicillin-stable oxacillin, and first- and second-generation cephalosporins. The expression of GES-1 and -2 confers higher resistance to the third-generation cephalosporins than is the case with GES-5, but the organism that expresses the latter enzyme has instead gained resistance to cephemycins. Most alarming is the increase in resistance toward carbapenems (imipenem, meropenem, ertapenem, and doripenem) by GES-5 (Table 1). We note that the elevated MICs for carbapenems within a laboratory \(E. coli\) strain (1 \(\mu\)g/ml for imipenem with GES-5) are considered acceptable clinically. However, the emergence of variants such as GES-5 in combination with other mechanisms of carbapenem resistance, such as the loss of certain porins, results in full blown resistance to carbapenems in clinical isolates (6, 22). These differences in MIC values highlight the importance of the residue at position 170 in determining the substrate specificity of these enzymes.

### TABLE 1

| MICs of carbapenem antibiotics for \(E. coli\) JM83 producing various GES enzymes | GES-1 | GES-2 | GES-5 |
|---------------------------------|-------|-------|-------|
| Antibiotic                      | Control |       |       |
| imipenem                       | 0.125  | 0.25  | 0.25  | 1     |
| meropenem                      | 0.031  | 0.031 | 0.031 | 0.5   |
| ertapenem                      | 0.004  | 0.031 | 0.031 | 0.25  |
| doripenem                      | 0.031  | 0.031 | 0.031 | 0.25  |

* Parental \(E. coli\) JM83 strain harboring the vector pHF016 containing no \(\beta\)-lactamase gene.

### TABLE 2

| Kinetic parameters for hydrolysis of imipenem by GES enzymes | GES-1 | GES-2 | GES-5 |
|-------------------------------------------------------------|-------|-------|-------|
| \(k_{\text{cat}}\) (s\(^{-1}\)) | 0.0059 ± 0.0003 | 0.012 ± 0.001 | 0.44 ± 0.01 |
| \(K_m\) (\(\mu\)M) | 1.9 ± 0.7 | 0.7 ± 0.5 | 1.5 ± 0.3 |
| \(k_{\text{cat}}/K_m\) (\(\text{M}^{-1}\) s\(^{-1}\)) | \(3 \pm 1\) \(\times\) \(10^4\) | \(2 \pm 1\) \(\times\) \(10^6\) | \(2.9 \pm 0.6\) \(\times\) \(10^5\) |
| \(K_m\) (nm) | 11 ± 2 | 224 ± 15 | 491 ± 45 |
| \(k_1\) (s\(^{-1}\)) | 3 \(\times\) \(10^2\) | 2 \(\times\) \(10^4\) | 3 \(\times\) \(10^5\) |
| \(k_1\) (s\(^{-1}\)) | 3 \(\times\) \(10^1\) | 5 \(\times\) \(10^3\) | 1.4 \(\times\) \(10^4\) |
| \(k_1\) (s\(^{-1}\)) | 0.007 ± 0.001 | 0.021 ± 0.001 | 0.032 ± 0.001 |
| \(k_1\) (s\(^{-1}\)) | 0.03 ± 0.01 | 0.028 ± 0.001 | 0.45 ± 0.01 |

* Not measured experimentally.

### Steady-state Kinetic Parameters and Dissociation Constants—

The GES-1, -2, and -5 enzymes were purified to homogeneity for kinetic analyses. Class A \(\beta\)-lactamases (including GES enzymes) are acylated by \(\beta\)-lactam antibiotics at Ser-70, a step that is facilitated by Lys-73 (23). Glu-166 and Asn-170 (the residue that varies in GES-1, -2, and -5) anchor the hydrolytic water molecule within the active site. This water promotes deacylation of the acyl-enzyme intermediate to complete substrate turnover. We evaluated the kinetics of imipenem turnover in detail (Table 2). Turnover of imipenem by GES-1 and -2 was poor compared with the case of GES-5. The significant effect on steady-state parameters is manifested on \(k_{\text{cat}}\) resulting in a roughly two orders of magnitude increase in \(k_{\text{cat}}/K_m\) for hydrolysis of imipenem going from GES-1 to -5. Enzyme saturation for substrate turnover (as judged by \(K_m\)) is achieved at low micromolar concentrations, but enzyme acylation was sufficiently sluggish to allow evaluation of the dissociation constants for the non-covalent enzyme-imipenem complexes (\(K_s\)). The \(K_s\) value for imipenem was 10 nM for GES-1 but significantly higher for GES-2 and -5, although it was still in the high nanomolar range. The value for \(K_m\) is greater than the \(K_s\) for each GES variant. This reveals that acylation (\(k_1\)) is more rapid than dissociation of the substrate from the non-covalent ES complex (\(k_{-1}\)), contrary to what is generally assumed for reactions following Michaelis-Menten kinetics (24).
Imipenem Resistance in GES β-Lactamases

![Chemical structures of imipenem and the product of imipenem hydrolysis.](image)

from a kinetic standpoint, due to the 225-fold difference in the acylation rate constants for the two isomers. This assertion is also supported by the fact that the measured $k_{cat}$ is greater than the rate constant describing the slower phase. Alternatively, two distinct enzyme populations might exist that experience acylation by imipenem with different rate constants. We propose that the rapid phase represents acylation of GES-5 under steady-state conditions and therefore that the acylation rate constant is 75-fold larger than $k_{cat}$ and hence is not rate-limiting for this enzyme variant. This represents a remarkable 5000- and 1500-fold enhancement of the rate of acylation of the GES-5 enzyme over that of GES-1 and -2, respectively, by imipenem.

We evaluated the deacylation rate constant ($k_d$) for the turnover of imipenem by monitoring recovery of enzyme activity (Table 2 and supplemental Fig. 2) (21). We note that we prepared the product of imipenem hydrolysis (an inseparable and interconvertible mixture of $\Delta^1$ and $\Delta^2$ tautomers, Fig. 1) by chemical synthesis. Analysis of this product with the GES variants revealed the dissociation of the non-covalent enzyme-product complex is readily achieved ($K_p > 4 \text{ mM}$) and would not affect the recovery of enzymatic activity. The GES-1 acyl-enzyme intermediate experiences deacylation with a rate constant of $0.03 \pm 0.01 \text{ s}^{-1}$, confirming that acylation by imipenem is indeed rate-limiting for this enzyme. The GES-2 acyl-enzyme intermediate undergoes deacylation with a rate constant of $0.028 \pm 0.001 \text{ s}^{-1}$, very close to the value for the acylation event, which reveals that neither acylation nor deacylation is entirely rate-limiting for this variant. The upper limit of detection by this method is $0.03 \text{ s}^{-1}$, which is smaller than the value of $k_{cat}$ for hydrolysis of imipenem by GES-5. Thus, we were unable to evaluate $k_d$ for GES-5 by this method, although the value for this rate constant can be estimated using Equation 5 and our experimental values for $k_{cat}$ and $k_d$ (Table 2). The deacylation rate constant for GES-5 is virtually identical to $k_{cat}$, and $k_d$ is larger than $k_{cat}$, indicating that this step is now rate-limiting, in contrast to the cases of GES-1 and -2. The GES-5 variant has experienced a 15-fold enhancement in the deacylation rate constant, over that of GES-1 and -2, which is necessary for the manifestation of imipenem resistance. Despite the fact that the magnitude of the enhancement for the acylation step is significantly larger than it is for deacylation, it would appear that the enhanced rate of acylation in conjunction with the more modest augmentation in $k_d$ are the critical events for the emergence of carbapenemase resistance in GES-5.

Microscopic Rate Constants for Substrate Binding and Release—Although direct evaluation of microscopic rate constants for substrate binding ($k_+$) and substrate release ($k_-$) are not possible, these rate constants were estimated using our experimental data and Equation 5–8 (Table 2). The GES-1 variant, containing Gly at position 170, has the highest affinity for imipenem ($K_d$), primarily due to a very small rate constant for substrate release ($k_-$). It is interesting to note that each substitution at position 170, from Gly to Asn and finally Ser (GES-1, -2, and -5, respectively), progressively results in 10-fold increases in the rate constant for imipenem binding ($k_+$) and a progressive 100-fold increase in the rate constants for imipenem release. This leads to a decreased affinity for imipenem from GES-1 to GES-2 to GES-5. This is desired for an enzyme that becomes a more competent catalyst as the loss of affinity for the substrate (larger $K_d$) avoids the undesired prospect of substrate inhibition.

Structural Comparison and Molecular Dynamics Simulations—Comparison of the structures of GES-1 (Fig. 2A) and other carbapenemases to class A β-lactamases, which do not hydrolyze carbapenem antibiotics, reveals subtle differences in their active sites. The x-ray structure provides only a snapshot of the enzyme, whereas in reality the protein structure should experience dynamic motion in solution, which may explain its ability to hydrolyze carbapenem antibiotics. The Ω-loop motif, which includes both residues 166 (critical for deacylation) and 170, assumes different conformations in various class A enzymes (Fig. 2) and, at least in the case of a subset of the TEM mutants, it has been argued to be a mobile element (27–29). Both TEM-1 (Fig. 2B) and NMC-A β-lactamases (Fig. 2C) are able to bind carbapenem antibiotics; however, only NMC-A is able to efficiently turn them over. We have previously shown that movement of the Ω-loop in NMC-A facilitates hydrolysis of carbapenem antibiotics (25). We wondered whether the Ω-loop within the active site of GES-1 is a mobile element. If so, this could explain how at least GES-1 could bind and slowly hydrolyze carbapenems. If indeed mobile, this factor might be critical for the GES-5 enzyme, as it has an enhanced rate of deacylation for imipenem. A 6-ns dynamics simulation revealed mobility in the Ω-loop (Glu-166 Ca moved into the active site at least by −1 Å, Fig. 2D). The dynamic simulations also revealed the existence of a water molecule between residues Glu-166 and Gly-170 within the active site of GES-1, presumably the deacylation water molecule. This suggests that as in the case of NMC-A, movement of the Ω-loop may facilitate enzyme deacylation.

Conclusion—A single amino acid substitution at position 170 of GES enzymes has a dramatic impact on the turnover of the carbapenem antibiotic imipenem. The GES-1 enzyme with Gly-170 has a high affinity for imipenem but is unable to efficiently turn it over due to the low rate constants for acylation and deacylation. Substitution to Asn-170, in GES-2, results in the canonical residue at this position for typical class A β-lactamases. This variant shows no improvement in the rate constant for deacylation, but the rate constant for acylation is enhanced such that neither is rate-determining. The GES-5 enzyme, with Ser-170, shows an enhancement...
of both microscopic rate constants; however, the defining alteration in the kinetic profile of GES-5 is the 5000- and 1500-fold increase in the rate constant for acylation over that of GES-1 and GES-2, respectively. This results in the alteration of the rate-determining step of the reaction from acylation to deacylation and reveals that the enhanced rate of deacylation is responsible for the increased resistance to imipenem. It is not obvious at the present, from a structural perspective, why the residue at position 170 should have an effect on the rate of acylation and/or deacylation. Elucidation of these questions awaits the future availability of x-ray information for the complexes of these enzymes with carbapenem antibiotics.

REFERENCES

1. Fisher, J. F., Meroueh, S. O., and Mobashery, S. (2005) *Chem. Rev.* **105**, 395–424
2. Babic, M., Hujer, A. M., and Bonomo, R. A. (2006) *Drug Resist. Updat.* **9**, 142–156
3. Walther-Rasmussen, I., and Haiby, N. (2007) *J. Antimicrob. Chemother.* **60**, 470–482
4. Page, M. G. (2008) *Clin. Microbiol. Infect.* **14**, Suppl. 1, 63–74
5. Queenan, A. M., and Bush, K. (2007) *Clin. Microbiol. Rev.* **20**, 440–458
6. Nordmann, P., and Poirel, L. (2002) *Clin. Microbiol. Infect.* **8**, 321–331
7. Nordmann, P., Cuzon, G., and Naas, T. (2009) *Lancet Infect. Dis.* **9**, 228–236
8. Bae, I. K., Lee, Y. N., Jeong, S. H., Hong, S. G., Lee, J. H., Lee, S. H., Kim, H. I., and Youn, H. (2007) *Diagn. Microbiol. Infect. Dis.* **58**, 465–468
9. Vourli, S., Giakkoupi, P., Miriagou, V., Tzelepi, E., Vatopoulos, A. C., and Tzouvelekis, L. S. (2004) *FEMS Microbiol. Lett.* **234**, 209–213
10. Poirel, L., Le Thomas, I., Naas, T., Karim, A., and Nordmann, P. (2000) *Antimicrob. Agents Chemother.* **44**, 622–632
11. Poirel, L., Weldhagen, G. F., Naas, T., De Champs, C., Dove, M. G., and Nordmann, P. (2001) *Antimicrob. Agents Chemother.* **45**, 2598–2603
12. Giakkoupi, P., Tzouvelekis, L. S., Tsakris, A., Loukova, V., Sofianou, D., and Tzelepi, E. (2008) *Antimicrob. Agents Chemother.* **44**, 2247–2252
13. Wachino, J., Doi, Y., Yamane, K., Shibata, N., Yagi, T., Kubota, T., and Arakawa, Y. (2004) *Antimicrob. Agents Chemother.* **48**, 2905–2910
14. Wachino, J., Doi, Y., Yamane, K., Shibata, N., Yagi, T., Kubota, T., Ito, H., and Arakawa, Y. (2004) *Antimicrob. Agents Chemother.* **48**, 1960–1967
15. Poirel, L., Brinas, L., Fortineau, N., and Nordmann, P. (2005) *Antimicrob. Agents Chemother.* **49**, 3593–3597
16. Mavroidi, A., Tzelepi, E., Tsakris, A., Miriagou, V., Sofianou, D., and Tzouvelekis, L. S. (2001) *J. Antimicrob. Chemother.* **48**, 627–630
17. Ambler, R. P., Coulson, A. F., Frère, J. M., Ghuyens, J. M., Joris, B., Forsman, M., Levesque, R. C., Tiraby, G., and Waley, S. G. (1991) *Biochem. J.* **276**, Pt 1, 269–270
18. Smith, C. A., Caccamo, M., Kantardjieff, K. A., and Vakulenko, S. (2007) *Acta Crystallogr. D Biol. Crystallogr.* **63**, 982–992
19. Clinical and Laboratory Standards Institute. (2006) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically: Approved Standard*, 7th Ed., Clinical and Laboratory Standards Institute, Wayne, Pennsylvania
20. Zafaralla, G., Manavathu, E. K., Lerner, S. A., and Mobashery, S. (1994) *Biochemistry* **33**, 3847–3852
21. Taibi, P., and Mobashery, S. (1995) *J. Am. Chem. Soc.* **117**, 7600–7605
22. Kim, S. Y., Park, Y. J., Yu, J. K., Kim, H. S., Park, Y. S., Yoon, J. B., Yoo, J. Y., and Lee, K. (2007) *Diagn. Microbiol. Infect. Dis.* **57**, 85–91
23. Meroueh, S. O., Fisher, J. F., Schlegel, H. B., and Mobashery, S. (2005) *J. Am. Chem. Soc.* **127**, 15397–15407
24. Fersht, A. (1999) *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*, pp. 103–131, W.H. Freeman, New York
25. Mourey, L., Miyashita, K., Swaren, P., Bulchvey, A., Samama, J. P., and Mobashery, S. (1998) *J. Am. Chem. Soc.* **120**, 9382–9383
26. Ratcliffe, R. W., Wildonger, K. J., Dimichele, L., Douglas, A. W., Hajdu, R., Goegelman, R. T., Springer, J. P., and Hirshfield, J. (1989) *J. Org. Chem.* **54**, 653–660
27. Vakulenko, S. B., Taibi-Tronche, P., Toth, M., Massova, I., Lerner, S. A., and Mobashery, S. (1999) *J. Biol. Chem.* **274**, 23052–23060
28. Taibi-Tronche, P., Massova, I., Vakulenko, S. B., Lerner, S. A., and Mobashery, S. (1996) *J. Am. Chem. Soc.* **118**, 7441–7446
29. Palchik, T., Le, Q. Q., Venkataram, K. V., L’Rocco, M., and Otera, H. (1994) *Mol. Microbiol.* **12**, 217–229
30. Maveyraud, L., Mourey, L., Kotra, L. P., Pedelacq, J. D., Guillet, V., Mobashery, S., and Samama, J. P. (1998) *J. Am. Chem. Soc.* **120**, 9748–9752