The Different Inhibition Mechanisms of OXA-1 and OXA-24 \(\beta\)-Lactamases Are Determined by the Stability of Active-Site Carboxylated Lysine

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**Background:** OXA-1 and OXA-24 are class D \(\beta\)-lactamases that resist clinically-used inhibitors.

**Results:** Spectroscopic methods and kinetic measurements show that penem drug candidates are good inhibitors of OXA-1 but are rapidly hydrolyzed by OXA-24.

**Conclusions:** An active site water in OXA-24 aids the reversible carboxylation of Lys84 enabling many reaction cycles.

**Significance:** Understanding the mechanism of class D \(\beta\)-lactamases is vital for drug development.

**ABSTRACT**

The catalytic efficiency of class D \(\beta\)-lactamases depends critically on an unusual carboxylated lysine as the general base residue for both the enzyme's acylation and deacylation steps. Microbiological and biochemical studies on the class D \(\beta\)-lactamases OXA-1 and OXA-24 showed that the two enzymes behave differently when reacting with two 6-methylidene penems (penem 1 and penem 3): the penems are good inhibitors of OXA-1 but act more like substrates for OXA-24. UV difference (UVD) and Raman spectroscopy revealed the respective reaction mechanisms are different. The penems form an unusual intermediate, a 1,4-thiazepine derivative in OXA-1 and undergo deacylation followed by the decarboxylation of Lys70, rendering OXA-1 inactive. This inactivation could not be reversed by the addition of 100 mM NaHCO\(_3\). In OXA-24, under mild conditions (E: I \(=\ 1: 4\)), only hydrolyzed products were detected and the enzyme remained active. However, under harsh conditions (E: I \(=\ 1: 2000\)), OXA-24 was inhibited via decarboxylation of Lys84, yet the enzyme could be reactivated by the addition of 100 mM NaHCO\(_3\). We conclude that OXA-24 not only decarboxylates with difficulty but also recarboxylates with ease; in contrast, OXA-1 decarboxylates easily but recarboxylates with difficulty. Structural analysis of the active site indicates a crystallographic water molecule may play an important role in carboxylation in OXA-24 (an analogous water molecule is not found in OXA-1), supporting the suggestion that a water molecule in OXA-24's active site can lower the energy barrier for carboxylation significantly.

\(\beta\)-Lactamase production is the most important mechanism by which Gram-negative pathogens including *Acinetobacter baumannii* and *Pseudomonas aeruginosa* become resistant to \(\beta\)-lactam antibiotics. Based on their protein sequence similarities, they are divided into four major classes (Class A-D) (1). Class A, C and D enzymes involve an active-site serine to hydrolyze \(\beta\)-lactams, whereas class B enzymes are zinc-dependent hydroases. Unlike the majority of class A enzymes which have been extensively studied, class D \(\beta\)-lactamases confer a higher-level
resistance to a broad spectrum of β-lactam inhibitors and are the least understood class (2,3).

The class D β-lactamases are characterized by the presence of a unique carboxylated lysine in the active site that participates in catalysis. While carboxylated lysine has also been found in other enzymes, such as rubisco (4), urease (5) and phosphotriesterase (6), there it mainly serves in a structural role. The carboxylated lysine in class D enzymes plays a similar role as the general base (Glu166) in SHV-1 class A β-lactamase (7). The formation of the carboxylated lysine is reversible (8). Low pH or mutation of hydrophobic residues surrounding the carboxylated lysine, such as Val117 (OXA-1) or Trp154 (OXA-10) results in decarboxylation of that lysine and loss of enzyme's activity, notably deacylation (9-12); while the addition of bicarbonate can reactivate the enzyme by recarboxylation of the lysine (8,12,13).

OXA-1 and OXA-24 are two class D enzymes exhibiting resistance to the clinically available β-lactamase inhibitors (tazobactam, sulbactam and clavulanate). They are both monomeric and are related based on three aspects: 1) ~30% sequence homology; 2) similar folded structures; 3) highly conserved active-site residues (>95%) (Using PDB entry: OXA-1, 1M6K (2); OXA-24, 3G4P (14)). However, they show differing affinities for β-lactam-based inhibitors.

OXA-1 is the most common of the class D β-lactamases and is found in up to 10% of Escherichia coli and Pseudomonas aeruginosa (2,15). Its closely-related variants (e.g. OXA-15, OXA-18, OXA-19), due to point mutations and plasmid transfer, have arisen with enhanced capability to hydrolyze imipenem, aztreonam and third-generation cephalosporins such as cefotaxime and ceftriaxone (15,16). Found in Acinetobacter baumannii, OXA-24 is a class D carbapenem-hydrolyzing enzyme that also possesses extended-spectrum cephalosporinase activity (3,17). Both OXA-1 and OXA-24 cause serious problems in nosocomial infections such as bloodstream infections, wound infections, and ventilator-associated pneumonia (18,19). Thus, the need to develop potent inhibitors of these enzymes is an urgent priority; to achieve this, it is imperative to understand the properties of these enzymes and how they work.

In this article, the catalytic properties of OXA-1 and OXA-24 are evaluated using two methyldene penems (penems 1 and 3, Figure 1). Bethel et al. in 2008 proposed that penems inactivate OXA-1 β-lactamase efficiently by forming an unusual acyl-enzyme complex (20). Here, our results show that penem inhibitors have a high affinity for both OXA-1 and OXA-24 enzymes. However, they are effective inhibitors of OXA-1 but act more like substrates for OXA-24.

EXPERIMENTAL PROCEDURES

Genetic constructs and host strains- The blaOXA-1 gene was cloned from plasmid RGN238 into pET12a(+)-KM as described previously (2). Plasmid RGN238blaOXA-1 was maintained in E. coli DH10B cells (Invitrogen, Carlsbad, CA). This host strain was used for minimum inhibitory concentration (MIC) determinations. For protein purification, blaOXA-1 was cloned in the modified vector pET12a(+) -KM described previously, and was expressed in E. coli BL21(DE3) cells (Stratagene, La Jolla, CA) (16).

For large-scale protein expression and β-lactamase characterization, the blaOXA-24 gene was cloned into the pET24a (+) vector (Novagen, Madison, WI) according to the following method. Using the Gene-Amp XL PCR kit (Applied Biosystems), high-fidelity amplification of blaOXA-24 without leader peptide sequence from the OXA-24/pIM-1-RA clone designed by Héritier et al. (22) was performed with primers OXA-24FOR and OXA-24REV, listed in Table 1. The cycling conditions used were 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min for 25 cycles, after which there was final extension at 72 °C for 10 min. A restriction digest of the pET24a (+) vector was done using NdeI and BamHI. The amplification product was purified using the QIAquick gel.
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extraction kit (Venlo, Netherlands) and digested using NdeI and BamHI. This product was ligated to the digested pET24a (+) vector and electroporated into E. coli DH10B. The resulting construct was sequenced with pET24a (+) primers T7 promoter primer and T7 terminator primer. After sequencing verification, the construct was transformed into E. coli BL21(DE3) cells for protein expression.

For MIC determinations, blaOXA-24 was cloned into the pBC SK (+) phagemid vector (Stratagene, La Jolla, CA) as described previously (23). blaOXA-24/pBC SK (+) sequence was verified using M13 Universal and M13 Reverse primers.

Inhibitors- The inhibitors penem 1 and penem 3 (Figure 1) were gifts from Wyeth Pharmaceuticals (Madison, NJ). Their chemical synthesis were described previously (24). A stock solution of the inhibitor at 20 mM in 10 mM HEPES buffer (pH 7.5) was prepared for "soak in" and "soak out" experiments with the protein crystals and for UV-absorbance studies. Only water was used in the rapid mix-rapid freeze experiment to avoid the interference of salt signal. Potency was verified using the colorimetric β-lactamase substrate nitrocefin (Becton Dickson, λ max = 482 nm; ε = 17,400 M -1 cm -1 ).

Antibiotic Susceptibility- The MICs of E. coli DH10B expressing blaOXA-1 or blaOXA-24 β-lactamases were determined in Mueller-Hinton (MH) agar supplemented with 20 mM NaHCO 3 using a Steers replicator which delivered 10 µl of MH broth containing 10 4 colony-forming units/spot. The penem inhibitors were tested at the concentration of 4 µg/ml partnered with piperacillin (Sigma, St. Louis, MO) at concentrations of 1-2048 µg/ml. As a comparator inhibitor, tazobactam (Chem-Impex, Wood Dale, IL) was used at the concentration of 4 µg/ml. Breakpoints for susceptibility and resistance were defined by the Clinical Laboratory Standards Institute and interpreted with criteria published in 2005 (CLSI standard M100-S15) (25,26).

Protein Isolation, Purification, and Crystallization- OXA-1 or OXA-24 were purified as described previously (20,27). Concentration of the protein was measured by Bio-Rad's protein assay, and the purity of the enzyme was evaluated by SDS-PAGE. Following purification, OXA-1 β-lactamase was crystallized using the protocol described in Sun et al. with protein concentration of 9 mg/ml (2). Crystals were grown by the hanging drop vapor diffusion method in a crystallization solution containing 0.05 M HEPES (pH 7.5) and 15% PEG 8000. OXA-24 β-lactamase was crystallized using the protocol of Bou et al (14). Briefly, OXA-24 was concentrated to 6 mg/ml in 10 mM HEPES buffer (pH 7.5). Crystals were grown by the hanging drop vapor diffusion method in a crystallization solution containing 0.1 M HEPES (pH 7.5), 0.1 M sodium acetate and 28% PEG 2000.

Ultraviolet difference (UVD) spectroscopy- In our protocol, each spectrum included absorbance at wavelengths (λ) from 200 nm to 600 nm. The spectrum of only β-lactamase (20 µM) or inhibitor (20 or 80 µM) was taken separately. Then OXA-1 and OXA-24 (20 µM for both) reacted with penem 1 (20 or 80 µM) in a ratio of 1: 1 or 1: 4 (E: I) and UV-absorbance spectra were taken at 30 second intervals, with the length of the experiment being 30 min. To obtain UV difference spectra, an apo β-lactamase spectrum was subtracted from the protein-inhibitor complex spectra at varying time intervals following addition of inhibitor.

Nitrocefin (NCF) assay uses nitrocefin as a chromogenic substrate to monitor the enzyme activity in solution. When OXA-1 or OXA-24 react with NCF, NCF is hydrolyzed and turns red (λ max = 482 nm); when the enzyme is inhibited, NCF is unreacted and remains yellow (λ max = 382 nm).

Raman Spectroscopy- The Raman microscope system has been described previously (28,29). A single OXA-1 or OXA-24 crystal was transferred from the mother liquor solution to a 4 µl drop of 0.05 M HEPES (pH 7.5) and 15% PEG 8000 (for OXA-1) or a drop of 0.1 M HEPES (pH 7.5), 0.1 M NaOAc and 28% PEG 2000 (for OXA-24). After obtaining spectra of the apo protein crystals, inhibitors were soaked into the drop to achieve a final volume of 5 µl and a final inhibitor concentration of 5 mM. Spectra were then acquired every 2–3 min after addition of the inhibitors. To obtain difference spectra, an apo β-lactamase spectrum was subtracted from the protein-inhibitor spectra at varying time intervals following addition of inhibitor (27).
To study the reaction in solution at early time points, we used a slightly modified KinTek instrument model RQF-3 (30). Reactions were initiated by mixing OXA-1 or OXA-24 enzyme (2.5 mg/ml) with penem inhibitors in a 1: 2 molar ratio and quenched at 1 s. The sample after reactions was examined using Raman microscope (31). For longer time scale reaction, we also used hand-mixing system which incubated enzyme with inhibitor at 1: 2 ratio for 5 s and 30 s and then quenched the reaction by injecting into the isopentane solution surrounded by liquid nitrogen.

**Kinetics** - Steady state kinetics were performed on an Agilent 8453 diode array spectrophotometer (Palo Alto, CA) in 50 mM Na phosphate buffer (pH 7.2) supplemented with 20 mM NaHCO\(_3\). \(v_\text{max}\) and \(K_m\) were determined from initial steady-state velocities for nitrocefin (NCF). The kinetic parameters were obtained using iterative non-linear least-squares fit of the data to the Henri-Michaelis equation using Origin 8.1 (OriginLab, Northampton, MA) according to Equation 1:

\[
v = \frac{V_{\text{max}} [S]}{(K_m + [S])} (1)
\]

The overall mechanism for \(\beta\)-lactamase-inhibitor reaction is shown in Scheme 1. We determined the \(K_i\) for the penems by measuring initial steady-state velocities of inhibitor against the indicator substrate NCF (100 \(\mu\)M). Assuming a competitive mode of inhibition under these conditions, initial velocity \((v_0)\) measurements immediately after mixing yield a \(K_i\) which closely approximates \(K_m\), as represented by Equation 2:

\[
v_0 = \frac{(V_{\text{max}}[S])}{K_{\text{cat}}(1 + K_i) + [S])} (2)
\]

\(K_i\) values were corrected for nitrocefin affinity \((K_m = 8.3 \mu\text{M for OXA-1, } K_m = 28 \mu\text{M for OXA-24})\) according to Equation 3:

\[
K_i(\text{corrected}) = \frac{K_i(\text{observed})}{(1 + [\text{NCF}]/K_{m\text{NCF}})} (3)
\]

IC\(_{50}\) defined as the inhibitor concentration resulting in a reduction of NCF (100 \(\mu\)M) hydrolysis by 50%, was determined by measurements of initial velocities after 5 min pre-incubation of enzyme with inhibitor.

Turnover numbers \((t_n)\) or partition ratios \((k_{\text{cat}}/k_{\text{nact}})\) \((k_{\text{cat}})\) refers to hydrolytic efficiency for inhibitors, as shown in Scheme 1) were determined as the ratio of inhibitor concentration to enzyme concentration necessary to decrease enzyme activity by 95% (32). The turnover numbers were determined after a 24 h incubation with increasing concentrations of the inhibitor. Incubations were done in a final volume of 300 \(\mu\)l and 25 \(\mu\)l of this reaction mixture were added to a 1 ml final volume to determine the residual enzyme activity using 100 \(\mu\)M nitrocefin.

**Quantum Mechanical Calculations** - \textit{Ab initio} quantum mechanical calculations were performed on CWRU’s cluster facility to predict the Raman spectra of penems and model intermediate compounds using Gaussian 03 (33). Calculations were performed at the DFT level using the 6-31+G(d) basis set. DFT calculations were performed with Becke’s three parameters hybrid method using the correlation functional of Lee, Yang, and Parr (B3LYP) (34,35). The vibrations giving rise to the most intense calculated peaks could be visualized using "GaussView", revealing which molecular vibrations contribute to the peaks.

**RESULTS**

a) **Kinetic data for penem 1 and 3 reacting with OXA-1 and OXA-24**

**Antibiotic susceptibility.** To first determine whether penem inhibitors can be used as effective partners with clinical antibiotics, we performed microbiological assays to evaluate their ability to lower the MICs. To establish a comparison, we used piperacillin, a broad-spectrum penicillin family member, with the penems at a concentration of 4 \(\mu\)g/ml. We also used tazobactam at the same concentration as a comparator \(\beta\)-lactamase inhibitor. Tazobactam in combination with piperacillin became available in clinic in the United States in 1993 and does extend piperacillin’s activity against most class A \(\beta\)-lactamase producing strains. Against \textit{E. coli} DH10B lacking OXA-1 or OXA-24 expression, the piperacillin MICs are 8 \(\mu\)g/ml, well within the susceptible range for piperacillin (CLSI guidelines) (36). In the bacterial strain where OXA-1 or OXA-24 is expressed, a high-level piperacillin resistance is observed (Table 2, piperacillin MIC is 512 \(\mu\)g/ml for OXA-1, 1024 \(\mu\)g/ml for OXA-24). When tazobactam was combined with piperacillin at the concentration of 4 \(\mu\)g/ml, we did not detect reduction in MICs for OXA-24 (1024 \(\mu\)g/ml, Table 2), and only slight reduction for OXA-1 (256 \(\mu\)g/ml, and no significant inhibition with piperacillin). This is consistent with the
observation that the current clinically used β-lactamase inhibitors (tazobactam, sulbactam and clavulanate) are not effective against class D β-lactamases (14,20,27). Before measuring the inhibitory activity of penem inhibitors combined with piperacillin, we first tested whether penem 1 or penem 3 possess any intrinsic antibiotic activity against bacterial strains. The results showed the MICs for penem 1 or penem 3 alone are >1024 µg/ml, indicating that they alone do not bear any inhibitory activity.

Penems combined with piperacillin resulted in significant differences between OXA-1 and OXA-24. In OXA-1, a noticeable reduction in MICs by penem 1 or penem 3 was observed (512 to 8 µg/ml, both). However, in OXA-24, the MIC is not affected in the presence of penem 1 or penem 3, which shows that the two inhibitors are not effective against OXA-24 β-lactamase.

**Kinetic parameters.** In order to further demonstrate that OXA-1 and OXA-24 β-lactamases behave differently with penem inhibitors, we performed kinetic assays to observe the properties and activities of penems 1 and 3. Table 3 shows the $K_i$ and $IC_{50}$ of the penem compounds with the enzymes OXA-1 and OXA-24. The data suggest that penem 1 and penem 3 are good inhibitors against both OXA-1 and OXA-24 because their $K_i$ and $IC_{50}$ values are very low (at nM level). However, closer examination shows that the $IC_{50}$ value is much lower than $K_i$ in OXA-1 while higher in OXA-24. This suggests that, in OXA-24, the two penem inhibitors to some extent undergo subsequent hydrolysis before forming the stable acyl-enzyme complex. Thus, next we aimed to measure the turnover number for both enzymes. In Table 3, the results show that $t_n$ for OXA-24 is ~450 times higher than for OXA-1 (900 vs 2). Considering that the periplasmic concentration of the OXA-10 β-lactamase in two clinical strains of *Pseudomonas* is about 4-15 µM (8), if OXA-1 or OXA-24 are at a similar concentration level as OXA-10, it would not be possible to inhibit OXA-24 under physiological conditions because of the high amounts of penems required. In summary, penem 1 and 3 are effective inhibitors for OXA-1 but not for OXA-24.

**b) Spectroscopic evidence for different reaction schemes in OXA-1 and OXA-24**

_Ultraviolet difference (UVD) spectroscopy: the role of carboxylated lysine._ Further evidence that OXA-1 and OXA-24 react differently with penem inhibitors is also obtained by UVD spectroscopy that has been widely used to provide insight into the nature of reactive intermediates or products formed during β-lactamase inactivation processes (37-39). We reacted penem 1 with OXA-1 or OXA-24 at different ratios to see whether the reaction is stoichiometric. The data for penem 3 are not shown but they are similar to penem 1.

Figure 2 shows the reaction between penem 1 and OXA-1 or OXA-24 at 1: 1 or 1: 4 ratio (E: I). The peak at 280 nm represents the unreacted compounds and is assigned to an electronic transition located in the conjugated ring and the methylenic double bond at C6 position, extending to the carbonyl group in the β-lactam ring. At 1: 1 ratio, all penem 1 molecules have been consumed because the 280 nm peak disappears, only leaving the product spectrum (Figure 2A). However, at 1: 4 ratio, almost three fourths of the penem 1 remained unreacted in OXA-1 (red line, Figure 2B) and, at this time, the enzyme is no longer active against nitrocefin (not shown). In OXA-24, the spectrum for 1: 4 ratio shows a similar pattern as the 1: 1 ratio that all penem 1s are hydrolyzed (blue line, Figure 2B). The enzyme is still active.

The unique peaks of the hydrolyzed-product spectra in OXA-1 (255 and 375 nm, Figure 2A) and OXA-24 (351 nm, Figure 2A) suggest the reaction undergoes different pathways. Based on the studies by Bethel et al. (20), we propose a reaction mechanism for penem 1 and OXA-1 (Scheme 2, modified from Bethel et al. (20)). Previous studies of compound BRL 42715 (Figure 1), a similar structure to penem 1, showed its reaction with a base, sodium methoxide in methanol, results in the formation of a seven-membered thiazepine with chromophores at 253 and 370 nm (40), which is consistent with the peaks in UVD spectroscopy of OXA-1. In addition, the NMR studies by Bethel et al. also support the conclusion that a similar intermediate, the 1,4-thiazepine derivative, is formed during the reaction between penem 1 and OXA-1 (20). Both these serve as the experimental foundation for our proposed mechanism in Scheme 2 and support the existence of seven-membered thiazepine in the reaction between penem 1 and OXA-1.
For OXA-24, in terms of the turnover number (900 for penem 1), the situation mostly mimics the reaction pathway for penicillin hydrolyzed by OXA-24 β-lactamase (14). Under normal conditions, penem inhibitors are treated as substrates of the enzyme. Thus, we propose another mechanism for penem 1 and OXA-24 (Scheme 3, adapted from the mechanism for penicillin hydrolysis (14)). In contrast to the reaction in OXA-1, the carboxylated Lys84 in the active site of OXA-24 utilizes a catalytic water molecule to decarboxylate the Ser81. As a result, the penem 1 inhibitor is hydrolyzed, the enzyme is regenerated since Lys84 is not decarboxylated (as discussed below) and hydrolyzes the next arriving inhibitor molecule.

In order to confirm the role of carboxylation and decarboxylation of the active-site lysine, we undertook assays with nitrocefin (NCF) and bicarbonate. The nitrocefin assay shows that OXA-24 can be inhibited by a high concentration of penem 1 (E: I = 1: 2000), although the addition of 100 mM NaHCO₃ can reactivate the enzyme because nitrocefin is hydrolyzed again (Figure 3A). A recent study in our laboratory shows that one penem sulfone inhibitor, SA-1-204 (Figure 1), can effectively inhibit OXA-24 by decarboxylating the Lys84 in the active site, yet the enzyme is recarboxylated and becomes active again after adding 100 mM NaHCO₃ as a source of CO₂ molecules in the solution (27). These findings together indicate that, in OXA-24, a high concentration of penem 1 causes the decarboxylation of Lys84, which has been shown to be critical for the enzyme’s decacylation (10,27).

In contrast, the nitrocefin assay shows that a low concentration of penem 1 effectively inhibits OXA-1, but nitrocefin does not react in mixture of OXA-1 and penem 1 (E: I = 1: 4) after treatment with 100 mM NaHCO₃ (Figure 3B). Considering that OXA-1 and OXA-24 belong to class D β-lactamases that use a carboxylated lysine side chain (Lys70 and Lys84, respectively) to aid catalysis, we hypothesize that penem 1 can also cause the essentially irreversible decarboxylation of OXA-1.

**Raman studies of penem 1-OXA-1/OXA-24 reactions in solution.** Using the rapid mix-rapid freeze protocol developed in our laboratory (30), we examined the reaction mixture between OXA-1 or OXA-24 with penem 1, 1 second after mixing. A ratio of 1: 2 (E: I) was used, we expect for OXA-1 to see the inhibited complex (tₙ is 2, Table 3), but for OXA-24 to see predominantly hydrolyzed product since tₙ is about 900 (Table 3). These predictions are supported by the UVD spectra discussed in the previous section.

Figure 4 compares the Raman spectrum of free penem 1 in aqueous solution with the spectrum of the freeze-dried reaction mixtures obtained 1 second after mixing and flash freezing. We first employed *ab initio* quantum mechanical calculations to help interpret the spectrum of the substrate (upper trace, Figure 4), which serves as a basis for the following analysis. Based on Gaussian calculations, the peak at 1687 cm⁻¹ in the spectrum of unreacted penem 1 is assigned to the methylenic double bond coupled to the carbonyl group in the β-lactam ring (Table 4), which is expected to change markedly when the β-lactam ring opens or the hybridization at C6 changes. Another characteristic feature is at 1757 cm⁻¹, due to the carbonyl group (C=O) in the intact lactam ring. The disappearance of this peak suggests the opening of the lactam ring due to acylation of the enzyme (41).

For OXA-1 reacting with penem 1, we see evidence for two species. Quantum mechanical calculations indicate that the band at 1630 cm⁻¹ is from species 4 in Scheme 2 and is due essentially to the methylenic double bond stretch coupled to the ester bond (–O-C(=O)-C=C-) in acyl-enzymes. (Table 4), although this mode is partially delocalized over adjacent conjugated bonds. Chemical structures including this acrylic group (R₁–O-C(=O)-C=C-R₂) all give rise to intense Raman peak around 1628 cm⁻¹ (data not shown). This assignment is strengthened by the observation that a similar mode occurs at 1645 cm⁻¹ for the reaction with OXA-24 (Figure 4). The latter mode is due to hydrolyzed species E and/or F in Scheme 3 (see below). The Carey group reported in the 1980s that the ethylenic stretch in α, β conjugated molecules, such as cinnamic acid and furylacryloyl acid, increases by 10-15 cm⁻¹ upon ionization of the acid group (see Table II in (42)). Species E and F (Scheme 3) are the ionized form and species 4 (Scheme 2) is the neutral ester form, respectively. Important evidence for the second species comes from the broad unresolved band around 1500 cm⁻¹ (Figure 4). The quantum
mechanical calculations for the seven-membered ring seen in Scheme 2 show two intense features near 1510 and 1492 cm$^{-1}$ (Table 4). These features arise from the double bonds in the seven-membered ring and are not resolved in Figure 4. Meanwhile, we do not detect a mode characterizing an ester near 1725 cm$^{-1}$. This argues that we are detecting the species with the ionized -COO$^-$ (species 9) and not the ester-like acyl enzyme (species 6). However, we cannot definitively argue in favor of species 9 since in some instances the Raman spectrum of acrylic acid ester, the C=O feature has low intensity. Interestingly, a characteristic C-S stretch is also present at 715 cm$^{-1}$, giving additional support to the formation of the seven-membered ring. It is noteworthy that Ke et al. have detected the same seven-membered ring species in the X-ray structure of SHV-1 class A β-lactamase complexed with penem 1 (43).

The reason we have evidence for two species from the OXA-1 reaction is that with a $t_n$ of 2, we expect to produce a population of product species 9 in Scheme 2 and a population of covalently inhibited stable acyl-enzyme, species 4 in reaction scheme 2. This analysis predicts that Lys70 is decarboxylated after the first cycle and thus, in the second cycle, species 4 is bound irreversibly. An underlying hypothesis is that acylation cannot occur without the participation of Lys70 but that this residue is essential for deacylation.

The solution data for OXA-24 and penem 1 are expected to be very different since the $t_n$ is high for OXA-24 (900, Table 3), we should see a lot of penem 1 transformed into hydrolyzed product. This expectation is confirmed by the Gaussian calculations. The latter show that species F (Scheme 3) has an intense Raman band near 1645 cm$^{-1}$ as seen in Figure 4. This is due to the C=C stretch in molecule F where -COO$^-$ shifts new C=C 10-15 cm$^{-1}$ to higher wavenumber than in the neutral molecule C, as discussed above and listed in Table 4. The calculations also reproduce the intense Raman band near 716 cm$^{-1}$ from the thiazole five-membered ring of species F (Scheme 3) and support the presence of the thiazole ring.

**Raman Analysis of penem 1 reaction with OXA-1/OXA-24 single crystals.** Whereas the solution studies had enzyme: inhibitor ratio of 1:2, the crystals suspended in a hanging drop containing inhibitor have access to a huge excess of inhibitors - 10 mM in a 5 µl drop. For OXA-1 and penem 1, it is again likely that the enzyme goes through a maximum of 2 cycles.

Figure 5A shows the Raman difference spectra of penem 1 reaction in OXA-1 single crystal (underneath the spectrum of unreacted penem 1 in aqueous solution). The results show that the reaction occurs slowly because there is no intense Raman signal at 3 min (Figure 5A), indicating that penem 1 molecule has not entered the crystal and the active site of OXA-1 by the time. At 15 min, some new peaks appear, e.g. at 1656 cm$^{-1}$. At 30 min, another peak at 1628 cm$^{-1}$ intensifies and remains stable up to 1 h, this is assigned below. The intense at 1656 cm$^{-1}$ is assigned to the protonated imine (C=NH$^+$) of species 5 (Scheme 2). This protonated imine due to the opening of the five-membered ring is a common intermediate in sulbactam, tazobactam and clavulanate reaction with SHV-1 β-lactamase, which also give rise to intense peak at 1656 cm$^{-1}$ (44). On the basis of the calculations discussed above, we predict the seven-member ring product (species 9, Scheme 2) will generate two intense peaks around 1490 and 1500 cm$^{-1}$. These were detected in solution (above) but they are not obvious in Figure 5A. The reason may be due to the low-abundance or fast-release. Considering that there are still intense substrate peaks together with the intermediate peaks under soak in conditions, in order to see what species is finally left in the active site of OXA-1, we performed a soak out experiment. By immersing the reacted crystal in holding solution containing no substrate we remove non-covalently bound substrates or products, leaving the covalently bound species in the active site. After 1 h incubation with penem 1, we transferred the crystal to a new hanging drop without any inhibitor molecule and took the spectrum of the crystal. In the soak-out experiment, the peaks at 1761, 1689 and 1654 cm$^{-1}$ disappear, only the 1629 cm$^{-1}$ peak remains, strongly suggesting that the species at 1629 cm$^{-1}$ represents an entity covalently bound in the active site (Figure 5A).

In accord with the solution studies (above), the 1629 cm$^{-1}$ feature is assigned to the stable acyl enzyme, species 4 in Scheme 2, that resists hydrolysis because Lys70 is decarboxylated in the first cycle of the reaction. Thus, it appears that
species 4 in reaction Scheme 2 is the final stable product in the crystal after 30 min soak in and 30 min soak out.

In the crystal reaction for OXA-24, we predict that reaction cycle will occur many times, since the \( t_n \) for penem 1 is \( t_n \approx 900 \) (Table 3). Figure 5B shows the reaction between penem 1 and OXA-24 single crystals. As for OXA-1, penem 1 enters the active site slowly because at 4 min, there are no intense substrate or intermediate peaks. At 15 min, a broad peak at 1628 cm\(^{-1}\) appears and another intense peak at 1647 cm\(^{-1}\) is present at 30 min. As described above, based on the quantum mechanical calculations, the peak at 1647 cm\(^{-1}\) is due to the released species F (Scheme 3), which is produced in large amounts due to multiple reaction cycles. Again, in order to remove the unreacted substrate and detect the covalently-bound species in the active site, the soak out experiment was conducted. Interestingly, we saw the 1628 cm\(^{-1}\) peak, the same peak as in the active site of OXA-1. This indicates that, after penem 1 reacts with OXA-1 or OXA-24, they both have the same species finally remaining, covalently bound, in the active site. We observe the 1628 cm\(^{-1}\) band due to species C in Scheme 3 because in OXA-24 Lys84 has finally decarboxylated after about 900 cycles, and acyl-enzyme species C can no longer be hydrolyzed. The same hypothesis applies as for OXA-1, acylation is still possible without a carboxylated lysine, but deacylation cannot occur, or occurs very slowly, at best.

**DISCUSSION**

The results discussed above indicate that OXA-1 and OXA-24 react differently with the penem inhibitors. The decaying lysine in both enzymes can be decarboxylated by penem 1; OXA-1 resists recarboxylation and thus cannot hydrolyze the acyl-enzyme. An unusual finding in our work is that, at modest conditions, penem 1 and 3 appear to be ineffective against OXA-24.

Based on the kinetic data, and the reactions in crystal and in solution, we propose that penem 1 causes decarboxylation of Lys70 in the active site of OXA-1 in the first cycle rendering the enzyme inactive. The next arriving molecule forms the acyl-enzyme complex (species 4, Scheme 2), where carboxylation of Lys70 is not essential for acylation (10,45), but does not react further because the enzyme loses the deacylation function following decarboxylation of Lys70. However, decarboxylation of Lys84 in the active site of OXA-24 occurs only under harsh condition. The enzyme remains active and hydrolyzes penem 1 again and again. The difference in the nitrocefin assay indicates that OXA-1 and OXA-24 differ in both decarboxylation and recarboxylation steps. OXA-24 not only decarboxylates with difficulty but also recarboxylates with ease. In contrast, OXA-1 decarboxylates easily but recarboxylates with difficulty. We now discuss evidence from the literature to explain the difference between OXA-1 and OXA-24.

Previous studies by the Mobashery group showed that the BlaR1 sensor, a signal transducer found in *Staphylococcus aureus* bacterium, has a serine and carboxylated lysine motif in the active site (46,47). Its protein sequence and overall folding indicate that it is evolutionarily related to class D β-lactamases. When the sensor reacts with antibiotics, it forms an acyl-enzyme complex and the Lys62 undergoes decarboxylation, switching the receptor to the "on" state and continuously inducing the expression of the β-lactamase (47-50). In order to find out why BlaR1 sensor decarboxylates easily compared to OXA enzymes, Birck *et al.* used the X-ray coordinates of OXA-10 as a model to calculate the energy barrier to decarboxylation. Before the decarboxylation occurs, the carboxylated lysine is protonated following active-site serine acylation (47,51,52). Based on their calculations on OXA-10 (Figure 6), if the protonation occurs on the oxygen in the -NH-COO\(^{-}\) of the side chain, there is a huge barrier (≈40 kcal/mol) for the decarboxylation of Lys70. However, if the protonation is on the ζ-nitrogen in the NH of the side chain, there is no barrier for the decarboxylation. Thus in BlaR1 sensor, if ζ-N protonation can be prevented, Lys62 will remain carboxylated and the acyl-enzyme can be hydrolyzed. Based on this consideration, Birck *et al.* changed the Lys62 in BlaR1 to S-(4-butoanoate) cysteine by chemical mutagenesis (53) to see whether it is sufficient to convert the BlaR1 sensor from a susceptible receptor to an antibiotic-resistant enzyme (51). The results show that the cysteine derivative does not undergo decarboxylation and the variant hydrolyzes the acyl-enzymes formed from a broad spectrum of
Different mechanisms of OXA-1 and OXA-24 β-lactamases

antibiotics (51). Kinetic studies indicate that this behavior is reproduced in OXA-10 (8). Returning to the OXA-1 and OXA-24 systems, we postulate that, in OXA-1, it is the ζ-nitrogen of Lys70 that undergoes protonation in the deacylation process and this brings about decarboxylation (Scheme 2, species 7,8,9). Even though the Ser67 is free now and can react with the next penem 1 molecule, forming the acyl-enzyme complex, the Lys70 is inactive and loses the deacylation function because of the decarboxylation. Previous studies have also shown that BlaR1 sensor shares more similar properties with OXA-1 than OXA-24 (47,54,55). In OXA-24, we postulate that it is the COO− that undergoes protonation (Scheme 3, species D), thus the enzyme is still active and can hydrolyze the next penem 1 molecule.

Regarding the recarboxylation process, we found that OXA-24 can be reactivated by the addition of 100 mM NaHCO3, but OXA-1 cannot. According to the calculation results by Schlegel and Mobashery’s groups on OXA-10 β-lactamase (21), the barrier for unassisted carboxylation of neutral lysine is as high as 30 kcal/mol. However, if the recarboxylation is assisted by a water molecule close enough (2.4 ~ 2.8 Å) to the lysine in the active site, the energy barrier is much lower (exothermic with a barrier of 14 kcal/mol). This led us to examine the active sites of both enzymes to determine if there is a water molecule that exists near the lysine in the active site of OXA-24 but not in OXA-1. Figure 7 shows the positions of the water molecules surrounding the lysine residue in the active of OXA-1 and OXA-24. In OXA-1, the closest water molecule is 4.6 Å away from Lys70; in OXA-24, it is 2.6 Å. Thus, there is a water molecule in OXA-24 that can be utilized to catalyze the recarboxylation. This water molecule, together with Trp167 can form H-bonds with CO2 group (2.6 and 2.9 Å, respectively), positioning the CO2 in the right place ready for the attack from ζ-nitrogen in Lys84 to form the carboxylated lysine seen in Figure 7. A detailed model for the reaction scheme during lysine carboxylation is shown in Li et al. (21). The X-ray structure of OXA-24 complexed with an inhibitor LN-1-255 also indicates that the bulky side group in the latter seems to force out the water molecule, leading to deacylation deficiency (14). Considering that the CO2 concentration is about 1.3 mM in cells (56), OXA-24 has easy access to the CO2 group and becomes fully carboxylated. This may explain, in part, why clinical strains harboring blaOXA-24 genes have multiple-drug resistance to β-lactams.

CONCLUSIONS

In conclusion, the two variants of class D β-lactamase OXA-1 and OXA-24 are found to react differently with 6-methylenedene penems. This is ascribed to the differential stability of the carboxylated lysine in the active site. The catalytic difference between OXA-1 and OXA-24 also provides us new insight into inactivation mechanism by OXA carbapenemases. Compared to OXA-1, OXA-24 is not only difficult to decarboxylate but also can easily be recarboxylated. This may be also consistent with their relative pathogenic effects as the pathogen harboring blaOXA-24 (A. baumannii) has been more problematic in the clinic. Based on these findings, a novel inhibitor design of class D β-lactamase should aim to accelerating the decarboxylation step and also importantly, retarding the recarboxylation step, unlike the traditional strategy in class A and C β-lactamases of blocking Glu166 (or its homologue) assisted hydrolysis.

REFERENCES

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FOOTNOTES

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2The abbreviations used are: OXA, oxacillinase; E: I, enzyme: inhibitor; M-H, Mueller-Hinton; CLSI, Clinical and Laboratory Standards Institute; NCF, nitrocefin; PEG, polyethylene glycol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; UVD, ultraviolet difference

FIGURE LEGENDS

Figure 1. Chemical structures of penem inhibitors (penem 1 and penem 3), comparators (tazobactam and BRL 42715), and penam sulfone inhibitor (SA-1-204).

Figure 2. UV difference spectrum of OXA-1 and OXA-24 reacting with penem 1. The intense peak at 280 nm represents unreacted penem 1 (black line). The concentration of enzyme is 20 µM for OXA-1 and OXA-24. The concentration of inhibitor is 20 µM (1: 1) or 80 µM (1: 4). After the enzyme is incubated with penem 1 inhibitor, the spectrum is recorded every 30 s for 30 min. The spectra shown in the figure are the UV-difference spectra at 1 min, ( the other spectra overlap together because the reaction occurs rapidly in solution). (A) OXA-1 or OXA-24 reacting with penem 1 at 1: 1 ratio (E: I); (B) OXA-1 or OXA-24 with penem 1 at 1: 4 ratio.
Figure 3. Recarboxylation of the lysine can restore the enzyme activity in OXA-24, but not in OXA-1. A, black line: 1 µM OXA-24 was incubated with 100 µM NCF in HEPES buffer (10 mM, pH 7.5); red line: 1 µM OXA-24 was first incubated with 2 mM penem 1, then 100 µM NCF was added; blue line: Reagents were added in the order of 1 µM OXA-24, 2 mM penem 1, 100 µM NCF and 100 mM NaHCO₃. B, black line: 1 µM OXA-1 was incubated with 100 µM NCF in HEPES buffer (10 mM, pH 7.5); red line: 1 µM OXA-1 was first incubated with 4 µM penem 1, then 100 µM NCF was added; blue line: Reagents were added in the order of 1 µM OXA-1, 4 µM penem 1, 100 µM NCF and 100 mM NaHCO₃.

Figure 4. Raman difference of spectra of reactions between penem 1 and OXA-1 or OXA-24 in solution. Unreacted penem 1 spectrum (upper trace, 10 mM, in H₂O). Enzyme (86 µM) and inhibitor (172 µM) were incubated at the ratio of 1: 2 (E: I). The reactions were quenched by liquid nitrogen after 1 s. The ice was then freeze-dried and characterized by Raman microscopy. 4(2), 9(2): species 4 and 9 in Scheme 2; C(3), F(3): species C and F in Scheme 3; C5-S1: C-S bond of the thiazole ring attached to the β-lactam ring.

Figure 5. Raman difference spectra of OXA-1 or OXA-24 single crystals reaction with penem 1. (A) Raman difference spectrum of OXA-1 and penem 1 at 3, 15, 30 min in the presence of PEG 8000. Control spectrum of penem 1 (upper trace) was first recorded in the presence of PEG. After penem 1 was soaked in, the spectra were taken at above indicated time points. (B) Raman difference spectrum of OXA-24 and penem 1 at 4, 15, 30 min in the presence of PEG 2000. Control spectrum of penem 1 (upper trace) was first recorded in the presence of PEG. After penem 1 was soaked in, the spectra were taken at above indicated time points.

Figure 6. QM/MM calculations using the X-ray coordinates of the OXA-10 active site reveal the protonation of the ζ-nitrogen leads to a barrierless decarboxylation of the lysine carbamate, (modified from Birck et al. ref 47).

Figure 7. The water position in the active site of OXA-1 (A) and OXA-24 (B). Water molecule is shown in red sphere. Selected interacting residues are labeled, and hydrogen bonds are indicated by dashed lines. The PDB entry: OXA-1 (1M6K), OXA-24 (3G4P).
Scheme 1. Reaction scheme of β-lactamase-inhibitor interactions.

\[
E + I \quad \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} \quad E : I \quad \overset{k_2}{\rightarrow} \quad E - I \quad \overset{k_3}{\rightarrow} \quad E - I^* \quad \overset{k_4}{\rightarrow} \quad E + P
\]

- \( k_{\text{cat}} \): Regeneration of active enzyme
- \( k_{\text{inact}} \): Irreversible inhibition
- M-M complex
- Acyl-enzyme
Scheme 2. Proposed mechanism for penem 1 and OXA-1.
Scheme 3. Proposed mechanism for penem 1 and OXA-24.
Different mechanisms of OXA-1 and OXA-24 β-lactamases

**Table 1. Primer sequences**

<table>
<thead>
<tr>
<th>Function and primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloning primers</td>
<td></td>
</tr>
<tr>
<td>OXA-24FOR</td>
<td>CATATGTCTATTTAAACTCCTTGA</td>
</tr>
<tr>
<td>OXA-24REV</td>
<td>GGATCCCTTTAATGATTTCCAGGA</td>
</tr>
<tr>
<td>OXA-24LDR</td>
<td>CATATGAAAAATTATATCCCTATATTC</td>
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<tr>
<td>Sequencing (Cy5-labeled) primers</td>
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<tr>
<td>M13 Universal</td>
<td>GTAAAACGACCGCCAG</td>
</tr>
<tr>
<td>M13 Reverse</td>
<td>CAGGAAACAGCTATGAC</td>
</tr>
<tr>
<td>T7 Promoter</td>
<td>TAATACGACTCCTATAGGG</td>
</tr>
<tr>
<td>T7 Terminator</td>
<td>GCTAGTTATTGCTCAGCGG</td>
</tr>
</tbody>
</table>
**Table 2.** Minimum inhibitory concentration of Laboratory Isolates.
According to the Clinical Laboratory Standards Institute, MIC breakpoints for piperacillin and piperacillin/tazobactam are: $\leq 8 \, \mu g/ml$, susceptible; 8-16 $\mu g/ml$, intermediate; $\geq 32 \, \mu g/ml$, resistant.

<table>
<thead>
<tr>
<th>MICs (µg/ml)</th>
<th>Laboratory Isolate</th>
<th>Piperacillin</th>
<th>Piperacillin/tazobactam*</th>
<th>Piperacillin/penem 1</th>
<th>Piperacillin/penem 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. coli</em> DH10B</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> bla\text{OXA}-1</td>
<td>512</td>
<td>256</td>
<td>8</td>
<td>8</td>
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<tr>
<td></td>
<td><em>E. coli</em> bla\text{OXA}-24</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
<td>1024</td>
</tr>
</tbody>
</table>

* The concentration of tazobactam, penem 1 and penem 3 is 4 $\mu g/ml$. 
**Table 3.** Kinetic parameters of inhibition.

\(K_i\) for the penems was determined by measuring initial steady-state velocities in the presence of a constant concentration of enzyme with increasing concentrations of inhibitors against nitrocefin (100 µM), the value was then corrected for nitrocefin affinity; \(IC_{50}\) was determined by measurements of inhibitor concentration that reduces the initial velocities by 50\% after 5 min pre-incubation of enzyme with inhibitor; \(t_n\) was determined as the ratio of inhibitor concentration to enzyme concentration necessary to decrease enzyme activity by 95\% after 24 h.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>OXA-1</th>
<th>OXA-24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_i) (nM)</td>
<td>(IC_{50}) (nM)</td>
</tr>
<tr>
<td>Penem 1</td>
<td>50 ± 8</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>Penem 3</td>
<td>380 ± 70</td>
<td>60 ± 5</td>
</tr>
</tbody>
</table>
Different mechanisms of OXA-1 and OXA-24 β-lactamases

Table 4. Raman peak assignments for the major peaks in penem 1 complexed with OXA-1 or OXA-24 difference spectra shown in Figure 4.

The structures of the proposed intermediates in the reaction pathway were sent to the HPCC at CWRU for calculating the theoretical Raman spectrum.

<table>
<thead>
<tr>
<th>Species</th>
<th>Observed Raman peaks (cm(^{-1}))</th>
<th>Calculated Raman peaks (cm(^{-1}))</th>
<th>Calculated relative Raman intensities</th>
<th>Peak assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXA-1(^a)</td>
<td>1630</td>
<td>1625</td>
<td>1628</td>
<td>152  methylenic double bond at C6</td>
</tr>
<tr>
<td></td>
<td>1440</td>
<td>1443</td>
<td>1448</td>
<td>43   C=O of thiazole ring</td>
</tr>
<tr>
<td></td>
<td>1373</td>
<td>1354</td>
<td>1367</td>
<td>46   -N=N=C- of bicyclic ring</td>
</tr>
<tr>
<td></td>
<td>715</td>
<td>716</td>
<td>723</td>
<td>3    -C-S between S1 and C5</td>
</tr>
<tr>
<td>OXA-24</td>
<td>1501</td>
<td>1492</td>
<td>1490</td>
<td>72   C=C of thiazepine ring</td>
</tr>
<tr>
<td></td>
<td>1440</td>
<td>1445</td>
<td>1445</td>
<td>33   C-H mode</td>
</tr>
<tr>
<td></td>
<td>1355</td>
<td>1365</td>
<td>1367</td>
<td>41   -N=N=C- of bicyclic ring</td>
</tr>
<tr>
<td></td>
<td>715</td>
<td>721</td>
<td>721</td>
<td>2    -C-S of thiazepine ring</td>
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<tr>
<td></td>
<td>1645</td>
<td>1642</td>
<td>1642</td>
<td>352  C=C stretch at C6</td>
</tr>
<tr>
<td></td>
<td>1466</td>
<td>1461</td>
<td>1461</td>
<td>16   C=C of thiazole ring</td>
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<tr>
<td></td>
<td>1443</td>
<td>1445</td>
<td>1445</td>
<td>46   C-H mode</td>
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<tr>
<td></td>
<td>1354</td>
<td>1365</td>
<td>1365</td>
<td>42   -N=N=C- of bicyclic ring</td>
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<tr>
<td></td>
<td>716</td>
<td>722</td>
<td>722</td>
<td>3    -C-S between S1 and C5</td>
</tr>
</tbody>
</table>

\(^a\) The values in this column represent the peaks from the spectrum of OXA-1/OXA-24 reaction with penem 1 in Figure 4.
Different mechanisms of OXA-1 and OXA-24 β-lactamases

Figure 1.

Tazobactam

SA-1-204

Penem 1

Penem 3

BRL 42715
Different mechanisms of OXA-1 and OXA-24 β-lactamases

Figure 2.
Figure 3.
Figure 4.

Different mechanisms of OXA-1 and OXA-24 β-lactamases
Different mechanisms of OXA-1 and OXA-24 β-lactamases

Figure 5.

A

B

Downloaded from http://www.jbc.org/ by guest on October 3, 2017
Figure 6.
Different mechanisms of OXA-1 and OXA-24 β-lactamases

Figure 7.

A

B

OXA-1

OXA-24
The Different Inhibition Mechanisms of OXA-1 and OXA-24 β-Lactamases Are Determined by the Stability of Active-Site Carboxylated Lysine

Tao Che, Christopher R. Bethel, Marianne Pusztai-Carey, Robert A. Bonomo and Paul R. Carey

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