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Construction and Characterization of Mutants of the TEM-1 β-Lactamase Containing Amino Acid Substitutions Associated with both Extended-Spectrum Resistance and Resistance to β-Lactamase Inhibitors

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Extended-spectrum TEM β-lactamases (ESBLs) do not usually confer resistance to β-lactamase inhibitors such as clavulanate or tazobactam. To investigate the compatibility of the two phenotypes we used site-directed mutagenesis of the bla_{TEM-1} gene to introduce into the TEM-1 β-lactamase amino acid substitutions that confer the ESBL phenotype: TEM-12 (Arg164→Ser), TEM-26 (Arg164→Ser plus Glu104→Lys), TEM-19 (Gly238→Ser), and TEM-15 (Gly238→Ser plus Glu104→Lys). These were combined with three sets of substitutions that confer inhibitor resistance: TEM-31 (Arg244→Cys), TEM-33 (Met69→Leu), and TEM-35 (Met69→Leu and Asn276→Asp). Introduction of the Arg244→Cys substitution gave rise to inhibitor-resistant hybrid enzymes that either lost ESBL activity (TEM-12, TEM-15, and TEM-19) or had reduced activity (TEM-26) against ceftazidime. In contrast, the introduction of Met69→Leu or Met69→Leu plus Asn276→Asp substitutions did not significantly affect the abilities of the enzymes to confer resistance to ceftazidime, although increased susceptibility to cefotaxime was observed with *Escherichia coli* strains that expressed the TEM-19 and TEM-26 β-lactamases. With the exception of the TEM-12 β-lactamase, introduction of the Met69→Leu substitution did not give rise to enzymes with increased resistance to clavulanate compared to that of the TEM-1 β-lactamase. However, introduction of the double substitution Met69→Leu plus Asn276→Asp in the ESBLs did give rise to low-level (TEM-19, TEM-15, and TEM-26) or moderate-level (TEM-12) clavulanate resistance. None of the hybrid enzymes were as resistant to clavulanate as the corresponding inhibitor-resistant TEM β-lactamase mutant, suggesting that active-site configuration in the ESBLs limits the degree of clavulanate resistance conferred.

Gram-negative bacteria may exhibit reduced susceptibility to β-lactam antibiotics by a number of mechanisms including reduced outer membrane permeability, target-site modification, and efflux of the β-lactam out of the cell (20, 23). However, by far the most common mechanism of resistance is the enzymatic inactivation of the β-lactam by a β-lactamase (18). There are many types of β-lactamases, which have been classified by their amino acid sequences and corresponding substrate profiles (6). The TEM-1 β-lactamase belongs to a functional group of broad-spectrum enzymes that are inhibited by clavulanate (6). This group includes enzymes such as the SHV-1 and OHIO-1 β-lactamases. Although the TEM-1 β-lactamase does not usually provide protection against extended-spectrum cephalosporins such as ceftazidime and cefotaxime or β-lactamase inhibitors like clavulanate and tazobactam (except in the case of TEM-1 overproduction), amino acid substitutions can alter the hydrolytic spectrum of the β-lactamase to encompass these compounds.

Extended-spectrum TEM β-lactamases (ESBLs) do not usually confer resistance to β-lactamase inhibitors, suggesting that the two phenotypes may be incompatible. In support of this suggestion, Imtiaz et al. (15) have shown that introduction of an amino acid substitution (Arg164→Ser) that confers on the TEM-1 β-lactamase the ability to efficiently hydrolyze ceftazidime leads to the loss of clavulanate resistance when introduced into the inhibitor-resistant β-lactamase TEM-31. However, recently a clinical *Escherichia coli* isolate that expressed a β-lactamase, TEM-50 (CMT-1), that conferred low-level resistance both to β-lactamase inhibitors and to extended-spectrum cephalosporins has been reported (22).

In order to investigate this phenomenon further we used site-directed mutagenesis of the TEM β-lactamase encoding gene to introduce into ESBLs amino acid substitutions known to confer inhibitor resistance. We found that the different amino acid substitutions gave rise to enzymes that conferred different resistance phenotypes. None of the substitutions conferred high-level resistance to both β-lactamase inhibitors and extended-spectrum cephalosporins, although the double amino acid substitution (Met69→Leu, Asn276→Asp) in the TEM-12 β-lactamase did give rise to an ESBL with a moderate level of clavulanate resistance.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* CJ236 [*dut-1 ung-1 thi-1 relA1*; pCJ105 (Cm^r)] and *E. coli* MV1190 [Δ(*lac-proAB*) *thi supE* Δ(*srl-recA*)*306*::Tn10(Tet^r); (F′:*traD36 proAB lacI*^{q}ZΔM15)] were used in this study. The plasmid vector pTZ18U was used as the initial source of the bla_{TEM} gene. All bacteria were grown in Luria-Bertani (LB) broth or on LB agar (Oxoid, Basingstoke, United Kingdom) containing the appropriate antibiotic (chloramphenicol, 20 μg/ml; amoxicillin, 100 μg/ml; or tetracycline, 10 μg/ml).

Antibiotics and reagents. The following companies kindly supplied antibiotic powders of known potencies: Bristol Meyers Squibb (cefepime and aztreonam); American Cyanamid (piperacillin and tetracycline); Glaxo Group Research Ltd. (ceftazidime and cephaloridine); Roussel Laboratories Ltd. (cefotaxime and chloramphenicol); and SmithKline Beecham (amoxicillin, clavulanate, temocillin, and ticarcillin). Nitrocefin was obtained from Oxoid.

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TABLE 1. Oligonucleotides used in site-directed mutagenesis experiments and in DNA sequencing

Procedure and oligonucleotide	Sequence (5′-3′)[a]	Codon change and nucleotide position[b]
Mutagenesis		
Ile86→Val	GGCGTCAACACGGGATAAT	ATT→GTT
Val184→Ala	ATTGCGGCAGGCATCGTGG	GTA→GCC
Met69→Leu	TAAAAGTGCTCAGCATTGGAAAAC	ATG→CTG
Asn276→Asp	TCTGTCTATCTCGTTCATCC	AAT→GAT
Arg244→Cys	ATGATACCGCAAGACCC	CGC→TGC
Glu104→Lys	GTGAGTATTTAACCAAGTC	GAG→AAA
Gly238→Ser	CACGCTCACTGGCTCCAGATTTAT	GGT→AGT
Arg164→Ser	GTTCCCAAGAATCAAGGC	CGT→TCT
Sequencing		
f-TEM2F	GTATGAGTATTCAACATTTCCGTGTCG	205-231
f-TEM2R	ACCAATGCTTAATCAGTGAGGCA	1064-1042
f-TEMi	ACTGTCATGCCATCCGTAAGA	556-536
f-TEM2i	CTGCGGCCAACTTACTTCTGACAA	598-621

[a] Altered bases are underlined.
[b] Nucleotide positions are according to Sutcliffe (27).

Susceptibility testing. MICs were determined by agar dilution on Diagnostic Sensitivity Test Agar (CM261; Oxoid) with an inoculum of about 10^4 organisms per spot as described previously (24). *E. coli* NCTC 10418 was used as the control strain.

Site-directed mutagenesis. Site-directed mutagenesis was performed with the reagents contained within the Muta-Gene Phagemid In Vitro Mutagenesis kit (version 2) from Bio-Rad (Hemel Hempstead, United Kingdom). The procedures used in this kit are based on the method originally described by Kunkel et al. (17). Oligonucleotides were designed with the aid of oligonucleotide design software (PrimerSelect; DNAStar) and were based on the sequence of the bla_{TEM-1} gene reported by Sutcliffe (27). The oligonucleotides were custom made by Pharmacia Biotech (St. Albans, United Kingdom) (Table 1).

DNA sequencing. In order to confirm that mutations had been introduced, plasmid DNA was extracted with a Qiagen QIAprep kit (Qiagen Ltd., Crawley, United Kingdom) and was sequenced in both directions with fluorescein-labelled primers (Table 1). DNA sequencing was performed with the reagents contained in a cycle sequencing kit (RPN 2438) from Amersham Life Sciences (Little Chalfont, United Kingdom) by following the manufacturer's instructions. The annealing temperature for the cycle sequencing reactions was 60°C, and the DNA sequence was determined with an automated DNA sequencer (Pharmacia Biotech).

Determination of IC_{50}s. Each strain was grown at 37°C in brain heart infusion broth (Oxoid) for 16 h, with shaking (200 rpm). The cells were harvested by centrifugation and were resuspended in 0.5 ml of sterile distilled water, and the β-lactamase was released by sonication. Sonication was performed for 20 s with a W-385 sonicator (Heat Systems; Ultrasonics, Inc., Farmingdale, N.Y.) with the following settings: 5-s cycle time, 50% duty cycle, and a 1.5 output control setting. β-Lactamase activity was measured by monitoring the rate of nitrocefin hydrolysis (10 μM) at 482 nm in a Biochrom 4060 spectrophotometer (Pharmacia Biotech). All assays were performed in 0.1 M phosphate buffer (pH 7.0) and at 37°C. In order to take into account the different levels of β-lactamase activities within the samples the activity of each sample was standardized to give an absorbance change of 0.15 per min. Samples were preincubated for 10 min at 37°C with various concentrations (0.01 to 50 μM) of the β-lactamase inhibitor before the β-lactamase activity was determined with nitrocefin (10 μM) as the reporter substrate. The concentration of β-lactamase inhibitor required to inhibit 50% of the β-lactamase activity (IC_{50}) was then determined graphically.

RESULTS

Mutagenesis. Phagemid pTZ18U conveniently encodes a bla_{TEM} gene, and use of pTZ18U thus negates the need to subclone the bla_{TEM} gene from another source. However, the bla_{TEM} gene from pTZ18U is not identical to bla_{TEM-1} as the result of two nucleotide changes, G^{244}→A and C^{545}→T, that were introduced to remove *Pst*I and *Hinc*II restriction sites, respectively. While the resulting amino acid substitutions, Ile84→Val and Ala184→Val, have been regarded as neutral (22a), Chaibi et al. (8) have demonstrated that the catalytic efficiency of the "artificial" TEM β-lactamase was one-half to one-third lower than that of the TEM-1 β-lactamase. Consequently, in this study we initially converted the artificial bla_{TEM} into bla_{TEM-1} and subsequently used this gene as the template for the construction of the TEM mutants. Four ESBL enzymes (TEM-12, TEM-15, TEM-19, and TEM-26) were constructed together with three β-lactamase-inhibitor-resistant mutants (TEM-31, TEM-33, and TEM-35) (Table 2). In order to investigate whether the amino acid substitutions found in β-lactamase inhibitor-resistant mutants could confer inhibitor resistance if introduced into ESBL enzymes, the three sets of amino acid substitutions that confer inhibitor resistance were engineered into the extended-spectrum antibiotic-resistant TEM β-lactamases by altering the gene-coding sequence. The amino acid substitutions corresponded to those found in the TEM-31 (Arg244→Cys), TEM-33 (Met69→Leu), and TEM-35 (Met69→Leu and Asn276→Asp) β-lactamases. In all cases the introduced nucleotide changes in the bla_{TEM} gene were confirmed by DNA sequencing.

Phenotypic characterization of TEM-1 β-lactamase and mutant derivatives. (i) TEM-1 and ESBL enzymes. The MICs of ampicillin and ticarcillin in the presence of clavulanate (2 μg/ml) and piperacillin in the presence of tazobactam (4 μg/ml) for *E. coli* MV1190 expressing the TEM-1 β-lactamase were relatively high (Table 2). This could be accounted for by the large quantity of the TEM-1 β-lactamase expressed as a result of the high copy number of the pTZ18U plasmid carrying the bla_{TEM-1} gene (Table 3). Despite this, because the TEM-1 β-lactamase and the mutant enzymes in this study shared the same genetic background, comparisons between the mutant enzymes and the TEM-1 β-lactamase could still be made.

The TEM-12, TEM-15, and TEM-26 β-lactamases were found to confer 16- to 128-fold higher levels of resistance to ceftazidime than the TEM-1 β-lactamase, confirming that these enzymes were indeed ESBLs (Table 2). Although the TEM-19 β-lactamase did not confer increased levels of resistance to ceftazidime, a 16-fold increase in the level of resistance to cefotaxime was observed. Cefepime was found to be less effective against *E. coli* MV1190 strains that expressed the TEM-12 and TEM-26 β-lactamases, and with the exception of TEM-19, the ESBLs conferred higher levels of resistance to aztreonam than the TEM-1 β-lactamase did. *E. coli* MV1190 expressing either of the four ESBL enzymes was found to be more susceptible to the penicillin–β-lactamase inhibitor combinations than *E. coli* MV1190 expressing the TEM-1 β-lactamase. None of the ESBL enzymes conferred increased resistance to temocillin. Measurement of the β-lactamase activities

TABLE 2. MICs of β-lactams and β-lactamase inhibitor combinations for *E. coli* MV1190 producing the TEM-1 and mutant TEM β-lactamases

Strain or amino acid changes from TEM-1[a]	Designation	MIC (μg/ml)[b]												
		AMX	AMX + CLA[c]	TIC	TIC + CLA[c]	PIP	PIP + TZB[d]	CLD	CTX	CAZ	CAZ + CLA[c]	FEP	AZM	TEM
TEM-1[e]	TEM-1	>1,024	256	>1,024	256	128	32	64	0.06	0.5	0.25	0.12	0.25	4
M69L	TEM-33	>1,024	>1,024	>1,024	1,024	128	64	16	0.06	0.5	0.25	≤0.06	0.25	4
M69L, N276D	TEM-35	>1,024	>1,024	1,024	512	128	32	16	0.06	0.25	0.25	≤0.06	0.12	4
R244C	TEM-31	1,024	512	64	64	8	4	2	0.06	0.25	0.12	≤0.06	0.12	4
G238S	TEM-19	>1,024	16	1,024	16	32	≤1	32	1	0.5	0.25	0.12	0.5	4
G238S, M69L		>1,024	32	512	16	32	≤1	32	0.25	0.25	0.25	0.12	0.25	8
G238S, M69L, N276D		>1,024	128	512	32	32	≤1	32	0.12	0.25	0.25	0.25	0.12	4
G238S, R244C		1,024	128	64	16	8	2	4	0.06	0.25	0.25	≤0.06	0.12	4
G238S, E104K	TEM-15	>1,024	16	1,024	32	32	≤1	32	8	8	0.25	0.25	4	8
G238S, E104K, M69L		>1,024	32	1,024	64	32	≤1	32	4	4	0.25	0.25	1	4
G238S, E104K, M69L, N276D	TEM-50	>1,024	128	1,024	128	32	≤1	32	4	4	0.5	0.5	2	4
G238S, E104K, R244C		1,024	64	256	64	8	≤1	4	0.06	0.25	0.25	0.25	1	4
R164S	TEM-12	>1,024	32	1,024	32	64	2	32	0.12	8	0.25	1	1	8
R164S, M69L		>1,024	256	512	64	32	≤1	8	0.12	8	0.25	0.5	1	4
R164S, M69L, N276D		>1,024	1,024	512	128	32	2	8	0.06	8	2	0.5	0.5	4
R164S, R244C		1,024	256	16	8	4	2	2	0.06	0.25	0.25	≤0.06	0.12	4
R164S, E104K	TEM-26	>1,024	16	>1,024	32	64	≤1	8	1	64	0.5	1	16	8
R164S, E104K, M69L		>1,024	64	1,024	64	64	2	8	0.5	64	2	1	16	4
R164S, E104K, M69L, N276D		>1,024	256	512	128	32	2	8	0.12	64	8	1	4	4
R164S, E104K, R244C		512	64	128	32	32	8	2	0.06	4	1	0.25	1	8
E. coli MV1190 (recipient strain)		8	4	2	2	≤1	≤1	2	0.06	0.12	0.12	≤0.06	0.12	4

[a] M69L refers to an amino acid substitution of leucine for methionine at position 69 in the TEM-1 β-lactamase protein sequence. The single amino acid codes for the other substitutions are as follows: N, asparagine; D, aspartate; G, glycine; R, arginine; C, cysteine; S, serine; E, glutamate; and K, lysine.

[b] AMX, amoxicillin; CLA, clavulanate; TIC, ticarcillin; PIP, piperacillin; TZB, tazobactam; CLD, cephaloridine; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; AZM, aztreonam; TEM, temocillin.

[c] Fixed concentration of clavulanate (2 μg/ml).

[d] Fixed concentration of tazobactam (4 μg/ml).

[e] TEM with amino acid modifications engineered to be identical to natural TEM-1.

TABLE 3. β-Lactamase activities and clavulanate and tazobactam IC_{50}s for TEM-1 β-lactamase and TEM mutant enzymes

Amino acid change from TEM-1[a]	Designation	β-Lactamase activity[b]	IC_{50} (μM)	
			Clavulanate	Tazobactam
TEM-1	TEM-1	5,330	0.08	0.03
M69L	TEM-33	586	2	0.5
M69L, N276D	TEM-35	1,190	12	0.6
R244C	TEM-31	514	10	1.7
G238S	TEM-19	2,950	0.001	0.002
G238S, M69L		853	0.03	0.02
G238S, M69L, N276D		2,360	0.38	0.04
G238S, R244C		485	1.5	0.3
G238S, E104K	TEM-15	29	0.002	0.004
G238S, E104K, M69L		319	0.07	0.01
G238S, E104K, M69L, N276D	TEM-50	952	0.3	0.02
G238S, E104K, R244C		696	1.1	1.2
R164S	TEM-12	25	0.02	0.02
R164S, M69L		49	0.25	0.2
R164S, M69L, N276D		1,440	1.8	0.3
R164S, R244C		937	4.5	5
R164S, E104K	TEM-26	523	<0.01	0.06
R164S, E104K, M69L		211	0.08	0.3
R164S, E104K, M69L, N276D		458	0.35	0.2
R164S, E104K, R244C		54	3.5	2

[a] See footnote *a* to Table 2 for a key to the amino acid substitutions.
[b] Activities are expressed as nanomoles of nitrocefin hydrolyzed per minute per milligram of protein.

of the ESBLs with nitrocefin as the reporter substrate indicated that the ESBL enzymes had lower levels of activity against nitrocefin than the TEM-1 β-lactamase; this was also true for the other mutant TEM β-lactamases (Table 3).

(ii) Inhibitor-resistant enzymes. The substitutions Arg244→Cys, Met69→Leu, and Met69→Leu plus Asn276→Asp in the TEM-1 β-lactamase gave rise to enzymes with resistance to clavulanate combined with resistance to amoxicillin or ticarcillin. Substitution of a Cys residue at position 244 of the TEM-1 β-lactamase also resulted in an enzyme (TEM-31) that conferred lower levels of resistance to penicillins and cephaloridine than the levels conferred by TEM-1 (Table 2). In contrast, a single Met69→Leu substitution and a double substitution, Met69→Leu plus Asn276→Asp, in the TEM-1 β-lactamase did not greatly affect the MICs of piperacillin, although a fourfold reduction in resistance to cephaloridine was observed (Table 2). None of the inhibitor-resistant enzymes conferred resistance to extended-spectrum cephalosporins, aztreonam, or temocillin.

(iii) Substitution of Cys for Arg at position 244 in ESBLs. Introduction of the Arg244→Cys substitution into the ESBL enzymes had an effect similar to that in TEM-1. Like the TEM-31 β-lactamase, the resulting hybrid enzymes conferred lower levels of resistance to penicillins and cephaloridine than their respective parent enzymes did. However, the MICs of penicillin-inhibitor combinations were elevated for the strain with TEM-26 plus the Arg244→Cys substitution. In addition, the amino acid substitution resulted in enzymes that either had lost (TEM-12, TEM-15, and TEM-19) or had a reduced ability (TEM-26) to confer resistance to ceftazidime. The MICs of cefepime and aztreonam were reduced 4- and 16-fold, respectively, for *E. coli* MV1190 expressing the hybrid TEM-26 β-lactamase compared to the MICs for the strain expressing the TEM-1 β-lactamase. All the hybrid enzymes with the Arg244→Cys substitution were found to be more resistant to clavulanate and tazobactam inhibition than the TEM-1 β-lactamase, with the IC_{50}s for the hybrid enzymes being comparable to those for the naturally occurring inhibitor-resistant TEM β-lactamases (Table 3).

(iv) Substitution of Leu for Met at position 69 in ESBLs. The IC_{50}s of clavulanate for the parental ESBL enzymes were lower than those of the TEM-1 β-lactamase, indicating that the ESBL enzymes were more susceptible to clavulanate inhibition than the TEM-1 β-lactamase. Introduction of a Leu residue at position 69 in the ESBLs resulted in hybrid enzymes that conferred increased levels of resistance to both clavulanate and tazobactam compared to the level of resistance conferred by their respective parental ESBL enzymes (Table 3). In the case of the TEM-12 β-lactamase, the amino acid substitution gave rise to a hybrid enzyme that was less susceptible to clavulanate inhibition than the TEM-1 β-lactamase. For the other ESBLs, however, the substitution resulted in hybrid enzymes for which clavulanate IC_{50}s were similar to those for the TEM-1 β-lactamase. Tazobactam was found to be equally effective against the TEM-1 β-lactamase and the hybrid ESBLs with a Gly238→Ser substitution (TEM-15 and TEM-19). However, tazobactam was less effective against hybrid ESBLs with the Arg164→Ser substitution (TEM-12 and TEM-26) (Table 3).

In contrast to the Arg244→Cys substitution, introduction of a Leu residue at position 69 in the ESBLs resulted in hybrid enzymes that retained the ability to confer resistance to ceftazidime and, in the case of the TEM-12 hybrid enzyme, that had increased levels of resistance to ceftazidime in combination with clavulanate. However, the amino acid substitution in the TEM-19 β-lactamase gave rise to a hybrid enzyme that conferred a lower level of resistance to cefotaxime than the parent enzyme did.

(v) Substitution of Leu for Met at position 69 and Asp for Asn at position 276 in ESBLs. Introduction of the double amino acid substitution Leu-69 and Asp-276 in the ESBLs gave rise to hybrid enzymes that were more resistant to clavulanate inhibition than hybrid ESBL enzymes with a single Leu-69 substitution. In the case of the TEM-12 β-lactamase, this gave rise to an enzyme for which the IC_{50} of clavulanate was similar to that for the inhibitor-resistant enzyme TEM-33. For the other hybrid ESBLs, however, the IC_{50}s of clavulanate were intermediate between that for the TEM-1 β-lactamase and those for the inhibitor-resistant enzymes. The IC_{50}s of tazobactam were similar for the hybrid enzymes with single or double amino acid substitutions. The double substitution did not greatly affect the ability of the enzymes to confer resistance to ceftazidime, although increased susceptibility to cefotaxime was apparent with *E. coli* MV1190 expressing the hybrid derivatives of the TEM-19 and TEM-26 β-lactamases. Two of the hybrid enzymes, TEM-12 and TEM-26, showed a markedly reduced susceptibility to ceftazidime combined with their susceptibility to clavulanate. As a consequence of the double amino acid substitution, extended-spectrum resistant variants of TEM-12, TEM-15, and TEM-26 β-lactamases that also conferred increased levels of resistance to β-lactamase inhibitors were constructed. However, the levels of clavulanate resistance

conferred by the hybrid ESBLs were not as high as that conferred by the corresponding inhibitor-resistant TEM β-lactamase TEM-35.

DISCUSSION

Substitution of Cys for Arg at position 244. In this study we replaced the Arg at position 244 in the TEM-12, TEM-15, TEM-19, and TEM-26 β-lactamases with a Cys residue in order to investigate whether the amino acid substitution would give rise to inhibitor-resistant ESBLs. In each case the substitution conferred increased levels of resistance to β-lactamase inhibitors, but the substitution also gave rise to enzymes that conferred lower degrees of resistance to penicillin and cephalosporins. Both these observations are consistent with a disruption of the Arg244 hydrogen-bonding arrangement predicted to occur in the TEM-1 β-lactamase (26, 30). Since the Cys residue at position 244 would be unable to form a hydrogen bond to the common carboxylate group of β-lactam antibiotics, this probably explains why the MICs of both penicillins and cephalosporins were affected by the amino acid substitution. This would be especially pertinent if, as suggested by Zafaralla et al. (30), the binding energy of Arg244 is used to lower the activation energy of the hydrolytic reaction.

The resistance to β-lactamase inhibitors conferred by the hybrid enzymes in this study is understandable in light of the essential role that the Arg244 residue plays in maintaining in position the water molecule (Wat399) believed to be important in the inactivation of β-lactamase by clavulanate (14, 28). In naturally occurring variants of the TEM-1 and TEM-2 β-lactamases, as in our mutants, substitution of Cys, Ser, or His residues at position 244 has given rise to inhibitor-resistant enzymes (1, 3, 4, 29). The shorter side chains of the substituted amino acids in the inhibitor-resistant variants are thought to be unable to form a hydrogen bond with Wat399, which is displaced as a consequence and which is unable to act as a proton source in the inactivation process (14, 16, 19). However, our results contrast with those of Imtiaz et al. (15), who reported that a substitution of a Ser for Arg at position 244 in the TEM-12 β-lactamase (also derived from TEM-1) neither conferred inhibitor resistance nor significantly affected the enzyme's ability to hydrolyze ceftazidime. Why the two different amino acid substitutions gave rise to two different effects is not clear. Imtiaz et al. (15) have suggested that an alteration of the topology of the active site that is caused by the Arg164→Ser substitution in the TEM-12 β-lactamase may have resulted in a different clavulanate binding arrangement that promoted a repositioning of the water molecule close to the site of inactivation. Consistent with this suggestion we found that the four ESBLs in this study were more sensitive to clavulanate inhibition than the TEM-1 β-lactamase. If a different clavulanate binding arrangement does occur in the hybrid enzymes, the results of this study show that the nature of the residue at position 244 is still important in dictating whether the enzyme is resistant to clavulanate or not. Thus, it would appear that the Ser residue, but not the Cys residue, either performs a role similar to that of the Arg residue in the hybrid enzymes or, through structural rearrangement, promotes another residue to perform a similar function.

Substitution of Leu for Met at position 69. Unlike the TEM-1 β-lactamase, in which substitutions of Leu, Val, or Ile for the Met at position 69 have all given rise to inhibitor-resistant enzymes (9, 11, 25, 31), substitution of a Leu residue for the Met residue at this position in the four ESBLs did not give rise to clavulanate-resistant enzymes. This probably can be explained by a different binding arrangement of the clavulanate molecule in the active site of the hybrid ESBL enzymes compared to that in the TEM-1 β-lactamase. As noted previously the parental ESBLs were more sensitive to clavulanate inhibition than the TEM-1 β-lactamase, suggesting that alterations within the active site enhanced the inhibitory action of clavulanate. Although the hybrid ESBLs were not resistant to clavulanate, they were less sensitive to clavulanate inhibition than their respective parent enzymes were. The substitutions at position 69 are thought to cause slight alterations to the active-site structure of the TEM-1 β-lactamase, resulting in deformation of the oxyanion hole and a less favorable binding orientation of the clavulanate molecule (10). This suggests that the clavulanate molecule still interacts with the oxyanion hole but possibly in a different manner.

Substitutions of Met69→Ile or Met69→Val in the SHV-5 β-lactamase and Met69→Ile in an OHIO-1 β-lactamase mutant bearing a Gly238→Ser substitution have all given rise to enzymes that were less susceptible to inhibition by clavulanate than their respective parent enzymes (2, 12). These mutant enzymes exhibited reduced penicillinase activity and, in the case of the SHV enzymes, a reduced ability to hydrolyze cephalothin and cefotaxime (2, 12). In contrast, substitution of a Leu residue at position 69 in the extended-spectrum TEM β-lactamases in this study did not significantly affect the ability of the enzymes to confer resistance to penicillins and ceftazidime, although reduced levels of resistance to cefotaxime were noted with the TEM-19 hybrid enzyme. These variations may or may not be related to the different nature of the substituted residues in each case. While all three residues may exert a hydrophobic effect, only the branched residues Val and Ile are thought to produce additional steric constraints. The smaller impact of the Leu69 substitution on the structure of the TEM-32 β-lactamase has been used to explain the lower clavulanate K_i value for this enzyme compared with that for the TEM-1 β-lactamase with Met69→Ile or Met69→Val substitutions (9). Whether the substitution of Val or Ile into position 69 of the ESBLs examined in this study would give rise to hybrid enzymes with greater degrees of clavulanate resistance has yet to be determined. However, in light of the reduced penicillinase activity of the SHV and OHIO enzymes, a similar reduction in resistance to penicillins and possibly cephalosporins may also be observed.

Substitution of Asp for Asn at position 276 plus Leu for Met at position 69. Amino acid substitutions at position 276 have been found naturally only in combination with changes at position 69 in the TEM β-lactamase (5, 13, 22, 31), although the change can confer inhibitor resistance in the absence of a substitution at position 69 (7, 21, 28). Recently, Sirot et al. (22) have reported on a natural variant of the TEM-15 β-lactamase, designated TEM-50, with amino acid substitutions, Met69→Leu and Asn276→Asp, found in the inhibitor-resistant β-lactamase TEM-35. An *E. coli* strain expressing the TEM-50 β-lactamase displayed susceptibilities to β-lactams, including ceftazidime and cefotaxime, that were between those for strains expressing the TEM-15 or TEM-35 β-lactamases. In our study we artificially constructed the TEM-50 β-lactamase together with mutants of the TEM-12, TEM-19, and TEM-26 β-lactamases. In contrast to Sirot et al. (22), we found that the MIC of ceftazidime for *E. coli* MV1190 expressing the TEM-50 β-lactamase was only twofold lower than the MIC of ceftazidime for the same strain expressing the TEM-15 β-lactamase. A possible explanation for this difference may have been the exceptionally high level of β-lactamase expressed from the high-copy-number plasmid pTZ18U harboring the TEM-coding gene used in this study. Such high levels of β-lactamase expression may have

masked small differences in hydrolytic activities between the enzymes.

In agreement with Sirot et al. (22), we found that the TEM-50 β-lactamase conferred low levels of resistance to clavulanate. We also demonstrated that when the double amino acid substitutions were introduced into the TEM-19, TEM-12, and TEM-26 β-lactamases the resulting enzymes also conferred increased levels of resistance to clavulanate and, in the case of the TEM-12 and TEM-26 derivatives, retained ceftazidime resistance. Indeed, these two hybrid mutants showed considerably reduced levels of susceptibility to the ceftazidime-clavulanate combination. Previous studies have shown that clavulanate is more potent against strains that produce inhibitor-resistant TEM β-lactamases with a single substitution (Asn276→Asp) than against those that have double substitutions (Met69→Leu and Asn276→Asp) (5, 7). Similarly, we demonstrate that double substitutions within the four ESBLs in this study also resulted in hybrid enzymes that conferred greater resistance to clavulanate than the levels of resistance conferred by those with the single Met69→Leu substitution. Furthermore, consistent with the study of Caniça et al. (7) on inhibitor-resistant TEM β-lactamases, we found tazobactam to be more potent than clavulanate against strains producing inhibitor-resistant enzymes with double substitutions. Thus, there appears to be a correlation between the inhibitor resistance phenotypes conferred by the single (Met69→Leu) and double (Met69→Leu and Asn276→Asp) substitutions in the TEM-1 β-lactamase and those conferred by the same substitutions in the extended-spectrum TEM β-lactamases.

In conclusion, of the hybrid enzymes constructed, the hybrid of the TEM-12 β-lactamase conferred the greatest reduction in sensitivity to clavulanate while it retained the ability to confer resistance to ceftazidime. As with all the hybrid enzymes, including those with the Arg244→Cys substitutions, the level of resistance to penicillin-clavulanate combinations that was conferred (Table 2) and the reduction in the degree of sensitivity to inhibition by clavulanate (Table 3) were not as high as those for equivalent inhibitor-resistant TEM β-lactamases. This suggests that the altered active-site configuration in the ESBL enzymes limits the degree of clavulanate resistance conferred by the ESBL-inhibitor hybrid enzymes. Whether this is due to a different binding arrangement of the clavulanate molecule in the active site of the extended-spectrum TEM β-lactamases or some other factor has yet to be determined.

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