

# Mutant TEM $\beta$ -Lactamase Producing Resistance to Ceftazidime, Ampicillins, and $\beta$ -Lactamase Inhibitors

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**A derivative of the TEM-1  $\beta$ -lactamase producing clinically significant levels of resistance to ceftazidime and  $\beta$ -lactamase inhibitors in the presence of penicillins was generated following five rounds of DNA shuffling and selection. This complex mutant enzyme contained three amino acid substitutions including those of residues 104 and 276 that are known to produce extended-spectrum resistance and, correspondingly, resistance to  $\beta$ -lactamase inhibitors. Although the Glu104Lys substitution by itself produced low levels of ceftazidime resistance, additional amino acid replacements in the enzyme with the triple mutation resulted in further enhancement of resistance to ceftazidime. Kinetic studies of the purified  $\beta$ -lactamase enzyme with the triple mutation indicated enhancement of the catalytic efficiency for turnover ( $k_{cat}/K_m$ ) of ceftazidime. The increases in the  $K_i$  values of both clavulanic acid and tazobactam for the enzyme with the triple mutation were consistent with the observed bacterial resistance to the reversibility of  $\beta$ -lactam resistance with these inhibitors.**

Five decades after their discovery,  $\beta$ -lactams, like penicillins and cephalosporins, are still the most widely used antibiotics for treatment of nosocomial and community-acquired infections. The emergence and subsequent wide dissemination of bacteria that are resistant to a variety of  $\beta$ -lactam antibiotics pose a serious threat to the future effective use of these antimicrobial agents (9, 30). Notwithstanding the fact that several mechanisms could be implicated, the principal mechanism of resistance to  $\beta$ -lactams in clinical bacterial isolates is the production by these organisms of specific enzymes,  $\beta$ -lactamases of four functional classes: classes A, B, C, and D (8).

Class A  $\beta$ -lactamases are the most common among clinical isolates. Major representative members of the class A  $\beta$ -lactamases in gram-negative microorganisms are the TEM and SHV types, which are most common in *Escherichia coli* and *Klebsiella pneumoniae* (20). In the 1970s and 1980s, novel  $\beta$ -lactams, such as the expanded-spectrum cephalosporins, were developed to counter the growing problem of  $\beta$ -lactamase-mediated  $\beta$ -lactam resistance in gram-negative bacilli. Since the expanded-spectrum cephalosporins are poor substrates for the prototypic TEM or SHV enzymes, they retain excellent activity against bacteria producing these  $\beta$ -lactamases. Another strategy used to overcome resistance to penicillins mediated by class A  $\beta$ -lactamases has been the development of  $\beta$ -lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam. These  $\beta$ -lactam drugs have poor antibacterial activities by themselves. However, they covalently bind to the active sites of class A  $\beta$ -lactamases and inhibit them. Therefore,  $\beta$ -lactamase inhibitors in combination with penicillins are active against penicillin-resistant bacteria.

Under selective pressure from the use of expanded-spectrum cephalosporins in the 1980s and 1990s, bacteria have

evolved to overcome the threat of these drugs. Strains resistant to expanded-spectrum cephalosporins have emerged (12, 34). These strains typically bear the TEM- or SHV-type  $\beta$ -lactamases with improved activity against expanded-spectrum  $\beta$ -lactams. These so-called extended-spectrum  $\beta$ -lactamases (ESBLs) were found to have from one to a few amino acid substitutions at positions 104, 164, 238, and 240 (11, 21, 23, 27, 28, 42). The TEM and SHV  $\beta$ -lactamases have also evolved in response to the use of  $\beta$ -lactamase inhibitors. Multiple clinical isolates resistant to penicillin- $\beta$ -lactamase inhibitor combinations have been reported (2, 6, 10, 19, 25). Examination of the  $\beta$ -lactamase genes has revealed that replacement of one of four different amino acid residues in the enzyme is responsible for the resulting phenotype (5, 18, 32, 36). Substitutions of critical residues at positions 69, 130, 244, and 276 were able to produce the inhibitor-resistant TEM (IRT) phenotype by themselves (1–3, 14) or in combination with other replacements (7, 18, 44). Thus, the parental TEM-1  $\beta$ -lactamase can readily evolve to resist inhibition or to confer a broadened  $\beta$ -lactam resistance spectrum, and such mutants are readily selected by growth in the presence of  $\beta$ -lactamase inhibitors or expanded-spectrum cephalosporins, respectively. Although several clinical isolates have been reported to combine both of these phenotypes, none of them produce clinically significant levels of resistance to both  $\beta$ -lactamase inhibitors and expanded-spectrum cephalosporins.

In the study described here we used shuffling of the TEM-1  $\beta$ -lactamase gene to construct mutant enzymes that would produce resistance to both expanded-spectrum cephalosporins and  $\beta$ -lactamase inhibitors. DNA shuffling is a powerful technique that allows one to randomly recombine multiple mutations introduced by PCR, thus mimicking and dramatically accelerating evolution. Mutant enzyme that contained three amino acid substitutions and that expressed the double ESBL and IRT phenotype was selected. The contributions of individual mutations to the resistance phenotype were evaluated.

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## MATERIALS AND METHODS

**Bacterial strains and media.** *E. coli* JM83 {F<sup>-</sup> *ara*  $\Delta$ (*lac-proAB*) *rpsL* (Str<sup>r</sup>) [ $\phi$ 80 *dlac*  $\Delta$ (*lacZ*)M15] *thi*} was used as the recipient strain for plasmids and as the host for the determination of susceptibilities to antibiotics (New England Biolabs). *E. coli* BMH 71-18 *mutS* {*thi supE*  $\Delta$ (*lac-proAB*) [*mutS*::Tn10] [F<sup>-</sup> *proAB lacI*<sup>q</sup>Z $\Delta$ M15]} was used as the intermediate host in site-directed mutagenesis experiments (Clontech). *E. coli* BL21(DE3) [F<sup>-</sup> *ompT hsdS<sub>B</sub>* (r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) *gal dem* (DE3)] was the host for target gene expression (Novagen). Luria-Bertani (LB) medium, Mueller-Hinton broth, or Terrific Broth (Difco) was used as the growth medium for all bacterial strains. Bacto Agar (Difco) was added at a concentration of up to 1.5% for the preparation of solid medium.

**Reagents, antibiotics, and susceptibility testing.** Restriction endonucleases, DNA-modifying enzymes, T4 DNA polymerase, and *Taq* DNA polymerase were purchased from New England Biolabs or BRL; *Pfu* Turbo DNA polymerase was purchased from Stratagene; and DNase I was purchased from Sigma. Inorganic chemicals were from Sigma or Gibco BRL. Plasmid DNA was isolated with a QIAprep Spin Miniprep kit (Qiagen Inc.). DNA manipulations were performed by standard procedures as described elsewhere (31). Kanamycin, ampicillin, piperacillin, cefotaxime, and ceftriaxone were obtained from Sigma. Ceftazidime was kindly supplied by Glaxo, cefepime and aztreonam were supplied by Bristol-Myers Squibb, imipenem was supplied by Merck Sharp & Dohme, clavulanic acid was supplied by SmithKline Beecham, and tazobactam was supplied by Wyeth-Ayerst. MICs were determined by a twofold dilution method in Mueller-Hinton broth with 96-well microtiter plates. The same bacterial host, *E. coli* JM83, was used for all MIC determinations.

**Plasmids.** Plasmids pTZ19-3 and pTZ19-4 (41) were used for DNA shuffling and site-directed mutagenesis of the TEM  $\beta$ -lactamase gene, respectively. Plasmid pSV105 was constructed to facilitate the fusion of the TEM  $\beta$ -lactamase gene with the leader sequence of outer membrane protein A (OmpA). This plasmid is based on the pUC19 vector and harbors the 63-bp OmpA leader sequence that was amplified by PCR from the chromosome of *E. coli* JM83 with two oligonucleotide primers: primer ompA-dir (AAACATATGAAAAAGACA GCTATCGCGA [the *NdeI* site is italicized]) and primer ompA-rev (GGCCTG CGTACGGTAGCGAAAC). The *NaeI* restriction site was subsequently introduced by site-directed mutagenesis at the 3' end of the *ompA* leader. Plasmid pET24 a+ (Novagen) was used to produce high levels of expression of the TEM  $\beta$ -lactamase genes.

**Site-directed mutagenesis.** Site-directed mutagenesis of the TEM-1  $\beta$ -lactamase gene was performed with double-stranded DNA of pTZ19-4 with a Transformer Site-Directed Mutagenesis kit (Clontech). Two primers were used for this purpose: selection primer GCATAAGCTATTGCCATTCTC, which mutates the recognition sequence for a unique *HindIII* restriction endonuclease site in the kanamycin resistance gene of the plasmid, and one of the mutagenic primers that allows introduction of the desired mutation into the TEM-1  $\beta$ -lactamase gene. The *NaeI* restriction site at the 3' end of the *ompA* leader was introduced in a similar way. Following mutagenesis, DNA was electroporated into recipient strain *E. coli* JM83, and mutants were selected on agar supplemented with 30  $\mu$ g of kanamycin per ml.

**DNA shuffling.** DNA shuffling of the TEM-1  $\beta$ -lactamase gene was performed by a previously described procedure (38), with some modifications. We used two custom-synthesized primers (primer TEMBam [GTGCGCGGATCCCCTATTT G] and primer TEMHind [AGTAAACTTGGTCTGAC]) to amplify the TEM-1  $\beta$ -lactamase gene from the vector, pTZ19-3. A total of 35 cycles of PCR with the *Taq* DNA polymerase was performed under mutagenic and nonmutagenic conditions (in 20 microtubes for each condition). The PCR mixture for nonmutagenic conditions contained (per 100  $\mu$ l) 0.2 mM dATP and dGTP, 0.4 mM dTTP and dCTP, 2.5 mM MgCl<sub>2</sub>, 1 $\times$  PCR buffer (Bethesda Research Laboratories, Inc.), 50 ng of DNA, 50 pmol of each primer, and 2.5 U of *Taq* DNA polymerase. The PCR mixture for mutagenic conditions was the same as that for nonmutagenic conditions, except that it contained a higher concentration (7 mM) of MgCl<sub>2</sub> and also contained 0.5 mM MnCl<sub>2</sub>. The following PCR program was used in a Perkin-Elmer Cetus 480 thermal cycler: 30 to 35 cycles at 94°C for 60 s, 94°C for 30 s, 60°C for 30 s, and 73°C for 60 s. The DNA from all tubes was combined, and primers were removed from the PCR product with the QIAquick PCR Purification kit (Qiagen). About 4 to 5  $\mu$ g of DNA was digested with DNase I to produce fragments of 50 to 200 bp. About 2 to 3  $\mu$ g of the purified fragments was

subjected to 30 to 45 cycles of PCR with no primers. This allowed the reassembly of small DNA fragments into full-sized genes. About 200 ng of the reassembled product was further amplified (20 cycles) with primers TEMBam and TEMHind to yield the full-sized  $\beta$ -lactamase gene. After digestion with *Bam*HI and *Hind*III and purification with the QIAquick PCR Purification kit (Qiagen), 300 to 400 ng of DNA of the  $\beta$ -lactamase gene was recloned into the pTZ19-3 vector. The resulting construct was used to electroporate competent *E. coli* JM83 cells, and mutants were selected for resistance to ceftazidime. To eliminate mutations that are not related to the resulting phenotype, we performed the so-called back-shuffling procedure when the DNA of mutant 1 has been reshuffled with a large excess (20 times) of wild-type DNA.

**Construction of mutant  $\beta$ -lactamases.** The enzyme with the triple mutation Glu104Lys, Ser268Gly, and Asn276Asp was constructed by substituting the 300-bp *XmnI*-*Bam*HI fragment in mutant 6 for the same fragment from the gene for the wild-type enzyme. Substitutions Lys104 for Glu104, Gly268 for Ser268, and Asp276 for Asn276 in the TEM- $\beta$ -lactamase were introduced by site-directed mutagenesis of the TEM-1  $\beta$ -lactamase gene of plasmid pTZ19-4 by using the following mutagenic primers: primer Glu104Lys (GAATGACTTGGTTGA ATACTCACCAGTC), primer Ser268Gly (CTACACGACGGGGGTCAGG CAACTATG), and primer Asn276Asp (CTATGGATGAACGAGATAGACA GATCGC), respectively. All combinations of amino acid substitutions in the TEM  $\beta$ -lactamase were constructed by combining the various restriction fragments of the TEM  $\beta$ -lactamase gene containing individual mutations. Specifically, the mutant with the Glu104Lys and Ser268Gly double mutation was constructed by ligating the  $\sim$ 620-bp *PstI*-*Bam*HI fragment from the pTZ19-3 derivative that contains a mutation that results in the Glu104Lys substitution with the large *PstI*-*Bam*HI fragment of another pTZ19-3 derivative that contains a mutation that results in the Ser268Gly substitution in the TEM-1  $\beta$ -lactamase. Similarly, the mutant with the Glu104Lys and Asn276Asp double mutation was constructed by ligating the  $\sim$ 620 bp *PstI*-*Bam*HI fragment that contains a mutation that results in the Glu104Lys substitution with the large *PstI*-*Bam*HI fragment of the pTZ19-3 derivative that contains a mutation that results in the Asn276Asp substitution in the TEM-1  $\beta$ -lactamase. The mutant with the double Ser268Gly and Asn276Asp replacements was constructed by replacing the  $\sim$ 620-bp *PstI*-*Bam*HI fragment in mutant 2 (that was obtained in the course of shuffling of the TEM-1  $\beta$ -lactamase gene) with the *PstI*-*Bam*HI fragment of the TEM-1  $\beta$ -lactamase gene.

**Cloning of TEM  $\beta$ -lactamase genes into expression vector.** To facilitate  $\beta$ -lactamase secretion into the growth medium, we fused the genes for the mature TEM-1  $\beta$ -lactamase and the  $\beta$ -lactamases with triple mutations with the leader sequence of the OmpA protein. DNA fragments encoding the mature TEM-1  $\beta$ -lactamase and the mutant  $\beta$ -lactamase were amplified from plasmid pTZ19-3 by 20 cycles of high-fidelity PCR with *Pfu* Turbo DNA polymerase. Two primers, primer TEM-Mdir (CACCCAGAAACGCTGGTG) and primer TEM-Mrev (GAGTAAGCTTGGTCTGACAG), were used for this purpose. PCR products were purified from the agarose gel, digested with *Hind*III, and ligated into the *NaeI*-*Hind*III sites of pSV106. The entire TEM  $\beta$ -lactamase genes and the OmpA leader were sequenced. TEM  $\beta$ -lactamase genes were cut out with the *NdeI* and *Hind*III endonucleases, cloned into the *NdeI*-*Hind*III sites of expression vector pET24a, and introduced by transformation into the recipient strain, *E. coli* BL21(DE3).

**DNA sequencing.** Sequencing of the double-stranded plasmid DNA was performed with the Sequenase DNA sequencing kit (version 2.0; U.S. Biochemicals) and a set of custom-made internal primers by the procedure described by manufacturer.

**Purification of TEM  $\beta$ -lactamases.** For enzyme purification, overnight cultures of *E. coli* BL21(DE3) in LB medium supplemented with kanamycin at 20  $\mu$ g/ml were diluted 100-fold in Terrific Broth (Difco) supplemented with 20  $\mu$ g of kanamycin per ml, 0.5 M sorbitol, and 2.5 mM betaine. After 4 to 5 h of incubation with shaking (260 to 280 rpm) at 37°C (until the optical density of the culture at 600 nm reached 0.6), isopropyl- $\beta$ -D-thiogalactopyranoside was added to a final concentration 0.4 mM and the cultures were incubated overnight at 25°C. The cells were pelleted by centrifugation, and the supernatants containing the enzyme were concentrated with an Amicon ultrafiltration device (membrane molecular weight cutoff, 10,000). Concentrated proteins were reconstituted in 0.01 M Tris buffer (pH 7.0) and loaded onto a DEAE anion-exchange column (Sigma) equilibrated with the same buffer. The enzymes were eluted by a linear gradient (10 mM Tris-100 mM Tris) and were homogeneous, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining.

**Determination of amounts of active enzymes.** Cells producing the wild-type  $\beta$ -lactamase and the  $\beta$ -lactamase with the triple mutations were grown at 37°C overnight in LB broth supplemented with 30  $\mu$ g of kanamycin per ml. After

TABLE 1. Mutants of the TEM-1  $\beta$ -lactamase conferring resistance to ceftazidime

| Residue in TEM-1 | Amino acid substitution |          |          |          |          |          |
|------------------|-------------------------|----------|----------|----------|----------|----------|
|                  | Mutant 1                | Mutant 2 | Mutant 3 | Mutant 4 | Mutant 5 | Mutant 6 |
| Ala42            | Gly                     | Gly      | Gly      | Gly      | Gly      | Gly      |
| Ile47            | Val                     | Val      | Val      | Val      | Val      | Val      |
| Glu104           | Lys                     | Lys      | Lys      | Lys      | Lys      | Lys      |
| Pro167           | Ser                     | Ser      |          | Ser      | Ser      |          |
| Leu169           | Pro                     |          |          | Pro      | Pro      |          |
| Ala184           | Val                     |          |          | Val      |          |          |
| Gly238           | Ser                     |          | Ser      |          | Ser      |          |
| Glu240           | Gly                     |          | Gly      |          | Gly      |          |
| Ser268           | Gly                     | Gly      | Gly      | Gly      |          | Gly      |
| Asn276           | Asp                     | Asp      | Asp      | Asp      | Asp      | Asp      |
| Lys288           | Glu                     |          |          |          |          |          |

20-fold dilution into the same medium, cells were grown for another 2 h and the cell density was adjusted spectrophotometrically at 600 nm. The cells were pelleted by centrifugation, suspended in 100 mM sodium phosphate buffer (pH 7.0), and subjected to sonication. The cell debris was then removed by centrifugation at 20,000  $\times g$ . Enzyme concentrations were determined from the rates of hydrolysis of ampicillin at a saturating concentration.

**Enzyme kinetics.**  $\beta$ -Lactamase activity against ampicillin and ceftazidime and the inhibition profile with clavulanic acid and tazobactam were determined spectrophotometrically with a Hewlett-Packard 8453 diode array spectrophotometer in 100 mM phosphate buffer (pH 7.0) at room temperature. Calculations of kinetic constants were performed with Microsoft Excel software. The hydrolysis of the  $\beta$ -lactam bond was monitored at 240 nm for ampicillin ( $\Delta\epsilon = 538 \text{ M}^{-1} \text{ cm}^{-1}$ ) and at 260 nm for ceftazidime ( $\Delta\epsilon = 10,500 \text{ M}^{-1} \text{ cm}^{-1}$ ). Kinetic parameters for the substrates were determined by the Hanes-Wolf method or with the integrated Michaelis-Menten equation (13).

Determination of the inhibition parameters was carried out by monitoring the hydrolysis of penicillin G at 240 nm ( $\Delta\epsilon = 570 \text{ M}^{-1} \text{ cm}^{-1}$ ) at room temperature in 100 mM sodium phosphate buffer (pH 7.0). These experiments were performed under conditions of excess substrate concentrations, as described by Koerber and Fink (24).

Inactivation rate constants for clavulanic acid and tazobactam with both the wild-type and mutant enzymes were calculated as described previously (40). Enzyme (1.6  $\mu\text{M}$ ) and inhibitor (200 to 800  $\mu\text{M}$  for clavulanic acid and 20 to 160  $\mu\text{M}$  for tazobactam) were incubated at 4°C, and at appropriate times small aliquots were removed from this mixture and diluted 50 times to an assay mixture containing 2 mM penicillin G. The highest steady-state rates during the course of substrate hydrolysis were used to calculate the remaining activity.

The dissociation constants ( $K_s$ ) of clavulanic acid and tazobactam for the wild-type enzyme and the enzyme with the triple mutation were calculated by the method of Dixon (15). Two concentrations of the penicillin G substrate were used, and a series of assay mixtures containing both the substrate and inactivators at various concentrations (2 to 24  $\mu\text{M}$  for clavulanic acid and 0.4 to 2  $\mu\text{M}$  for tazobactam) were prepared in 100 mM sodium phosphate buffer (pH 7.0). An aliquot of the stock solution of the enzyme was added to obtain final concentrations of 6 nM for both the wild-type enzyme and the enzyme with triple mutations, followed by the immediate measurement of enzyme activity. Rates were measured for the first 5% of substrate turnover.

Partition ratios ( $k_{\text{cat}}/k_{\text{inact}}$  [where  $k_{\text{inact}}$  is the rate constant for inactivation]) for clavulanic acid and tazobactam were determined for both enzymes by the titration method (33). Several buffered mixtures containing various molar ratios ( $[I]/[E]$ ) of each of the inhibitors (I) with each of the enzymes (E) were incubated at 4°C overnight (ca. 20 h). The molar ratio ( $[I]/[E]$ ) for clavulanic acid varied from 1 to 200 for the wild-type enzyme and 1 to 500 for the mutant enzyme. For tazobactam the molar ratio varied from 1 to 400 for the wild-type enzyme and 1 to 600 for the mutant enzyme. The remaining activity of the enzyme was assayed under conditions of excess penicillin G (2 mM).

Rate constants for the recovery of enzyme activity ( $k_{\text{rec}}$ ) from the transiently inhibited species for the wild-type and mutant enzymes incubated with each of the inhibitors were measured at the molar ratio ( $[I]/[E]$ ) that gave the longest interval of time before the steady-state rate of hydrolysis of penicillin G was reached. Calculations of the constants were performed by the method of Glick et al. (17).

## RESULTS

**Mutant  $\beta$ -lactamases obtained by DNA shuffling and their phenotypic and genotypic characterization.** In an effort to select a mutant  $\beta$ -lactamase that confers resistance to both expanded-spectrum cephalosporins and  $\beta$ -lactamase inhibitors, we first subjected the TEM-1  $\beta$ -lactamase gene to DNA shuffling and selected transformants of *E. coli* for growth on agar with increasing concentrations of ceftazidime. After five successive rounds of DNA shuffling we were able to obtain hundreds of colonies on the agar plate with 128  $\mu\text{g}$  of ceftazidime per ml. We checked 48 colonies from this plate for resistance to ampicillin or ceftazidime in the presence of 2  $\mu\text{g}$  of clavulanic acid per ml. Two of them exhibited resistance to the combination of ceftazidime and clavulanic acid. Determination of the sequences of the  $\beta$ -lactamase genes from these clones revealed that they were identical and had a total of 16 mutations, with 11 resulting in amino acid replacements in the inferred TEM-1  $\beta$ -lactamase sequence and with 5 being silent mutations. This mutant (designated mutant 1) not only had substitutions in the TEM-1  $\beta$ -lactamase at positions that are known to contribute to ceftazidime resistance in clinical isolates and laboratory mutants (Glu104Lys, Pro167Ser, Gly238Ser, Glu240Gly), but it also had an Asn276Asp substitution, whose presence is known to produce resistance to inactivation by clavulanic acid (Table 1). Although mutant 1 had gained marked resistance to ceftazidime (256 times the level of resistance produced by TEM-1) and ceftazidime in combination with clavulanic acid, the MICs of the other expanded-spectrum cephalosporins like cefepime, cefotaxime, and ceftriaxone rose only fourfold (Table 2). Additionally, mutant 1 lost most of the ampicillin resistance conferred by the parental TEM-1 enzyme.

To minimize the number of mutations in the  $\beta$ -lactamase while retaining the ability to confer ceftazidime resistance, we back-shuffled the *bla* gene of mutant 1 and selected transformants for growth on agar with various concentrations (8, 16, 32, 64, and 128  $\mu\text{g}/\text{ml}$ ) of ceftazidime. We tested 60 transformants (12 from each concentration) for resistance to ceftazidime, cefepime, cefotaxime, and ampicillin (data not shown) and sequenced the *bla* genes from 10 of these transformants that produced various levels of resistance to antibiotics. Sequence analysis revealed five different clones that had smaller

TABLE 2. MICs of  $\beta$ -lactams conferred by TEM-1 and its mutant derivatives

| Strains producing TEM-1 and mutant $\beta$ -lactamases | MIC ( $\mu$ g/ml) <sup>a</sup> |           |      |           |      |      |      |
|--|--------------------------------|-----------|------|-----------|------|------|------|
|  | AMP                            | AMP + CLA | CAZ  | CAZ + CLA | FEP  | CTX  | CRO  |
| None ( <i>E. coli</i> JM83)                            | 4                              | 2         | 0.06 | 0.03      | 0.03 | 0.03 | 0.03 |
| TEM-1  | 16,000                         | 250       | 0.5  | 0.25      | 0.25 | 0.06 | 0.06 |
| Mutant 1   | 64                             | 32        | 250  | 16        | 2    | 0.25 | 0.25 |
| Mutant 2   | 8,000                          | 250       | 128  | 8         | 2    | 0.25 | 0.25 |
| Mutant 3   | 8,000                          | 16        | 250  | 8         | 2    | 0.25 | 0.25 |
| Mutant 4   | 32                             | 16        | 128  | 8         | 2    | 0.25 | 0.25 |
| Mutant 5   | 32                             | 16        | 64   | 16        | 0.25 | 0.12 | 0.06 |
| Mutant 6   | 32,000                         | 4,000     | 16   | 4         | 4    | 0.25 | 0.25 |

<sup>a</sup> Abbreviations: AMP, ampicillin; CLA, clavulanic acid (at 2  $\mu$ g/ml); CAZ, ceftazidime; FEP, cefepime; CTX, cefotaxime; and CRO, ceftriaxone.

numbers of amino acid substitutions in the TEM  $\beta$ -lactamase gene than mutant 1 (Table 1, mutants 2 to 6). The mutants that we obtained by back-shuffling were checked not only for their susceptibilities to ceftazidime and other  $\beta$ -lactam antibiotics but also for the reversibility of ampicillin and ceftazidime resistance by clavulanic acid. As seen in Table 2, all of the mutants retained resistance to ceftazidime alone and in combination with clavulanic acid, but for most mutants the ceftazidime MICs were lower than that for mutant 1. Mutants 2, 3, and 6 regained high-level resistance to ampicillin. In addition, mutant 6 was the only one that had a high level of resistance to ampicillin in the presence of clavulanic acid.

**Construction of specific mutant enzymes and susceptibility testing.** In order to further minimize the number of mutations in the TEM-1  $\beta$ -lactamase, the enzyme with the triple mutation (E104K, S268G, N276D) was first constructed. Although the ampicillin and ceftazidime MICs for this mutant were two-fold lower than those for the mutant with quintuple mutations (mutant 6), its level of resistance to ampicillin paralleled that produced by the TEM-1  $\beta$ -lactamase and its level of resistance to ceftazidime was 16 times higher (Table 3). On the basis of the amino acid sequence of the enzyme with triple mutations, six additional mutant TEM  $\beta$ -lactamases were constructed (see experimental procedures above) to evaluate the contributions of individual amino acid substitutions and their combinations to the resulting resistance (Table 3).

All mutant  $\beta$ -lactamases exhibited high-level resistance to both ampicillin and piperacillin comparable to that conferred by the parental TEM-1 enzyme (Table 3). On the other hand,

the levels of susceptibility to ceftazidime varied among these mutants. The Glu104Lys mutation resulted in a fourfold higher ceftazidime MIC, and the additional presence of Ser268Gly or Asn276Asp enhanced the level of resistance further. The presence of both of the latter mutations together with Glu104Lys in the enzyme with the triple mutation raised the ceftazidime MICs even further. The variations of the MICs of cefepime paralleled the ceftazidime MIC profiles for the mutant strains, although the actual MICs of cefepime were generally lower. For ceftriaxone, cefotaxime, and aztreonam, the only mutant for which there was more than a fourfold rise in the MIC from the low MICs conferred by the parental TEM-1  $\beta$ -lactamase was that with the Glu104Lys and Ser268Gly substitutions, and the resulting MICs were still only 1  $\mu$ g/ml for all three antibiotics. The virtual absence of resistance to imipenem conferred by the TEM-1 enzyme was not affected by any of the mutations that we examined.

As expected, the presence of either clavulanic acid or tazobactam reduced the MICs of ampicillin and piperacillin conferred by the TEM-1  $\beta$ -lactamase (Table 4). These reductions of the MICs were enhanced by the presence of the Glu104Lys mutation in the enzyme with a single mutation. The presence of the Asn276Asp mutation, either alone or in the presence of other mutations, reduced the effect of the  $\beta$ -lactamase inhibitors in enhancing susceptibility to ampicillin and piperacillin. Since the Glu104Lys mutation produced resistance to ceftazidime, the effects of the inhibitors on ceftazidime susceptibility could be studied in the mutants bearing this mutation. Thus, the modest level of resistance to ceftazidime conferred by the

TABLE 3. MICs of various antibiotics conferred by mutant TEM  $\beta$ -lactamases

| Amino acid replacement(s)        | MIC ( $\mu$ g/ml) <sup>a</sup> |       |      |      |      |      |      |      |
|----------------------------------|--------------------------------|-------|------|------|------|------|------|------|
|                                  | AMP                            | PIP   | CAZ  | FEP  | CRO  | CTX  | ATM  | IPM  |
| <i>E. coli</i> JM83 <sup>b</sup> | 4                              | 1     | 0.06 | 0.03 | 0.03 | 0.03 | 0.03 | 0.12 |
| TEM-1 (none) <sup>c</sup>        | 16,000                         | 4,000 | 0.5  | 0.25 | 0.06 | 0.06 | 0.12 | 0.12 |
| E104K                            | 16,000                         | 2,000 | 2    | 0.5  | 0.25 | 0.25 | 0.5  | 0.12 |
| S268G                            | 16,000                         | 4,000 | 0.5  | 0.5  | 0.12 | 0.12 | 0.12 | 0.12 |
| N276D                            | 16,000                         | 4,000 | 0.5  | 0.25 | 0.03 | 0.03 | 0.06 | 0.12 |
| E104K, S268G                     | 16,000                         | 4,000 | 4    | 2    | 1    | 1    | 1    | 0.12 |
| E104K, N276D                     | 16,000                         | 2,000 | 4    | 1    | 0.06 | 0.06 | 0.25 | 0.12 |
| S268G, N276D                     | 16,000                         | 4,000 | 1    | 0.5  | 0.03 | 0.03 | 0.06 | 0.12 |
| E104K, S268G, N276D              | 16,000                         | 4,000 | 8    | 2    | 0.12 | 0.12 | 0.25 | 0.12 |
| A42G, I47V, E104K, S268G, N276D  | 32,000                         | 8,000 | 16   | 4    | 0.25 | 0.25 | 0.5  | 0.12 |

<sup>a</sup> Abbreviations: AMP, ampicillin; PIP, piperacillin; CAZ, ceftazidime; FEP, cefepime; CRO, ceftriaxone; CTX, cefotaxime; ATM, aztreonam; IPM, imipenem.

<sup>b</sup> Recipient strain *E. coli* JM83.

<sup>c</sup> Wild-type TEM-1  $\beta$ -lactamase.



TABLE 4. Susceptibilities of *E. coli* JM83 producing TEM-1 and its mutants to  $\beta$ -lactams and to  $\beta$ -lactam- $\beta$ -lactamase inhibitor combinations

| Amino acid replacement(s)       | MICs ( $\mu\text{g/ml}$ ) <sup>a</sup> |            |            |            |            |             |       |            |            |             |     |            |            |            |            |             |
|---------------------------------|--|------------|------------|------------|------------|-------------|-------|------------|------------|-------------|-----|------------|------------|------------|------------|-------------|
|                                 | AMP                                    | AMP + CLA2 | AMP + CLA4 | AMP + TAZ4 | AMP + TAZ8 | AMP + TAZ16 | PIP   | PIP + TAZ4 | PIP + TAZ8 | PIP + TAZ16 | CAZ | CAZ + CLA2 | CAZ + CLA4 | CAZ + TAZ4 | CAZ + TAZ8 | CAZ + TAZ16 |
| TEM-1 (none) <sup>b</sup>       | 16,000                                 | 250        | 64         | 4,000      | 2,000      | <32         | 4,000 | 1,000      | 500        | <16         | 0.5 | 0.25       | 0.25       | 0.5        | 0.5        | 0.06        |
| E104K                           | 16,000                                 | 64         | 16         | 2,000      | 500        | <32         | 2,000 | 500        | 32         | <16         | 2   | 0.5        | 0.5        | 1          | 1          | 0.12        |
| S268G                           | 16,000                                 | 250        | 125        | 4,000      | 2,000      | <32         | 4,000 | 2,000      | 500        | <16         | 0.5 | 0.25       | 0.25       | 0.5        | 0.5        | 0.06        |
| N276D                           | 16,000                                 | 2,000      | 500        | 4,000      | 2,000      | <32         | 4,000 | 1,000      | 500        | <16         | 0.5 | 0.5        | 0.5        | 0.5        | 0.5        | 0.06        |
| E104K, S268G                    | 16,000                                 | 125        | 64         | 4,000      | 1,000      | <32         | 4,000 | 2,000      | 500        | <16         | 4   | 1          | 1          | 4          | 2          | 0.5         |
| E104K, N276D                    | 16,000                                 | 1,000      | 250        | 4,000      | 2,000      | <32         | 2,000 | 1,000      | 500        | <16         | 4   | 2          | 2          | 4          | 2          | 1           |
| S268G, N276D                    | 16,000                                 | 2,000      | 500        | 4,000      | 2,000      | <32         | 4,000 | 2,000      | 500        | <16         | 1   | 1          | 1          | 1          | 1          | 0.12        |
| E104K, S268G, N276D             | 16,000                                 | 1,000      | 500        | 4,000      | 2,000      | <32         | 4,000 | 1,000      | 500        | 64          | 8   | 2          | 2          | 8          | 4          | 2           |
| A42G, I47V, E104K, S268G, N276D | 32,000                                 | 4,000      | 1,000      | 8,000      | 4,000      | 2,000       | 8,000 | 2,000      | 1,000      | 1,000       | 16  | 4          | 4          | 8          | 8          | 8           |

<sup>a</sup> Abbreviations: AMP, ampicillin; CLA, clavulanic acid; TAZ, tazobactam; PIP, piperacillin; CAZ, ceftazidime. The numbers after the abbreviation's indicate concentrations (in micrograms per milliliter).

<sup>b</sup> Wild-type TEM-1  $\beta$ -lactamase.

Glu104Lys mutant enzyme could be reversed by clavulanic acid or tazobactam. The presence of the Asn276Asp mutation, which alone confers resistance to inactivation of the enzyme by both inhibitors, reduced the effects of both inhibitors on the reduction of the level of ceftazidime resistance conferred by the Glu104Lys mutation. The enzyme containing just these two mutations (Glu104Lys and Asn276Asp) exhibited this stability of resistance to ceftazidime in the presence of clavulanic acid or tazobactam. The  $\beta$ -lactamase mutant with the triple mutation (Glu104Lys, Ser268Gly, and Asn276Asp), which conferred a somewhat higher level of resistance to ceftazidime alone, exhibited two times higher levels of resistance to ceftazidime in the presence of tazobactam and similar levels of resistance in the presence of clavulanic acid compared with those for the mutant with the double Glu104Lys and Asn276Asp mutations. Thus, the enzyme with the triple mutation conferred ceftazidime MICs of 2 and 8  $\mu\text{g/ml}$  in the presence of clavulanic acid (2 or 4  $\mu\text{g/ml}$ ) and tazobactam (4  $\mu\text{g/ml}$ ), respectively.

**Isolation of enzymes.** The experiments with enzymes extracted from cells by sonication revealed that there is 1.3-fold increase in the level of production of the enzyme with the triple mutation compared to that of the wild-type  $\beta$ -lactamase. The same experiment demonstrated that both enzymes were stable for at least 4 h at 4°C.

Expression of the enzyme with the OmpA leader in the presence of sorbitol and betaine has allowed us to isolate 40 mg of soluble TEM-1 and 30 mg of the  $\beta$ -lactamase with the triple mutation (more than 80% pure) directly from 400 ml of

culture medium. One-step ion-exchange chromatography was sufficient to further purify the enzymes to apparent homogeneity. Pure enzymes were stable at least for 3 months, as judged by an activity assay.

**Enzyme kinetics.** Table 5 reports the kinetics of substrate hydrolysis by the  $\beta$ -lactamase with the triple mutation in comparison with that by the TEM-1 enzyme. The catalytic efficiency ( $k_{\text{cat}}/K_m$ ) of the  $\beta$ -lactamase with the triple mutation against ampicillin declined 3.4-fold compared with that of TEM-1. On the other hand, the catalytic efficiency of the enzyme with the triple mutation against ceftazidime was 6.3 times higher than that of TEM-1.

Table 6 presents the effects of clavulanic acid and tazobactam on the purified  $\beta$ -lactamase with the triple mutation and the TEM-1  $\beta$ -lactamase. The  $K_i$  values of clavulanic acid were 7.8-fold higher for the enzyme with the triple mutation than for the wild-type enzyme, and those of tazobactam were comparable for both enzymes. With clavulanic acid the partition ratio ( $k_{\text{cat}}/k_{\text{inact}}$ ) of the enzyme with the triple mutation was 2.7 times higher than that of the TEM-1  $\beta$ -lactamase, and with tazobactam it was only slightly (18%) higher than that of the TEM-1  $\beta$ -lactamase.

## DISCUSSION

Our approach to the question of whether a TEM-derived mutant  $\beta$ -lactamase could confer extended-spectrum  $\beta$ -lactam resistance and resistance to  $\beta$ -lactamase inhibitors was based on the ability to generate numerous random mutations by PCR

TABLE 5. Specificity profile by comparison of kinetic parameters for ampicillin and ceftazidime for the wild-type  $\beta$ -lactamase and the  $\beta$ -lactamase with triple mutations

| Substrate   | $\beta$ -Lactamase  | $k_{\text{cat}}$ ( $\text{s}^{-1}$ ) | $K_m$ ( $\mu\text{M}$ ) | $k_{\text{cat}}/K_m$ ( $\text{M}^{-1} \text{s}^{-1}$ ) |
|-------------|---------------------|--------------------------------------|-------------------------|--|
| Ampicillin  | <sup>a</sup> TEM-1  | $1,320 \pm 150$                      | $54 \pm 9$              | $(2.5 \pm 0.5) \times 10^7$                            |
|             | Mutant              | $3,220 \pm 990$                      | $440 \pm 140$           | $(7.0 \pm 3) \times 10^6$                              |
| Ceftazidime | TEM-1               | $(1.3 \pm 0.4) \times 10^{-2}$       | $140 \pm 45$            | $(9.0 \pm 4.0) \times 10^1$                            |
|             | Mutant <sup>a</sup> | $\leq 0.05$                          | ND                      | $(5.90 \pm 0.04) \times 10^2$                          |

<sup>a</sup> Determined by integrated Michaelis-Menten equation at  $[S] \ll K_m$ .

<sup>b</sup> ND, not determined.

TABLE 6. Inhibition profiles of wild-type  $\beta$ -lactamase and  $\beta$ -lactamase with triple mutations for clavulanic acid and tazobactam

| $\beta$ -Lactamase | Inhibitor   | $k_{\text{cat}}$ ( $\text{s}^{-1}$ ) | $k_{\text{inact}}$ ( $\text{s}^{-1}$ ) | $K_i$ ( $\mu\text{M}$ ) | $k_{\text{cat}}/k_{\text{inact}}$ |
|--------------------|-------------|--------------------------------------|--|-------------------------|-----------------------------------|
| Wild type          | Clavulanate | $0.4 \pm 0.1$                        | $(2.0 \pm 0.2) \times 10^{-3}$         | $1.4 \pm 0.3$           | $180 \pm 30$                      |
|                    | Tazobactam  | $1.1 \pm 0.6$                        | $(2 \pm 1) \times 10^{-3}$             | $0.014 \pm 0.003$       | $550 \pm 120$                     |
| Mutant             | Clavulanate | $0.36 \pm 0.01$                      | $(7.5 \pm 0.2) \times 10^{-4}$         | $11 \pm 1$              | $480 \pm 7$                       |
|                    | Tazobactam  | $0.72 \pm 0.07$                      | $(1.1 \pm 0.2) \times 10^{-3}$         | $0.018 \pm 0.002$       | $650 \pm 18$                      |

mutagenesis and to randomly recombine them by DNA shuffling (38). DNA shuffling is known to be a powerful mutagenic technique that creates numerous combinations of mutations in a gene of interest, thus mimicking evolution and accelerating it in an observable time frame (39, 43). We first produced a pool of mutant TEM  $\beta$ -lactamase genes with numerous combinations of mutations that would produce high levels of resistance to ceftazidime. We anticipated that subsequent selection on ceftazidime or ampicillin in combination with the  $\beta$ -lactamase inhibitors would allow us to obtain the desired mutants with a double ESBL-IRT phenotype.

Mutant 1, generated by five successful rounds of DNA shuffling and selection, is a complex  $\beta$ -lactamase that combines three amino acid substitutions of the ESBL type (Glu104Lys, Gly238Ser, and Glu240Gly) with an IRT-specific mutation (Asn276Asp). It confers high levels of resistance to ceftazidime alone and in combination with clavulanic acid. On the other hand, mutant 1 produces very low levels of resistance to ampicillin. Analysis of amino acid substitutions in five derivatives of mutant 1 (mutants 2 to 6) suggests that the proline-for-leucine substitution at position 169 may be responsible for the loss of ampicillin resistance. Thus, all derivatives of mutant 1 with Leu169Pro substitutions (mutants 4 and 5) produce low levels of ampicillin resistance, while those with leucine at position 169 (mutants 2, 3, and 6) produce high levels of ampicillin resistance. At the same time, the Leu169Pro substitution in mutant 2 produces an eightfold increase in the level of resistance to ceftazidime in comparison to that of mutant 6, which lacks this mutation (Tables 1 and 2). As has been shown previously (26), some substitutions at position 169 in combination with other adjacent mutations in the omega loop of the TEM-1  $\beta$ -lactamase could produce resistance to ceftazidime, with a concomitant significant compromise in the resistance to ampicillin. Also, the Leu169Pro substitution by itself has not previously been shown to be able to confer such a phenotype, and our results support previous data showing the importance of residue 169 in ceftazidime and ampicillin resistance. On the other hand, poor levels of expression of TEM  $\beta$ -lactamases with substitutions at positions 161 to 170 could prevent the Leu169Pro mutation from appearing in clinical isolates (26).

Although mutant 1 and all its derivatives (mutants 2 to 6) produce high levels of resistance to ceftazidime and significant levels of resistance to ceftazidime in the presence of clavulanic acid, only mutant 6 conferred a high level of resistance to the combination ampicillin and clavulanic acid (Table 2). Analysis of the amino acid sequences of mutants 1 to 6 showed that they all possess the Glu104Lys substitution, which provides only an insignificant increase in the level of resistance to ceftazidime by itself. Mutants 1 to 5, in addition, harbor at least one of the four other mutations (Pro167Ser, Leu169Pro, Gly238Ser,

Glu240Gly) that are able, by themselves, to produce high-level resistance to ceftazidime. Analysis of data from various studies indicates that combinations of such mutations that produce an ESBL phenotype with high-level resistance with mutations that produce the IRT phenotype results in complex enzymes that are able to express only one of these phenotypes (4, 22, 37). Similarly, mutants 1 to 5 produce resistance only to ceftazidime. Among the amino acid substitutions that are present in the enzyme with the triple mutation, Glu104Lys is the only one that is known to produce the ESBL phenotype, and Asn276Asp is the only one that produces the IRT phenotype. While the  $\beta$ -lactam- $\beta$ -lactamase inhibitor resistance produced by the Asn276Asp substitution alone is clinically important, the Glu104Lys substitution produces only a low level of ceftazidime resistance. The other two replacements, Ser268Gly and Asn276Asp, which are neutral by themselves with respect to ceftazidime resistance, produce increases in the ceftazidime MICs when they are individually combined with Glu104Lys and especially produce increases in the ceftazidime MICs when they are both combined with Glu104Lys to produce the enzyme with the triple mutation. The enzyme with the quintuple mutation, which has two additional amino acid substitutions, Ala42Gly and Ile47Val, conferred another twofold rise in the MICs of ampicillin, piperacillin, and ceftazidime. The roles of these additional mutations merit further exploration.

In the presence of clavulanic acid (2  $\mu\text{g}/\text{ml}$ ), the ceftazidime MICs conferred by the enzyme with the triple mutation were lower than those in the absence of the inhibitor but were still eight times higher than that for the TEM-1-producing *E. coli* strain. Thus, the presence of clavulanic acid produced a relative decline of only one dilution in the ratios of the ceftazidime MICs conferred by the enzyme with the triple mutation to that conferred by TEM-1. In addition, the enzyme with the triple mutation produced higher ampicillin-clavulanic acid and piperacillin-tazobactam MICs, as well as higher ampicillin-tazobactam and ceftazidime-tazobactam MICs (Table 4).

On the enzymologic level, the increased catalytic efficiency of the enzyme with the triple mutation for ceftazidime in comparison with that of TEM-1 was consistent with the increase in the ceftazidime MIC. However, the decline in the catalytic efficiency of this enzyme for ampicillin was not reflected in the MICs of ampicillin conferred by the two enzymes. These differences could in part be explained by the higher level of expression of the enzyme with the triple mutation, as enzyme stability does not seem to be an issue in this case. Also, susceptibility testing with the conventional twofold dilutions might not reveal small differences. The increases in the  $K_i$  values of both clavulanic acid and tazobactam for the  $\beta$ -lactamase with the triple mutation are consistent with the observed resistance to the reversibility of  $\beta$ -lactam resistance with these inhibitors

in cell cultures. The partition ratios of the enzyme with the triple mutation with both clavulanic acid and tazobactam were greater than those of TEM-1, although the increase for tazobactam was quite modest.

Three clinical isolates (16, 29, 35) and several laboratory mutants (22, 37) that produce the so-called complex  $\beta$ -lactamases have been described to date. These complex enzymes possess, in various combinations, both the amino acid substitutions that produce extended-spectrum antibiotic and inhibitor resistance. All resultant mutant  $\beta$ -lactamases, with one exception, produce only one of the two resistance phenotypes. The only enzyme that conferred both the ESBL and the IRT phenotypes had the following combination of amino acid substitutions: Arg164Ser, Met69Leu, and Asn276Asp. This complex  $\beta$ -lactamase produces significant resistance to ceftazidime and, to some extent, to inhibition by clavulanic acid. At the same time the enzyme became susceptible to inhibition by tazobactam. Our enzyme with the triple mutation that also possesses the Asn276Asp substitution confers broad resistance to  $\beta$ -lactamase inhibitors.

Thus, despite the appearance of numerous ESBL- and IRT-producing bacteria and several microorganisms that produce complex  $\beta$ -lactamases, no clinical isolates that express both of these phenotypes (i.e., resistance to both expanded-spectrum cephalosporins and penicillins in combination with the  $\beta$ -lactamase inhibitors) at clinically relevant levels have been reported. Our results indicate that as few as three mutations in the common TEM-1  $\beta$ -lactamase are sufficient to produce this phenotype. The eventual appearance of such resistance in clinical isolates, especially in strains with decreased cell wall permeability, could compromise the utility of  $\beta$ -lactamase inhibitors to reverse this increasingly common type of resistance to extended-spectrum  $\beta$ -lactams. On the other hand, the construction of such mutant enzymes provides an opportunity to carry out structural studies to provide a greater understanding of the interaction between the mutations that account for the ESBL and IRT phenotypes in class A  $\beta$ -lactamases.

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