ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1997, p. 1940–1943 Vol. 41, No. 9
0066-4804/97/$04.00+0


# Construction and Characterization of an OHIO-1 β-Lactamase Bearing Met69Ile and Gly238Ser Mutations

ROBERT A. BONOMO,[1]* JAMES R. KNOX,[2] SUSAN D. RUDIN,[1] AND DAVID M. SHLAES[3]

*Research Service, U.S. Department of Veterans Affairs Medical Center, and Department of Medicine, Case Western Reserve University, Cleveland Ohio, 44106[1]; Department of Molecular and Cell Biology, University of Connecticut Storrs, Connecticut 06269[2]; and Infectious Disease Research, Wyeth Ayerst, Pearl River, NY 10965[3]*

Received 29 July 1996/Returned for modification 5 November 1996/Accepted 30 April 1997

**Amino acid changes that influence activity and resistance to β-lactams and β-lactamase inhibitors were explored by constructing the Gly238Ser and Met69Ile-Gly238Ser mutants of the OHIO-1 β-lactamase, a class A enzyme of the SHV family. The $K_m$ values of cefotaxime and ceftazidime for OHIO-1 and Met69Ile β-lactamases were ≥500 μM. The $K_m$ of cefotaxime for the Gly238Ser β-lactamase was 26 μM, and that of ceftazidime was 105 μM. The $K_m$ of cefotaxime for the Met69Ile-Gly238Ser β-lactamase was 292 μM, and that of ceftazidime was 392 μM. For the β-lactamase inhibitors clavulanate and sulbactam, the apparent $K_i$ values for the Met69Ile-Gly238Ser enzyme were 0.03 and 0.15 μM, respectively. Relative $V_{max}$ values indicate that the Met69Ile-Gly238Ser mutant of the OHIO-1 β-lactamase possesses cephalosporinase activity similar to that of the Gly238Ser mutant but diminished penicillinase activity. In an *Escherichia coli* DH5α strain that possesses a Met69Ile β-lactamase of the OHIO-1 family, the added Gly238Ser mutation resulted in a phenotype with qualities that confer resistance to expanded-spectrum cephalosporins and, to a lesser extent, β-lactamase inhibitors.**

The β-lactamase enzymes (EC 3.5.2.6) are the principal defense mechanism that bacteria possess to protect themselves against the lethal effects of β-lactam antibiotics. Kinetic, molecular modeling, and crystallographic analyses of class A β-lactamases are increasing our understanding of the intramolecular relationships that influence substrate binding and catalysis (10, 13, 14, 18). A detailed understanding of the mechanisms underlying this resistance is critical for future drug design.

The OHIO-1 β-lactamase is a class A enzyme of the SHV family (21). Single amino acid substitutions in the OHIO-1 and SHV-1 enzymes have been described. These substitutions permit the hydrolysis of oxyimino-cephalosporin substrates (Gly238Ser, Gly242Cys) or confer resistance to inactivation by β-lactamase inhibitors (Met69Ile) (ABL numbering system) (1, 3, 20). We have previously reported that the double mutant Met69Ile-Gly242Cys of the OHIO-1 β-lactamase is catalytically inactive (5). The construction of this mutant demonstrated that the combination of a Met69Ile and Gly242Cys substitution profoundly alters β-lactamase activity. To further explore the relationship between substitutions that confer resistance to expanded-spectrum cephalosporins and to β-lactamase inhibitors, the Gly238Ser and Met69Ile-Gly238Ser mutants of the OHIO-1 β-lactamase were constructed and characterized.

(This work was presented in part at the 34th Interscience Conference on Antimicrobial Agents and Chemotherapy, Orlando, Fla., 4 to 7 October 1994 [5a].)

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *Escherichia coli* DH5α [(*supE44 ΔlacU169*) (*Φ80 lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] and CJ236 *dut ung* [*dut-1 ung-1 thi-1 relA1*/pCJ105 (Cam$^r$F′)] were used in the experiments described here. The OHIO-1 and Met69Ile β-lactamase genes were directionally subcloned into the phagemid vector pBC SK(−) which encodes chloramphenicol resistance, and the vector was transformed into *E. coli* CJ236 and DH5α (5). Plasmids were isolated by using the Wizard Miniprep Kit (Promega Corp., Madison, Wis.). All bacteria were grown in Luria-Bertani (LB) broth and agar containing the appropriate antibiotic for selection (chloramphenicol at 20 μg/ml or ampicillin at 100 μg/ml and chloramphenicol at 20 μg/ml).

**Antibiotics and restriction enzymes.** The antibiotics used in this study and their sources were described previously (3–5, 21). The restriction enzymes used in this study were obtained from Promega and United States Biochemicals (Cleveland, Ohio) (4, 5).

**Site-directed mutagenesis.** Mutant β-lactamases containing the Gly238Ser and the Met69Ile-Gly238Ser substitutions were independently constructed and separately identified. The purified oligonucleotide used for site-directed mutagenesis was obtained from National Biosciences (Plymouth, Minn.). The sequence of the mutagenic oligonucleotide used in this experiment is 5′-CGCACCCCGTT CGCTAGCTCCGGTCTTA-3′.

Mutagenesis was performed by the uracil template method of Kunkel et al. (15) with the material supplied in the Altered Sites Kit (Promega) and by a modification of the protocol of McClary et al. (17). DNA sequencing was performed on double-stranded templates by using the Sequenase kit, version 2.0, from United States Biochemicals. Custom-made internal primers and the commercially available T3 and T7 primers from Stratagene (La Jolla, Calif.) were used. [$^{35}$S]dATP was purchased from Amersham Life Sciences (Arlington Heights, Ill.).

**Phenotypic characterization.** Antibiotic susceptibility testing by the agar dilution MIC method was performed with β-lactams and β-lactamase inhibitors. MICs were determined in LB agar with a replicator (Craft Machine, Inc., Chester, Pa.). Spots of $10^4$ CFU were plated onto LB plates containing antibiotics.

**Kinetic parameters.** β-Lactamase was liberated by osmotic shock from *E. coli* DH5α by an adaptation of the protocol described by Zafaralla et al. (26). Ammonium sulfate precipitation was next performed to concentrate and identify the fraction with the greatest activity. Specific activity was determined as micromolar nitrocefin hydrolyzed per minute divided by the total protein concentration ($\lambda$ = 482 nm, $\Delta\varepsilon$ = 17,400 $M^{-1}$ $cm^{-1}$). The 55 to 75% ammonium sulfate fraction (greatest specific activity) was resuspended in 20 μM sodium phosphate buffer (pH 7.4) and dialyzed. Uniform amounts of crude enzyme, as determined by hydrolysis of nitrocefin, were used to determine hydrolytic activity. β-Lactamase activity was detected with a spectrophotometer at 25°C in 20 μM phosphate buffer (pH 7.4). The protein concentration was determined by absorbance measurements at 280 nm. Analytical isoelectric focusing was used to confirm that the activity of only a single β-lactamase was present in these preparations.

Hydrolysis of the β-lactam substrates was performed at ambient temperature in an HP 8453 UV-visible spectrophotometer with a cell with a 1-cm path length.

* Corresponding author. Mailing address: Geriatric CARE Center, 12200 Fairhill Rd., Cleveland, OH 44120. Phone: (216) 844-7249. Fax: (216) 844-7254. E-mail: rab14@po.cwru.edu.

TABLE 1. MICs of the antibiotics tested[a]

| Drug[b] | MIC (μg/ml) | | | |
|---|---|---|---|---|
| | OHIO-1 | Met69Ile | Gly238Ser | Met69Ile-Gly238Ser |
| Amp | 2,048 | 1,024 | 8,192 | 512 |
| Pip | 512 | 512 | 128 | 64 |
| Mez | 512 | 512 | 512 | 128 |
| Lor | 8 | 4 | 128 | 16 |
| Tax | 0.03 | 0.03 | 16 | 0.12 |
| Taz | 0.06 | 0.06 | 8 | 0.5 |
| Tri | 0.016 | 0.016 | 8 | 0.06 |
| Atm | 0.032 | 0.032 | 2 | 0.032 |

[a] The host strain is *E. coli* DH5α. OHIO-1, Met69Ile, Gly238Ser, and Met69Ile-Gly238Ser represent *E. coli* DH5α strains harboring the respective β-lactamase directionally subcloned into the vector pBC SK(−), which encodes chloramphenicol resistance. All MICs were determined in the presence of chloramphenicol at 20 μg/ml.

[b] Amp, ampicillin; Pip, piperacillin; Mez, mezlocillin; Lor, cephaloridine; Tax, cefotaxime; Taz, ceftazidime; Tri, ceftriaxone; Atm, aztreonam.

Changes in absorbance and extinction coefficients were determined previously (21). Initial rates were determined from the first 5 to 10% of the reactions with multiple substrate concentrations. Various concentrations of substrates bracketing the $K_m$ were used. The $K_m$ and $V_{max}$ values were calculated by the Lineweaver-Burk method. For inhibitor kinetics the enzyme and clavulanate or sulbactam were preincubated for 10 min at 25°C before the addition of a uniform concentration of indicator substrate (nitrocefin). Standard plots of $1/v$ versus $I$ (Dixon) were prepared, and the apparent $K_i$ was calculated. All determinations of β-lactamase activity were performed in the same manner to ensure uniformity with previous experiments with the same enzymes and the same kinetic analysis (3–5, 20, 21).

## RESULTS

**Construction of Gly238Ser and Met69Ile-Gly238Ser mutants.** After in vitro mutagenesis of the OHIO-1 and Met69Ile β-lactamase genes, the Gly238Ser and Met69Ile-Gly238Ser mutations were individually identified by DNA sequencing of randomly selected *E. coli* DH5α transformants isolated on plates containing chloramphenicol at 20 μg/ml. The DNA sequences of the Gly238Ser and Met69Ile-Gly238Ser *bla* genes on at least one strand were confirmed for each of the mutants.

**Phenotypic characterization.** To explore the effect of the Gly238Ser and Met69Ile-Gly238Ser mutations on β-lactamase activity, we evaluated these activities in vitro by determining the MICs in a homogeneous genetic background, *E. coli* DH5α. All our strains possessed either the wild-type β-lactamase gene or one of the mutant genes subcloned directionally into the phagemid vector, pBC SK(−).

Agar dilution MICs (Table 1) demonstrated that the strain possessing the Gly238Ser mutation was resistant to β-lactams. The strain bearing this β-lactamase was more resistant to cefotaxime and ceftazidime than the parent strain or the double-mutant strain. The Met69Ile-Gly238Ser mutant β-lactamase-bearing strain of *E. coli* DH5α was also more resistant to cefotaxime and ceftazidime than the OHIO-1- and Met69Ile-bearing strains and was more susceptible to ampicillin than the Gly238Ser-bearing strain.

The strains were also tested with β-lactam–β-lactamase inhibitor combinations (Table 2). For ampicillin-clavulanate, the Met69Ile-Gly238Ser-bearing strain had intermediate resistance between the OHIO-1- and Gly238Ser-bearing strains. The Gly238Ser- and the Met69Ile-Gly238Ser-bearing strains were more susceptible to ampicillin-sulbactam than OHIO-1- and Met69Ile-bearing strains.

**Kinetic analysis.** An assessment of the impact of the substitutions Gly238Ser, Met69Ile, and Met69Ile-Gly238Ser on the kinetic behavior of OHIO-1 β-lactamase was performed (Tables 3 and 4). In general, the lowest $K_m$ values of the β-lactams tested was for the Gly238Ser β-lactamase. For the OHIO-1 and Met69Ile β-lactamases the $K_m$ values of oxyimino-cephalosporins, cefotaxime, and ceftazidime were greater than 500 μM. For the double mutant enzyme, Met69Ile-Gly238Ser, $K_m$ values of penicillin, piperacillin, cephaloridine, and cefoperazone were similar to those for the Gly238Ser mutant β-lactamase. The $K_m$ of cefotaxime for the Met69Ile-Gly238Ser β-lactamase was more than 10-fold greater than that for the Gly238Ser enzyme (292 versus 26 μM). Similarly, the $K_m$ of ceftazidime was approximately fourfold greater (392 versus 105 μM, respectively).

TABLE 2. MICs of ampicillin–β-lactamase inhibitor combinations[a]

| Strain | MIC (μg/ml) | |
|---|---|---|
| | Clav | Sul |
| OHIO-1 | 0.25 | 32 |
| Met69Ile | 4 | 128 |
| Gly238Ser | 0.015 | 4 |
| Met69Ile-Gly238Ser | 0.06 | 1 |

[a] See footnote *a* of Table 1. MICs were determined in the presence of ampicillin at 100 μg/ml and chloramphenicol at 20 μg/ml. Clav, clavulanic acid; sul, sulbactam.

The apparent $K_i$ of clavulanate for the Met69Ile-Gly238Ser enzyme was slightly greater than that for the Gly238Ser β-lactamase. These values are much less than the apparent $K_i$ of clavulanate for the OHIO-1 and Met69Ile β-lactamases (Table 3). In a similar manner, the Met69Ile-Gly238Ser mutant enzyme was more susceptible to inactivation by sulbactam than the wild-type or Met69Ile β-lactamase but was less susceptible to inactivation than the Gly238Ser enzyme.

Data summarizing the $V_{max}$ (hydrolysis) values of the enzymes are presented in Table 4. The OHIO-1 and Met69Ile β-lactamases hydrolyzed penicillin faster than cefoperazone and cefotaxime. The double mutant possessed activity against cephalosporins similar to that of the Gly238Ser mutant, but it had reduced activity against penicillins.

## DISCUSSION

The continued evolution in nature of β-lactamases that are able to inactivate each new generation of β-lactams threatens our ability to successfully treat many infections encountered in the clinic. The recent discovery of enzymes resistant to inactivation by β-lactamase inhibitors is also an extremely worrisome development (2, 6, 7, 9, 19, 22–24, 27). Hence, an in-depth understanding of the molecular interactions affecting structure-function relationships is necessary for future rational drug design (16).

The effect of point mutations on OHIO-1 β-lactamase activity in *E. coli* DH5α can be appreciated by examining the MICs. By MICs (Table 1), the Gly238Ser-bearing strain was resistant to all β-lactams, especially the oxyimino-cephalosporins. This increased cephalosporin resistance in strains bearing SHV enzymes has been observed previously (8, 10). It has been noted that the increase in cephalosporin resistance is much greater than that in strains with a similar mutation and bearing the extended-spectrum TEM enzymes (25). As suggested by Venkatachalam et al. (25), there may be important structural differences between the TEM and SHV β-lactamases. The Met69Ile-Gly238Ser strain was also more resistant to oxyimino-cephalosporins than the OHIO-1- and Met69Ile β-lactamase-bearing strains, but it was not as resistant as the

TABLE 3. Michaelis constants ($K_m$ or $K_i$) for OHIO-1 β-lactamase variants[a]

| Enzyme | $K_m$ (μM) | | | | | | | | $K_i$ (μM) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pen | Pip | Mez | Lor | Ncf | Per | Tax | Taz | Clav | Sul |
| OHIO-1 | 25 | 44 | 75 | 348 | 164 | 76 | >500 | >500 | 0.2 | 5 |
| Met69Ile | 124 | 5 | 154 | 83 | 104 | 22 | >500 | >500 | 15 | 182 |
| Gly238Ser | 15 | 15 | 12 | 22 | 5.3 | 12 | 26 | 105 | 0.01 | 0.03 |
| Met69Ile-Gly238Ser | 17 | 17 | 100 | 17 | 47 | 11 | 292 | 392 | 0.03 | 0.15 |

[a] Nitrocefin was used as our indicator substrate for the determination of the apparent $K_i$. Pen, benzylpenicillin; Per, cefoperazone; Ncf, nitrocefin; see footnote *b* of Table 1 and footnote *a* of Table 2 for definitions of the other abbreviations.

Gly238Ser-bearing strain. Furthermore, the Met69Ile-Gly238Ser-bearing strain was more susceptible to ampicillin.

The MICs of the β-lactamase inhibitors (clavulanate and sulbactam) in combination with ampicillin at 100 μg/ml (Table 2) indicate that the *E. coli* DH5α Gly238Ser mutant strains and the Met69Ile-Gly238Ser mutant strains are more susceptible to ampicillin-clavulanate and ampicillin-sulbactam than the OHIO-1- and Met69Ile-bearing strains. The enhanced susceptibility to β-lactamase inhibitors by strains bearing extended-spectrum enzymes seems to be dominant to the Met69Ile inhibitor-resistant mutation in the Met69Ile-Gly238Ser mutant strain. The presence of the Met-to-Ile substitution is only reflected by the modest increase in ampicillin-clavulanate resistance (Table 2). No phenotypic resistance to sulbactam is seen.

As described previously (20), the $K_m$ values of cefotaxime and ceftazidime for the Gly238Ser β-lactamase were lower than those for the OHIO-1 β-lactamase. The Met69Ile-Gly238Ser double mutant also hydrolyzed cefotaxime and ceftazidime, substrates not hydrolyzed by the wild type, Met69Ile, or the other double mutant, Met69Ile-Gly242Cys (5). Although the $K_m$ values of the substrates cefotaxime and ceftazidime for the Met69Ile-Gly238Ser enzyme were not as great as that for the Gly238Ser β-lactamase, the observed $K_m$ is consistent with the MIC data (Table 1). These data support the notion that the enhanced cephalosporinase activity observed in the SHV family of β-lactamases is predominantly influenced by the Gly238Ser substitution (10, 20, 25). Relative $V_{max}$ values (Table 4) indicate that the double-mutant enzyme, Met69Ile-Gly238Ser, has cephalosporinase activity similar to that of Gly238Ser but diminished penicillinase activity.

In keeping with our MIC findings, the two mutant β-lactamases, Gly238Ser and Met69Ile-Gly238Ser, were susceptible to inhibition by clavulanate and sulbactam. The Met69Ile-Gly238Ser mutant enzyme was slightly less susceptible to clavulanate inhibition than the Gly238Ser β-lactamase. The Met69Ile-Gly238Ser mutant enzyme was less susceptible to sulbactam inhibition than the Gly238Ser single mutant (Table 3). This variability may be due to differences in the inactivation chemistries of the two inhibitors (11, 12).

In summary, our studies demonstrated that the double mutant Met69Ile-Gly238Ser is catalytically active and possesses qualities intermediate to the properties of the individual Gly238Ser and Met69Ile mutants. The MIC and preliminary kinetic data indicate that the extended-spectrum cephalosporin resistance and the ability to hydrolyze oxyimino-cephalosporins is partially preserved and is dominant to the inhibitor-resistant phenotype. In sharp contrast, the previously described Met69Ile-Gly242Cys double mutant was catalytically impaired. In the OHIO-1 β-lactamase family of enzymes, the double mutant combinations attempted so far (Met69Ile-Gly238Ser [this study] and Met69Ile-Gly242Cys [5]) have not resulted in a phenotype that is able to hydrolyze oxyimino-cephalosporins and still be significantly resistant to inhibition by mechanism-based inactivators.

TABLE 4. Relative $V_{max}$ values for OHIO-1 β-lactamase variants

| Strain | $V_{max}$[a] | | | | | |
|---|---|---|---|---|---|---|
| | Lor | Per | Tax | Pen | Pip | Mez |
| OHIO-1 | 100 | 10 | <1 | 40 | 3 | 7 |
| Met69Ile | 100 | 38 | <1 | 330 | 132 | 16 |
| Gly238Ser | 100 | 44 | 33 | 322 | 11 | 33 |
| Met69Ile-Gly238Ser | 100 | 54 | 48 | 2 | 5.6 | 1 |

[a] All values are relative to that for cephaloridine, which set equal to 100. Definitions of the abbreviations are given in the footnotes to Tables 1 to 3.

## ADDENDUM IN PROOF

A recent report by Sirot et al. (D. Sirot, C. Recule, E. B. Chaibi, L. Bret, J. Croize, C. Chanal-Claris, R. Labia, and J. Sirot, Antimicrob. Agents Chemother. **41:**1322–1325, 1997) describes a mutant TEM enzyme (designated TEM-50) with the Met69Leu, Glu104 Lys, Gly238Ser, and Asn276Asp mutations. This enzyme also confers resistance to inhibitors and extended-spectrum cephalosporins.

## ACKNOWLEDGMENTS

This work was supported in part by the Claude D. Pepper Foundation and by the National Institute on Aging (grant 1 KO8 AG 00684-01 to R.A.B.). D.M.S. is a clinical investigator in the U.S. Department of Veterans Affairs.

We thank Susan Johnson for expert secretarial assistance and Louis B. Rice for critical reading of the manuscript.

## REFERENCES

1. **Ambler, R. P., A. F. W. Coulson, J. M. Frere, J. M. Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley.** 1991. A standard numbering scheme for the class A β-lactamases. Biochem. J. **276:**269–272. (Letter.)
2. **Blazquez, J., M. R. Baquero, R. Canton, I. Alos, and F. Baquero.** 1993. Characterization of a new TEM-type β-lactamase resistant to clavulanate, sulbactam, and tazobactam in a clinical isolate of *Escherichia coli*. Antimicrob. Agents Chemother. **37:**2059–2063.
3. **Bonomo, R. A., C. Currie-McCumber, and D. M. Shlaes.** 1992. OHIO-1 β-lactamase resistant to mechanism-based inactivators. FEMS Microbiol. Lett. **92:**79–82.
4. **Bonomo, R. A., C. G. Dawes, J. R. Knox, and D. M. Shlaes.** 1995. β-Lactamase mutations far from the active site influence inhibitor binding. Biochem. Biophys. Acta **1247:**121–125.
5. **Bonomo, R. A., C. G. Dawes, J. R. Knox, and D. M. Shlaes.** 1995. Complementary roles of mutations at positions 69 and 242 in a class A β-lactamase. Biochem. Biophys. Acta **1247:**113–120.

5a. **Bonomo, R. A., J. R. Knox, C. G. Dawes, and D. M. Shlaes.** 1994. Clavulanic acid (Clav) resistant double mutants of OHIO-1, a class A β-lactamase, abstr. C97, p. 138. *In* Program and abstracts of the 34th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.

6. **Brun, T., J. Peduzzi, M. M. Canica, G. Paul, P. Nevot, M. Barthelemy, and R. Labia.** 1994. Characterization and amino acid sequence of IRT-4, a novel

TEM-type enzyme with decreased susceptibility to β-lactamase inhibitors. FEMS Microbiol. Lett. **120:**111–117.
7. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother. **39:**1211–1233.
8. **Collatz, E., R. Labia, and L. Gutmann.** 1990. Molecular evolution of ubiquitous β-lactamases towards extended-spectrum enzymes active against newer β-lactam antibiotics. Mol. Microbiol. **4:**1615–1620.
9. **Henquell, C., C. Chanal, D. Sirot, R. Labia, and J. Sirot.** 1995. Molecular characterization of nine different types of mutants among 107 inhibitor-resistant TEM β-lactamases from clinical isolates of *Escherichia coli*. Antimicrob. Agents Chemother. **39:**427–430.
10. **Huletsky, A., J. R. Knox, and R. C. Levesque.** 1993. Role of Ser-238 and Lys-240 in the hydrolysis of third-generation cephalosporins by SHV-type β-lactamases probed by site-directed mutagenesis and three-dimensional modeling. J. Biol. Chem. **268:**3690–3697.
11. **Imtiaz, U., E. Billings, J. R. Knox, E. K. Manavathu, S. A. Lerner, and S. Mobashery.** 1993. Inactivation of class A β-lactamases by clavulanic acid: the role of arginine-244 in a proposed nonconcerted sequence of events. J. Am. Chem. Soc. **115:**4435–4442.
12. **Imtiaz, U., E. M. Billings, J. R. Knox, and S. Mobashery.** 1994. A structure-based analysis of the inhibition of class A β-lactamases by sulbactam. Biochemistry **33:**5728–5738.
13. **Knox, J. R.** 1993. Crystallography of penicillin-building enzymes, p. 36–49. *In* P. H. Bentley and R. Pondsford (ed.), Recent advances in the chemistry of anti-infective agents. Special Publication 119. Royal Society of Chemistry, London, United Kingdom.
14. **Knox, J. R.** 1995. Extended-spectrum and inhibitor-resistant TEM-type β-lactamases: mutations, specificity, and three-dimensional structure. Antimicrob. Agents Chemother. **39:**2593–2601.
15. **Kunkel, T. A., J. D. Roberts, and R. A. Zkour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
16. **Matagne, A., and J. M. Frere.** 1995. Contribution of mutant analysis to the understanding of enzyme catalysis: the case of class A β-lactamases. Biochem. Biophys. Acta **1247:**109–127.
17. **McClary, J. A., F. Whitney, and J. Geisselsoder.** 1989. Efficient site-directed in vitro mutagenesis using phagemid vectors. BioTechniques **7:**282–287.
18. **Raquet, X., J. Lamotte-Brasseur, E. Fonze, S. Goussard, P. Courvalin, and J. M. Frere.** 1994. TEM β-lactamase mutants hydrolyzing third-generation cephalosporins. A kinetic and molecular modelling analysis. J. Mol. Biol. **244:**625–639.
19. **Saves, I., D. Burlet-Schiltz, P. Swaren, F. Lefevre, J.-M. Masson, J.-C. Prome, and J. P. Samama.** 1995. The asparagine to aspartic acid substitution at position 276 of TEM-35 and TEM-36 is involved in the β-lactamase resistance to clavulanic acid. J. Biol. Chem. **31:**18240–18245.
20. **Shlaes, D. M., and C. Currie-McCumber.** 1992. Mutations altering substrate specificity in OHIO-1, an SHV-1 family β-lactamase. Biochem. J. **284:**411–415.
21. **Shlaes, D. M., C. Currie-McCumber, A. Hull, I. Behlau, and M. Kron.** 1990. OHIO-1 β-lactamase is part of the SHV-1 family. Antimicrob. Agents Chemother. **34:**1570–1576.
22. **Stapleton, P., P.-J. Wu, A. King, K. Shannon, G. French, and I. Phillips.** 1995. Incidence and mechanisms of resistance to the combination of amoxicillin and clavulanic acid in *Escherichia coli*. Antimicrob. Agents Chemother. **39:**2478–2483.
23. **Thomson, C. J., and S. G. B. Amyes.** 1992. TRC-1: emergence of a clavulanic acid-resistant TEM-β-lactamase in a clinical strain. FEMS Microbiol. Lett. **70:**113–117.
24. **Vedel, G., A. Belaaouaj, L. Gilly, R. Labia, A. Phillipon, P. Nevot, and G. Paul.** 1992. Clinical isolates of *Escherichia coli* producing TRI β-lactamases: novel TEM-enzymes conferring resistance to β-lactamase inhibitors. J. Antimicrob. Chemother. **30:**449–462.
25. **Venkatachalam, K. V., W. Huang, M. LaRocco, and T. Palzkill.** 1994. Characterization of TEM-1 β-lactamase mutants from positions 238 and 241 with increased catalytic efficiency for ceftazidime. J. Biol. Chem. **269:**23444–23450.
26. **Zafaralla, G., S. Manavathu, S. A. Lerner, and S. Mobashery.** 1992. Elucidation of the role of arginine-244 in the turnover processes of class A β-lactamases. Biochemistry **31:**3847–3852.
27. **Zhou, X. Y., F. Bordon, D. Sirot, M.-D. Kitzis, and L. Gutmann.** 1994. Emergence of clinical isolates of *Escherichia coli* producing TEM-1 derivatives or an OXA-1 β-lactamase conferring resistance to β-lactamase inhibitors. Antimicrob. Agents Chemother. **38:**1085–1089.