

# Phenotypic Study of Resistance of $\beta$ -Lactamase-Inhibitor-Resistant TEM Enzymes Which Differ by Naturally Occurring Variations and by Site-Directed Substitution at Asp<sup>276</sup>

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**At this time an amino acid substitution at position 276 in the TEM-1 enzyme is associated with an additional substitution at position 69 in natural  $\beta$ -lactamase-inhibitor-resistant (IRT)  $\beta$ -lactamases. The effect of the Asn<sup>276</sup>→Asp substitution on resistance was assessed with the Asn276Asp variant, generated by site-directed mutagenesis. The mutant was resistant to  $\beta$ -lactamase inhibitors, but the MICs of amoxicillin combined with clavulanic acid or tazobactam were strikingly different for *E. coli* strains producing the Asn276Asp variant and those producing naturally occurring IRTs with single or double substitutions. The inhibitory effects of clavulanic acid and tazobactam were the same in IRTs with substitutions at position 69 (IRT-5 and IRT-6). The effect of clavulanic acid on the MICs of amoxicillin for the Asn276Asp variant was greater than that of tazobactam. In IRTs with double substitutions, at positions 69 plus 276 (IRT-4, IRT-7, and IRT-8) or 69 plus 275 (IRT-14), tazobactam was a more potent inhibitor than clavulanic acid. The effect of the Asn<sup>276</sup>→Asp substitution on the values of the kinetic constants and the concentration required to inhibit by 50% the hydrolysis of benzylpenicillin confirms that this single mutation is responsible for resistance to  $\beta$ -lactamase inhibitors. Molecular modeling of the Asn276Asp mutant shows that Asp<sup>276</sup> can form two salt bonds with Arg<sup>244</sup> close to the penicillin-binding cavity. The addition of the Asp<sup>276</sup> mutation to that preexisting at position 69 confers a higher selective advantage to bacteria, as shown by the reduction in  $\beta$ -lactamase inhibitor efficiencies of the double variants. Therefore, the emergence of multiple mutations in TEM  $\beta$ -lactamases by virtue of the use of  $\beta$ -lactamase inhibitors increases selection pressure resulting in the convergent evolution of resistant strains.**

The  $\beta$ -lactam antibiotics are the most frequently prescribed antimicrobial agents in clinical practice. The enzymes of the  $\beta$ -lactamase family of gram-negative bacteria play a significant role in the development of resistance to  $\beta$ -lactam antibiotics. The frequent occurrence of  $\beta$ -lactamase genes in readily transmissible plasmids and their possible integration into bacterial chromosomes is of concern in the management of antimicrobial therapy in the community and in hospital centers. The evolution of extended-spectrum resistance is mediated by mutant derivatives of the TEM and SHV  $\beta$ -lactamases (17).

Strains resistant to inhibitors of  $\beta$ -lactamases and containing TEM-derived  $\beta$ -lactamases have been described (2, 3, 5, 7, 12, 19, 39, 42). These  $\beta$ -lactamase-inhibitor-resistant (IRT) TEM  $\beta$ -lactamases are encoded by *bla*<sub>IRT</sub> genes that carry mutations affecting the kinetic properties of the enzymes by altering the binding of both the  $\beta$ -lactams and the  $\beta$ -lactamase inhibitors (24). The structure-function relationships of some of these mutations have been investigated with variants generated in vitro (3, 16, 21, 29, 38). The effect of the sequence variation at position 276 of the TEM-1  $\beta$ -lactamase (Asn<sup>276</sup>→Asp) has been examined by this approach (38) to assess enzyme kinetics

and the resistance pattern in qualitative terms. This substitution has never occurred alone in natural variants and is always accompanied by substitution of the methionine at position 69 (Met<sup>69</sup>) in  $\beta$ -lactamase.

We studied the quantitative effects of the IRT variants with single and double substitutions on resistance to antibiotic-inhibitor combinations. We generated an Asn<sup>276</sup>→Asp variant (hereafter designated Asn276Asp) by site-directed mutagenesis. We compared its phenotypic characteristics with those of various naturally occurring IRTs (IRT-5, IRT-6, IRT-7, and IRT-8 and the reference IRTs IRT-4 [7] and IRT-14 [10]) and the in vitro-generated variants M69L (16) and W165R (33).

(This work was presented in part at the 16th Interdisciplinary Meeting on Anti-Infectious Chemotherapy, Paris, France, 1996 [11].)

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and phage.** The characteristics of the bacterial strains, plasmids, and phage used in this study are given in Table 1. The phagemid pBluescript-II KS(-) (Stratagene Cloning Systems, La Jolla, Calif.), which encodes the TEM-1  $\beta$ -lactamase, was used for site-directed mutagenesis. This phagemid was propagated in *Escherichia coli* RZ1032 to obtain the single-stranded template DNA for site-directed mutagenesis. Mutated DNA (pMBS276D) was introduced into the bacterial host *E. coli* XL-1 Blue, which was then tested for antibiotic susceptibility. The *E. coli* strains producing IRT-5 (P30), IRT-6 (P9), IRT-7 (P11), IRT-8 (P12), IRT-4 (7), and IRT-14 (10) and the control strain expressing the TEM-1 (R111)  $\beta$ -lactamase were used for MIC assays.

**Media and chemicals.** The bacteria used for site-directed mutagenesis were cultured in Luria-Bertani (Gibco BRL, Life Technologies, Paisley, Scotland) and 2× YT media (36). Brain heart infusion (Difco Laboratories, Detroit, Mich.) medium was used to culture bacteria for  $\beta$ -lactamase production. Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) was used for

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TABLE 1. Bacterial strains and plasmids used or studied in this work investigation

Strain, plasmid, or phage	Description, characteristics, or genotype	Reference or source
<i>E. coli</i> strains		
RZ1032	Host for pBluescript; HfrKL16 PO/45 [ <i>lysA</i> (61-62)] <i>dut-1 ung-1 thi-1 relA1</i> Zbd-279::Tn10, <i>supE44</i>	25
XL-1 Blue	Host for pBluescript with mutated $\beta$ -lactamase; <i>recA1 endA1 gyrA96 thi hsdR17</i> ( <i>rk<sup>-</sup>, mk<sup>+</sup></i> ) <i>supE44 relA1 <math>\lambda^-</math>, lac</i> mutant [F' <i>proAB lacI<sup>q</sup>ΔM15 Tn10</i> (Tet <sup>r</sup> )]	8
K-12 J53/R111	Amoxicillin-resistant strain producing TEM-1	27
P9	Clavulanate-resistant clinical strain producing IRT-6	12 and this work
P11, P12, P30	Clavulanate-resistant clinical strains producing IRT-5 (P30), IRT-7 (P11), and IRT-8 (P12)	Cochin Hospital, Paris, France (this work)
Plasmids		
pBluescript II KS(-) (pBSKS)	<i>E. coli</i> high-copy-number cloning vector; Ap <sup>r</sup> <i>ori</i> ColE1, <i>ori</i> M13 phage	GenBank accession no. X52329
pMBS276D	High-copy-number recombinant plasmid encoding <i>bla</i> gene with Asp <sup>276</sup> variation	This work
Phage R408		
	M13 helper phage for packaging Bluescript M13 vectors	35

the MIC assay. The  $\beta$ -lactams used in this study for determination of the MICs and the values of the kinetic constants were those used in previous work (10).

**Plasmid purification and bacterial transformation.** Plasmid DNA was isolated from *E. coli* by the alkaline lysis method (36). Competent *E. coli* cells (XL-1 Blue and RZ1032) were prepared and transformed with plasmid DNA by the calcium chloride method (15). Attempts to transfer the clinical plasmids encoding the *bla*<sub>IRT-5</sub>, *bla*<sub>IRT-6</sub>, *bla*<sub>IRT-7</sub>, and *bla*<sub>IRT-8</sub> genes to *E. coli* were unsuccessful.

**Single-stranded DNA preparation and site-directed mutagenesis of the TEM-1  $\beta$ -lactamase.** We used the numbering of amino acid residues in the  $\beta$ -lactamase sequence proposed by Ambler et al. (1). Single-stranded plasmid DNA was prepared as described previously (36). Site-directed mutagenesis was carried out as described by Kunkel et al. (25) with an oligodeoxyribonucleotide with the intended substitution (underlined): 5'-ATG AAC GAG ATA GAC AGA T-3'. *E. coli* XL-1 Blue colonies containing the plasmid DNA with the mutant *bla* gene were selected for ampicillin and tetracycline resistance (Ap<sup>r</sup> and Tc<sup>r</sup>, respectively).

**Identification and characterization of mutants.** Plasmids were isolated from resistant clones and were tested for the presence of the intended mutation with a diagnostic *Tsp509I* enzyme as described previously (13). Plasmids testing positive were further sequenced (37) to confirm the presence of the expected mutation in the plasmid DNA (pMBS276D).

**MIC assays and purification and IEF of  $\beta$ -lactamases.** Bacterial susceptibility to  $\beta$ -lactams was measured by the agar dilution test as described previously (10). Independent *E. coli* XL-1 Blue clones carrying pMBS276D or TEM-1 were grown overnight at 37°C in a large volume (3 to 5 liters) of brain heart infusion medium with continuous shaking. The preparation of cell-free lysates, enzyme purification (7, 40), reverse-phase high-performance liquid chromatography (34), analytical isoelectric focusing (IEF) (31), and  $\beta$ -lactamase detection (27) were carried out by published procedures. The enzymes used as controls in IEF were TEM-1 (R111) (27), TEM-2 (RP4) (32), IRT-1 (TEM-31), and IRT-2 (TEM-30) (40).

**Kinetic parameter value determinations.** The values of  $K_m$ ,  $K_i$  and  $V_{max}$  and the concentration required to inhibit by 50% the hydrolysis of benzylpenicillin (IC<sub>50</sub>) of the Asn276Asp  $\beta$ -lactamase were estimated at 37°C by computerized microacidimetry at pH 7.0 (26). The values of the kinetic parameters ( $V_{max}$  and  $K_m$ ) were derived by weighted linear regression of these data. If the  $V_{max}$  and  $K_m$  values were very low they were simply not reported or  $K_i$  was measured by competitive inhibition with a reporter substrate, benzylpenicillin (1,000  $\mu$ M), instead (9, 28). Purified  $\beta$ -lactamases were used for the  $k_{cat}$  assay. The quantitative effect of inhibitors on the activities of the wild-type or mutant  $\beta$ -lactamases was assessed by determining the IC<sub>50</sub>. Five concentrations of inhibitors were used: from 10 to 300  $\mu$ M for TEM-1 or 100 to 1,000  $\mu$ M for the mutant (clavulanic acid), from 0.5 to 100  $\mu$ M for TEM-1 or for the mutant (sulbactam), from 25 to 500  $\mu$ M for TEM-1 or for the mutant (tazobactam), and from 2.5 to 10  $\mu$ M for TEM-1 or for the mutant (brobactam). The  $\beta$ -lactamase was preincubated with the inhibitor for 10 min at 37°C in saline buffer at pH 7.0, and the assay was initiated by the addition of 1 mM benzylpenicillin. Inhibition data for clavulanic acid were plotted against the inhibitor: enzyme ratio to determine the partition ratio for inactivation from the extrapolated value for 100% inactivation. One unit of  $\beta$ -lactamase is the amount of enzyme that hydrolyzes 1  $\mu$ mol of benzylpenicillin per min at 37°C under these experimental conditions.

**Molecular modeling.** The crystal structure of the PC1  $\beta$ -lactamase is available from the Protein Data Bank, Brookhaven National Laboratory, Brookhaven, Conn., under the entry 3BLM (20), as the structure of TEM-1  $\beta$ -lactamase (1XPB) (18) and a closely related TEM-1  $\beta$ -lactamase (1BTL) (23). Molecular modeling of the Asp<sup>276</sup> TEM mutant was performed by using the Amber force field (41).

## RESULTS

**Isoelectric point.** The mutant  $\beta$ -lactamase encoded by pMBS276D was shown to have a pI of 5.2 by analytical IEF.

**Susceptibility to  $\beta$ -lactams of *E. coli* producing Asn276Asp  $\beta$ -lactamase.** The MICs for *E. coli* Asn276Asp are presented in Table 2. For *E. coli* XL-1 Blue carrying pMBS276D, the MICs of amoxicillin were eightfold higher and those of ticarcillin and piperacillin fourfold higher than those for the wild-type in the presence of clavulanate. Tazobactam did not reduce the MICs of these  $\beta$ -lactams. Intriguingly, we found a striking difference in the MICs for the TEM-1 encoded by R111 and that encoded by pBSKS in the presence of tazobactam. Both plasmids were thought to encode the wild-type TEM-1 enzyme. The MICs of mecillinam, imipenem, and cephalosporins for the wild-type TEM-1 (pBSKS) and the Asn276Asp variant were similar.

**Effect of inhibitor-amoxicillin combination on IRTs characterized by one or two amino acid substitutions.** In the presence of  $\beta$ -lactamase inhibitors, the MICs of amoxicillin were four-

TABLE 2.  $\beta$ -Lactam MICs for *E. coli* strains

Antibiotic <sup>a</sup>	MIC ( $\mu$ g/ml)			
	K-12 J53 TEM-1 (R111) <sup>b</sup>	XL-1 Blue <sup>c</sup>	XL-1 Blue TEM-1 (pBSKS) <sup>d</sup>	XL-1 Blue Asn276Asp (pMBS276D) <sup>e</sup>
Amx	≥8,192	4	≥8,192	≥8,192
Amx + Clav (2 $\mu$ g/ml)	64	4	256	2,048
Amx + Tazo (2 $\mu$ g/ml)	128	4	4,096	≥8,192
Amx + Tazo (4 $\mu$ g/ml)	64	4	2,048	4,096
Tic	≥8,192	2	≥8,192	≥8,192
Tic + Clav (2 $\mu$ g/ml)	128	2	256	1024
Tic + Tazo (2 $\mu$ g/ml)	256	2	≥8,192	≥8,192
Pip	128	0.5	512	256
Pip + Clav (2 $\mu$ g/ml)	2	0.5	2	8
Pip + Tazo (2 $\mu$ g/ml)	16	0.5	128	128
Mecillinam	8	0.125	64	32
Cephalothin	16	4	32	16
Cefoxitin	4	2	2	2
Cefotaxime	0.03	≤0.015	≤0.015	≤0.015
Imipenem	0.125	0.125	0.125	0.125

<sup>a</sup> Amx, amoxicillin; Tic, ticarcillin; Pip, piperacillin; Clav, clavulanic acid; Tazo, tazobactam.

<sup>b</sup> Strain producing TEM-1 (reference strain).

<sup>c</sup> Susceptible recipient strain.

<sup>d</sup> Strain producing TEM-1.

<sup>e</sup> Strain producing TEM-1 with Asp<sup>276</sup> substitution.

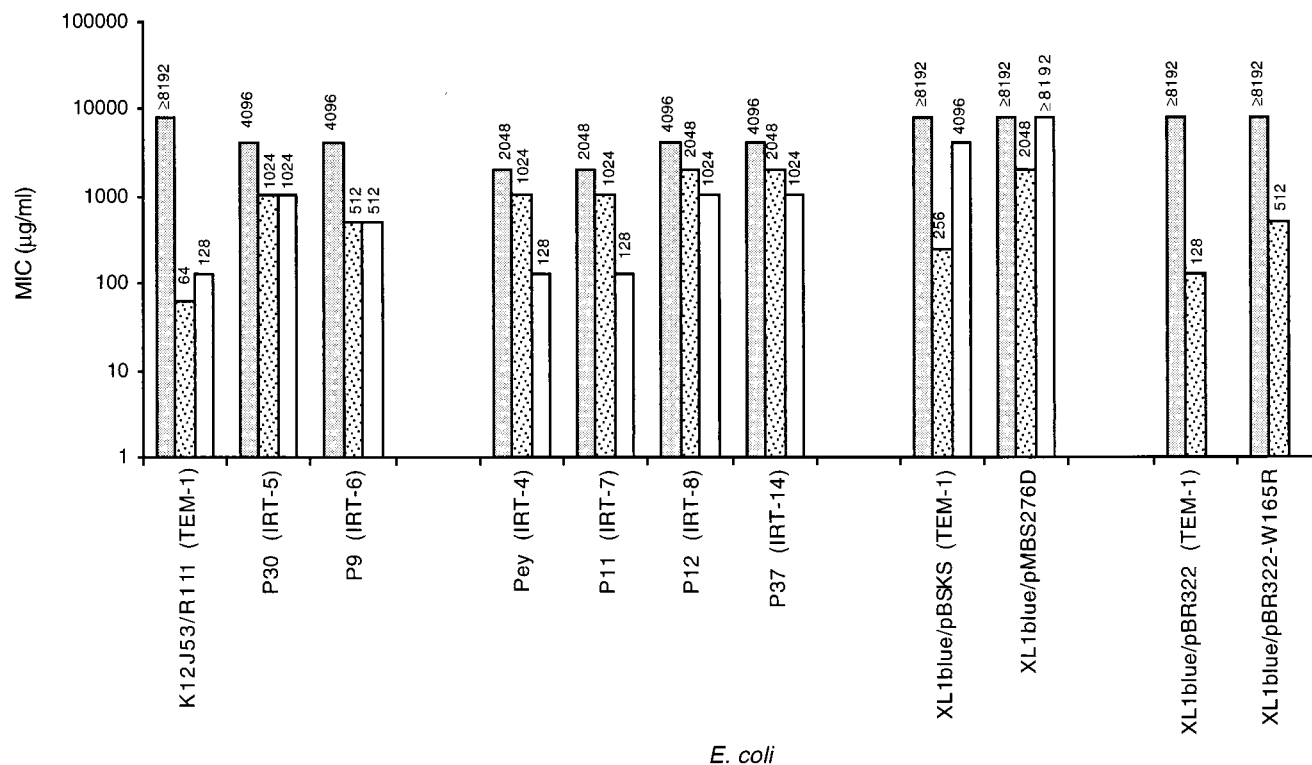


FIG. 1. MICs for *E. coli* producing TEM-1 wild-type enzymes, TEM-1 variant enzymes obtained by site-directed mutagenesis, and natural IRT enzymes. The effects of clavulanate and tazobactam combined with amoxicillin were determined. The following enzymes were used: wild-type TEM-1 enzymes R111 (this study), pBSKS (this study), and pBR322 (33); TEM-1 variants pMBS276D with Asn276Asp (this study) and pBR322 with Trp165Arg (33); natural IRT enzymes (this study) P30 producing IRT-5 (Met69Leu), P9 producing IRT-6 (Met69Val), P11 producing IRT-7 (Met69Val plus Asn276Asp), and P12 producing IRT-8 (Met69Ile plus Asn276Asp); and Pey producing IRT-4 (Met69Leu plus Asn276Asp) (7) and P37 producing IRT-14 (Met69Leu plus Arg275Gln) (10). □, amoxicillin; ▤, amoxicillin plus clavulanate; ▥, amoxicillin plus tazobactam.

fold lower for strains producing IRT-5 and eightfold lower for strains producing IRT-6. The effects of clavulanic acid and tazobactam on these single variants were the same (Fig. 1). The MICs of amoxicillin with clavulanic acid for the double variant IRT-7- and IRT-8-producing strains were reduced by half. In the presence of tazobactam, they were 16-fold lower for strains producing IRT-7 and 4-fold lower for strains producing IRT-8.

**Effects of the Asn<sup>276</sup>→Asp substitution in TEM-1  $\beta$ -lactamase on kinetic parameters.** The  $\beta$ -lactamase mutant Asn276Asp and the wild-type TEM-1  $\beta$ -lactamase (Asn<sup>276</sup>) were produced in *E. coli* with a high-level expression vector. They were extracted from *E. coli* and purified. The specific activities were 5,300 U/mg for the mutant and 2,400 U/mg for TEM-1. The kinetic parameters for the Asn276Asp and TEM-1  $\beta$ -lactamases were determined for 17  $\beta$ -lactams and are summarized in Tables 3 and 4. The chief results were as follows described below.

(i) **Penicillins.** The Asn276Asp enzyme hydrolyzed all penicillins tested except oxacillin. The  $k_{cat}$  values of the mutant enzyme were 1.5- to 2-fold higher than those of TEM-1. The  $K_m$  values of the mutant enzyme were four- to ninefold higher than those of the wild-type enzyme. Mecillinam was a poor substrate. Consequently, the  $k_{cat}/K_m$  values of the mutant enzyme for penicillins were reduced two- to sixfold.

(ii) **Cephalosporins.** The  $k_{cat}$  and  $K_m$  values of the enzyme were unaffected except for those for cephaloridine, for which the  $k_{cat}/K_m$  value of the mutant was three-fold higher than that of the wild type. Aztreonam, expanded-spectrum cephalosporins, and cefoxitin were not substrates for these  $\beta$ -lactamases. The catalytic efficiencies ( $k_{cat}/K_m$ ) of the Asn276Asp  $\beta$ -lactamase for penicillins were less than 50% of those of the TEM-1 enzyme (Table 4). The catalytic efficiencies of the Asn276Asp  $\beta$ -lactamase for mecillinam and cephalosporins were comparable to or higher than those of TEM-1. The IC<sub>50</sub>s for TEM-1

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TABLE 3. Kinetic parameters for the hydrolysis of  $\beta$ -lactam substrates by the wild-type (pBSKS/TEM-1) and mutant (Asp<sup>276</sup>)  $\beta$ -lactamases<sup>a</sup>

Substrate	Wild-type (TEM-1)			Mutant (Asn276Asp)		
	$k_{cat}$ (s <sup>-1</sup> ) <sup>b</sup>	$K_m$ ( $\mu$ M) <sup>c</sup>	$k_{cat}/K_m$ ( $\mu$ M <sup>-1</sup> · s <sup>-1</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$k_{cat}/K_m$ ( $\mu$ M <sup>-1</sup> · s <sup>-1</sup> )
Benzylpenicillin	1,160	28	41.2	1,980	105	18.9
Amoxicillin	1,080	32	33.8	1,620	276	5.9
Ticaracillin	139	13	10.7	269	113	2.4
Piperacillin	1,060	67	15.9	1,660	232	7.2
Mezlocillin	1,280	68	18.8	2,100	347	6.0
Mecillinam	822	1,730	0.48	436	793	0.55
Imipenem	— <sup>d</sup>	ND <sup>e</sup>	ND	—	198 <sup>f</sup>	ND
Cephalothin	312	341	0.9	273	389	0.7
Cephaloridine	1,630	1,020	1.6	1,830	397	4.6
Ceftazidime	—	ND	ND	—	261 <sup>f</sup>	ND
Cefoperazone	413	402	1.68	513	405	1.3

<sup>a</sup> The  $k_{cat}$  and  $K_m$  of Asn276Asp for oxacillin, aztreonam, cefotaxime, ceftriaxone, cefoxitin, and cefuroxime could not be measured (there was no detectable hydrolysis or  $V_{max}$  was <2% and  $K_i$  wash >500  $\mu$ M).

<sup>b</sup> The standard deviation s for the analysis were  $\pm$ 5%.

<sup>c</sup> The standard deviation s were  $\pm$ 10% for  $K_m$  values of <100  $\mu$ M and  $\pm$ 20% for  $K_m$  values of >100  $\mu$ M.

<sup>d</sup> —, no detectable hydrolysis.

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

<sup>f</sup>  $K_m$  was determined as  $K_i$  by the reporter substrate method. For more details, see Materials and Methods.

TABLE 4. Ratio of  $k_{cat}/K_m$  of mutant enzymes with Asp<sup>276</sup> and Leu<sup>69</sup> substitutions and of IRT-4 and IRT-14  $\beta$ -lactamases relative to that of the wild-type (pBSKS/TEM-1)<sup>a</sup>

Substrate	$k_{cat}/K_m$ ratio (%)				
	TEM-1	Asn276Asp	M69L <sup>b</sup>	IRT-4 Met69Leu, Asn276Asp <sup>c</sup>	IRT-14 (Met69Leu, Arg275Gln) <sup>d</sup>
Benzylpenicillin	100	46	44	10.1	7.8
Amoxicillin	100	18	26	13.6	11.6
Ticarcillin	100	22	12	3.9	1.3
Piperacillin	100	45	52	5.5	6.5
Mezlocillin	100	32	ND <sup>e</sup>	ND	ND
Mecillinam	100	115	ND	ND	ND
Cephalothin	100	78	21	1.8	13
Cephaloridine	100	288	24	21.8	58.2
Cefoperazone	100	77	68	ND	ND

<sup>a</sup> The value for wild-type pBSKS/TEM-1 was set equal to 100.

<sup>b</sup> Data are from reference 16.

<sup>c</sup> Data are from reference 7.

<sup>d</sup> Data are from reference 10.

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

and the mutant  $\beta$ -lactamases are given in Table 5. Clavulanic acid was 100 times more active than sulbactam against the mutant enzyme, 2.5 times more active than tazobactam, and 4 times less active than brobactam. The IC<sub>50</sub>s for the mutant were sevenfold higher with clavulanic acid, twofold higher with tazobactam, and similar to those for TEM-1 with sulbactam and brobactam. The inactivation of the mutant was progressive, with partition ratio of 950 for inactivation by clavulanic acid, compared to a ratio of 120 for the TEM-1  $\beta$ -lactamase.

**Molecular modeling.** Figure 2, based on the crystal structure of TEM-1  $\beta$ -lactamase (18, 23), shows the stereo view of the environment of Asp<sup>276</sup> and interactions with Arg<sup>244</sup> of the Asn276Asp mutant, in which two salt bonds can be formed between those amino acids.

## DISCUSSION

Previous work has stressed the importance of the Asn<sup>276</sup>→Asp change in  $\beta$ -lactamase for conferring resistance to clavulanic acid (38). This change also decreases the substrate affinity and catalytic efficiency of  $\beta$ -lactamase. However, this substitution has never been found alone in strains with naturally occurring IRT and is always accompanied by another substitution, at position 69, which contributes on its own to the IRT phenotype. Thus, site-directed mutagenesis at position 276 was used to investigate the role of this position in inhibitor-resistant phenotypes. The previous study evaluated only the qualitative effects of antibiotic-inhibitor combinations.

In this study we investigated the effects of the Asn<sup>276</sup>→Asp substitution, generated by site-directed mutagenesis of the *bla*<sub>TEM-1</sub> gene (the variant was Asn276Asp), on other func-

TABLE 5. IC<sub>50</sub>s for hydrolysis of benzylpenicillin

Inhibitor	IC <sub>50</sub> ( $\mu$ M) <sup>a</sup>	
	TEM-1	Asn276Asp
Clavulanic acid	0.02	0.13
Sulbactam	10	13.4
Tazobactam	0.15	0.33
Brobactam	0.02	0.03

<sup>a</sup> The standard deviation for the analysis was  $\pm 5\%$ .

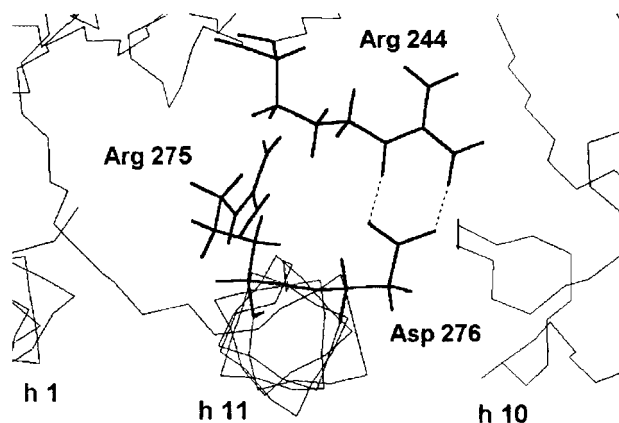


FIG. 2. Molecular modeling of the Asn276Asp mutant of TEM-1: stereo view of the environment of Asp<sup>276</sup> and interactions with Arg<sup>244</sup>. Hydrogen bonds are indicated by dashed lines. The backbones of  $\alpha$ -helices h1, h2, and h10 are shown (at the bottom) as thin lines.

tional characteristics of TEM-1  $\beta$ -lactamase and, in particular, on the MICs and IC<sub>50</sub>s. We also compared the properties of this enzyme with those of naturally occurring variants to assess the contribution of the amino acid change to the resistance phenotype.

The observed differences in susceptibility to clavulanate and tazobactam of *E. coli* bearing pBSKS and *E. coli* bearing R111 (Table 1) are probably due to the higher copy number of pBSKS, as observed previously for *E. coli* resistant to  $\beta$ -lactamase inhibitors (30). However, the Asn276Asp variant was more resistant than the wild type TEM-1 to clavulanate and tazobactam (Table 2). Interestingly, this enhanced resistance to  $\beta$ -lactamase inhibitors has already been observed by determination of the MICs for the Asn<sup>276</sup>→Gly variant of OHIO-1 (4). This substitution did not affect the level of resistance to penicillins and cephalosporins according to the observed MICs (Table 2). Clavulanate and tazobactam had different effects on naturally occurring IRT enzymes, depending on whether the strains had a single substitution (at position 69) or double substitutions (at both position 69 and position 275 or 276) in their  $\beta$ -lactamases (Fig. 1). Indeed, clavulanic acid was more potent against strains producing IRT enzymes with single substitutions than against those with double substitutions. Tazobactam was more potent than clavulanic acid against strains producing IRT enzymes with double substitutions. The double substitution (positions 69 and 276) was also present in IRT-10 along with a third substitution at position 165 (19), which was itself responsible for an IRT phenotype (W165R) (33) (Fig. 1). These observations might be in agreement with the mechanisms, proposed by Imtiaz et al. (22), that differentiate inhibition by clavulanic acid from that by tazobactam.

Some changes in the functional properties of the enzyme caused by the Asp<sup>276</sup> mutation may not be apparent, because determination of the MIC takes into account the whole response of bacteria. However, kinetic results were consistent with the MICs for the mutant enzyme. Higher  $K_m$  and  $k_{cat}$  values were recorded in these studies. This demonstrated the close relationship between enzyme properties and resistance to  $\beta$ -lactams. We also found that the Asn276Asp mutant enzyme had lower catalytic efficiencies ( $k_{cat}/K_m$ ) than TEM-1 due to the higher  $K_m$  values for penicillins (Table 3), suggesting that the substrates may interact less efficiently with the mutant enzyme. Our kinetic data were consistent with those previously reported for the same mutation in a different plasmid construct

and host bacterium (38). Single substitutions at positions 69 (mutant M69L) (16) and 276 (mutant Asn276Asp) independently caused similar reductions in the catalytic efficiency of TEM-1. There was a greater reduction in catalytic efficiency when mutations at both positions occurred together, as in mutant IRT-4 (7). There was a similar reduction in catalytic efficiency with the doubly substituted IRT-14 mutant, demonstrating the importance of the mutation at position 275 in conferring the IRT phenotype (10) (Table 4).

In the evolutionary process of IRTs, the amino acid substitution of the methionine at position 69 with leucine, valine, or isoleucine appears to be crucial in the first stages of the emergence of IRTs (12). The addition of the Asp<sup>276</sup> mutation to the preexisting mutation at position 69 should confer a higher selective advantage to the bacterium. Similar selection processes probably operate for the mutation at position 275 (Arg<sup>275</sup>→Gln) (a similar reduction in catalytic efficiency for IRT-14; Table 4), for which no site-directed mutagenesis or a functional assay has so far been done. The Trp<sup>165</sup>→Arg substitution, which accounts for resistance to inhibitors (29, 33), would further contribute to such a selection process. This substitution occurs with the two other substitutions (positions 69 and 276) in the naturally occurring IRT-10 (19). Thus, the strain producing the IRT-10 β-lactamase may have undergone stronger selection pressure than the others.

The possible mechanism of the resistance generated by the substitutions at positions 275 and 276 is discussed below. These amino acid positions are not located close to the conserved boxes in class A enzymes. In TEM-1, they are at the beginning of helix h11 (23). In the crystal structure of PC1 β-lactamase (20), as the structures of TEM-1 β-lactamase (18) and a closely related TEM-1 β-lactamase (23) show, Arg<sup>244</sup> is located on β sheet s-4 and its guanidinium side chain is close to the penicillin-binding cavity. In PC1, Asp<sup>276</sup> forms a salt bond with Arg<sup>244</sup>, whereas in TEM-1, Asn<sup>276</sup> forms hydrogen bonds with the guanidinium of Arg<sup>244</sup>. Molecular modeling of the Asp<sup>276</sup> TEM mutant suggests that the Asp<sup>276</sup> residue of the mutant forms a salt bond, as shown in Fig. 2, very similar to that observed with the PC1 enzyme. Nevertheless, the PC1 β-lactamase is susceptible to clavulanic acid. In fact, the interactions of clavulanic acid with the PC1 and TEM β-lactamases are not the same. With the PC1 enzyme, clavulanic acid forms a stable acyl enzyme, a *cis*-enamine susceptible to rearrangement into a decarboxylated *trans*-enamine (14). In the case of TEM enzymes, as shown with the TEM-2 enzyme, the acyl enzyme is not stable and acylation of Ser70 is followed by cross-linking of the enzyme with serine 130, as observed by electrospray ionization mass spectroscopy (6). Thus, in IRTs, it is credible that the formation of the acyl enzyme is still possible. This is shown in kinetic experiments with a transient inhibition, but the acyl enzyme almost totally reactivates because the level of cross-linking seems very low.

Therefore, IRT enzymes with double substitutions are the result of convergent evolution, because each substitution itself causes the overall resistance phenotype. There are differences in the behaviors of IRT enzymes with single and double substitutions toward different inhibitors. This highlights the importance of considering such enzyme sequence variations in strategies to design new inhibitor molecules.

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